CANCER VACCINES

An Interactive Qualifying Project Report

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ABSTRACT

Cancer vaccines are a type of therapy that uses a patient's immune system (or components thereof) to fight the patient's tumor. Several types of cancer vaccines have been developed, including the use of therapeutic antibody vaccines, dendritic cell vaccines, tumor-infiltrating lymphocyte vaccines, chimeric antigen receptor vaccines, and immune checkpoint vaccines. While early vaccine experiments often failed, recent studies have shown some spectacular successes. The goal of this IQP was to investigate the field of cancer vaccines, assessing its problems, identifying future trends, and making recommendations for moving the field forward. Our team performed a review of the current research literature, and conducted interviews with scientists and physicians who design or use these vaccines. We found the cancer vaccine field to be complex, but it provides a variety of approaches for potentially treating a patient's tumor. In some cases these approaches have already provided complete remissions for relapsing cancers that would be impossible to treat using any other approach. While each type of therapy can induce side-effects in a portion of the patients, we agree with our interviewees that the side-effects are usually minor, transient, and treatable, and are far less severe than attempting to save the patient's life from a relapsing fatal cancer.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

AUTHORSHIP

Author	Topics Covered
Johan Girgenrath	Therapeutic Antibody Vaccines
Leila Camplese	Dendritic Cell Vaccines
Michaela Hunter	Tumor-Infiltrating Lymphocyte Vaccines
Talal Hamza	Chimeric Antigen Receptor Vaccines
Cole Royer	Checkpoint Inhibitor Vaccines

PROJECT GOALS

Mission Statement and Objectives

Cancer is a collection of related diseases, all sharing the property of uncontrolled cell division. It can initiate almost anywhere in the human body, and has over one-hundred different variations. At the cellular level, cancer is a disease caused by DNA mutations and uncontrolled division of abnormal cells in the body. As a result of these cellular changes, cancer can be difficult to treat. With our increasing aging population, the *number* of people in the U.S. dying of cancer has risen over the years. But with more accurate and sensitive detection techniques, better drugs for blocking cell growth (using a variety of mechanisms), and more precise surgical tools for removing tumors, the death *rates* have actually declined since 1990. Tumor treatments vary widely, depending on the location and type. Treatment options include chemotherapy (the use of chemical drugs to block DNA replication or other key cancer processes, including taxanes, anthracyclines, platins), radiation (to kill rapidly dividing cells), surgery (to remove the tumor), targeted cancer therapy (drugs that interfere with specific molecules a tumor needs to grow), and biologic therapy (i.e. cancer vaccines, the subject of this IQP).

But despite the advances mentioned above, and the large amount of money provided by the "War on Cancer" (initiated by the National Cancer Act of 1971), cancer still remains the second leading cause of death in the U.S., following cardiovascular disease, so better therapies are needed. The subject of this IQP is a new form of targeted cancer therapy termed "**cancer vaccines**". This type of therapy uses the patient's own immune system, or components of the immune system (such as antibodies, T-cells, dendritic cells, etc.), to fight the patient's tumor. Several types of cancer vaccines have been developed, and each will be investigated in this IQP with respect to effectiveness and problems:
> 1) therapeutic antibodies against proteins present on the surface of tumor cells
>> (mono- specific antibodies, bi-specific antibodies, and antibody-drug conjugates),
> 2) dendritic cell vaccines,
> 3) tumor-infiltrating lymphocyte vaccines
> 4) chimeric antigen receptor vaccines
> 5) immune checkpoint vaccines

As is the case for all experimental therapies, cancer vaccines are approved for use in cancer patients only if their tumors do not respond to traditional therapies such as chemotherapy or radiation treatment. So, the patients assigned to cancer vaccine clinical trials are those with very poor prognosis. Under these drastic conditions, any successes are worth pursuing. While the early cancer vaccines did not work well, the past few years have shown some spectacular successes, including complete cancer remissions for highly refractive tumors. The topic of cancer vaccines has become one of the hottest topics in all of cancer research. Currently, researchers are racing to expand the use of immuno-therapies to benefit different types of cancer patients. Hundreds of clinical trials are now underway to see whether improved responses can be achieved by using **combination therapies**, each working with a different mechanism. Advances in rapid and affordable DNA sequencing technologies have allowed the identification of a patient's own tumor neo-antigens as targets, creating a form of **personalized medicine**. In addition, scientists have determined that the patient's **gut microbiome** (type and number of microbes present in the patient's GI tract) helps determine whether the patient will respond to therapy.

But these new vaccines still come with problems. They usually cause deleterious side-effects, they can be very expensive (especially for the personalized vaccines), and they do not work well in all patients. The overall goal of this IQP project is to document and evaluate this new technology, to determine which types of cancer vaccines work best, to document their problems, and help prioritize future directions.

The specific objectives are to:

1 **Develop** a comprehensive assessment of the scientific experiments that led to the development and use of cancer vaccines.
2 **Characterize** what key scientific stakeholders believe are the strengths, weaknesses, reliability, usefulness, and cost of this new technology, and any other concerns.
3 **Evaluate** all of the obtained evidence and prioritize the remaining problems.
4 **Recommend** potential solutions for remaining problems, and prioritize future experiments.

Notes on Project Risks

1. **Potential Risk to the Human Subjects:** The risks to the interviewees, if any, should be very minor. For this type of project, the information requested and disclosed in the interviews will be technical, or the interviewees own personal opinions. This type of information should not be harmful if disclosed. This project has no need to request or disclose proprietary information, such as technical secrets, as this is not needed to accomplish the goals of the project.

2. **Justification of Risk:** Interviews with human subjects (biomedical researchers) are required to obtain information for this project beyond a standard review of the existing literature, although the risk is minor due to the type of information disclosed.

3. **Risk Reduction:** For this project, risk to the interviewees will be reduced by informing the subjects that the interview is voluntary (so they can withdraw their response if they feel it might be risky to divulge the information asked for), they may end the interview at any time, and they can ignore any question they wish. If we plan to quote an individual in our report, we will first obtain the interviewee's permission. Any request for confidentiality will be honored by making an anonymous quote, or by not citing the information.

BACKGROUND

Section-1: Introduction to Cancer

Cancer Description and Causes

Cancer is a collection of related diseases, all sharing the property of uncontrolled cell division. It can initiate almost anywhere in the human body, and has over one-hundred different variations. The extra cells form growths called tumors. Some cancers form solid tumors, while others, such as leukemia, form diffuse tumors. *Malignant* tumors spread into (invade) nearby tissues, or can break off and travel to distant places in the body through the blood or the lymph system to form new tumors. *Benign* tumors do not invade nearby tissues. From the prostate gland, to the thyroid glands, the lungs, or the kidney, these malignancies symptomatically grow and strongly affect health.

At the cellular level, cancer is a disease caused by DNA mutations and uncontrolled division of abnormal cells in the body. It has several hallmarks, including: genomic instability, deregulated cell signaling causing cell division and growth, sustained cell proliferation, resistance to cell death, and evasion from the patient's immune system (Hanahan and Weinberg, 2011). As a result of these cellular changes, cancer can be difficult to treat.

Cancer Prevalence and Survival Rates

According to the Centers for Disease Control, in 2015 (their most recent data) cancer was the second leading cause of death in the United States, with 595,930 (22.7%) deaths, compared to 633,842 (24.1%) for the number-1 killer heart disease (CDC, 2018). The number of people in the US dying of cancer has risen over the years with our increasing aging population, but with improved cancer treatments the death *rates* have actually declined since 1990 (CBS, 2018). The gap between the number-1 killer and number-2 killer is decreasing, likely due to the improving survival rates for heart disease patients (CBS, 2018).

Cancer Survival Rates

Cancer survival rates vary widely, from 8%-18% for difficult to treat cancers, such as pancreatic, lung, or liver cancers, to greater than 65% for easier to treat cancers, such as cancers of the colon, breast, kidney, and prostate (that grow slowly and are easier to treat if detected early) (Howlader et al., 2017). Cancer survival rates have improved significantly in the past several decades due to more accurate and sensitive detection techniques, better drugs for blocking cell growth (using a variety of mechanisms), and more precise surgical tools to remove tumors.

Cancer Treatments

        Tumor treatments vary widely, depending on the location and type. Benign tumors in a safe area of the body that do not cause organ disruption are sometimes left alone and watched carefully. If a tumor begins uncontrolled growth, treatment options include chemotherapy (the use of chemical drugs to block DNA replication or other key cancer processes, including taxanes, anthracyclines, platins), radiation (to kill rapidly dividing cells), surgery (to remove the tumor), targeted cancer therapy (drugs that interfere with specific molecules a tumor needs to grow), and biologic therapy (i.e. cancer vaccines, the subject of this IQP).

Strong Need for New Cancer Drugs

        Despite the advances mentioned above, and the large amount of money provided by the "War on Cancer" (initiated by the National Cancer Act of 1971), cancer still remains the second leading cause of death (following cardiovascular disease), so better therapies are needed.

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Background Section-2:
Introduction to Immunology

This IQP focuses on the immune system, so a brief introduction to this topic will aid our understanding of how cancer vaccines work. The immune system is composed of a network of cells, tissues, and organs whose main purpose is to protect the body from disease and infection. Cells in the immune system recognize problems in the body, communicate with other cells, and react to perform beneficial functions. The immune system is divided into two major sub-divisions: **innate immunity** and **adaptive immunity**.

Main Divisions of the Immune System

Innate immunity is the portion of the immune system that is ready for *immediate response* when an infection is first detected. This system includes physical and chemical surface barriers (such as the skin, sweat, tears, saliva, respiratory tract mucous, stomach acid, and urine) which serve as an initial barrier to infection (Science Learning Hub, 2010). This system also includes the use of defensive cells, defensive proteins, inflammation, and fever. The cells involved in innate immunity include: natural killer cells (NKs), mast cells, eosinophils, basophils, and phagocytic cells [macrophages, neutrophils, and dendritic cells (DCs)]. These cells recognize molecular patterns present on the surface of bacteria and fungi, and act to engulf the pathogens (or aid other cells that engulf and kill them) (Vesely et al., 2011). DCs are also part of the body's adaptive immune system (see below).

If a pathogen is able to survive the body's innate defenses, the body will eventually react with a more advanced response to specifically target the pathogen. This *adaptive immune system* is also known as the **antigen-specific** immune response (Spurrell and Lockley, 2014). **Antigens** are short domains of amino acids or sugars that are viewed as foreign by the immune system. The adaptive cells of this system include antigen-presenting cells (APCs), B-lymphocytes (B-cells), and T-lymphocytes (T-cells). APCs make contact with a pathogen, internalize it, and process selected antigens for presentation on the cell surface. These presented antigens bind to immature pre-B-cells and pre-T-cells, which then begin to differentiate and commit to the antigen. Once these cells have matured, they are specific to the antigen that induced them: B-cells manufacture and secrete antibodies against the antigen into the blood, and cytotoxic T-cells identify infected cells and kill them. DCs, B-cells, and T-cells are all part of the cancer vaccine topic (National Cancer Institute, 2018). Some cancer vaccines use DCs isolated from a patient to prime them against a tumor-specific antigen, then perfuse the DCs back into the patient to induce B-cells and T-cells to eliminate the tumor. Other cancer vaccines isolate, prime, and perfuse T-cells back into the patient.

Antigen Presenting Cells (APCs)

As their name implies, APCs are a specialized type of white blood cell that present foreign antigens on their surface. These presented antigens are recognized by other components of the immune system, such as B-cells and T-cells, to help them commit to that specific antigen (Wellness, 2015). In this process, a foreign invader is detected by an APC and engulfed. Proteases inside the APC degrade foreign antigens on the invader surface into smaller peptides which are then transported to the APC surface where they combine with either an MHC type-I molecule (**professional presentation**) or a type-II molecule (**non-professional presentation**). This antigen-MHC complex is then recognized by B-cells and T-cells to help them commit to that particular antigen (Kimball's Biology Pages, 2013). Professional APCs

include dendritic cells, macrophages, or B-cells. These cells present their antigens using MHC-I, and are the only type to activate helper T-cells. Non-professional APCs include fibroblast cells, thymus epithelial cells, thyroid epithelial cells, glial cells, pancreatic beta cells, and vascular endothelial cells. These cells present antigens using MHC-II in a weaker type interaction (Garland Science, 2001).

Antibodies

One type of cancer vaccine involves injecting the patient with antibodies directed against the patient's tumor cells. **Antibodies** are proteins secreted by mature B-cells into the bloodstream that interact with foreign invaders to bring them to the attention of the immune system for elimination. Antibodies have a "Y" structure (**Figure-1**) comprised of two long chains and two short chains. The antibody constant regions (blue in the diagram) dictate which type of immune cell the antibody engages, while the variable domains (red in the diagram) interact with the foreign antigen.
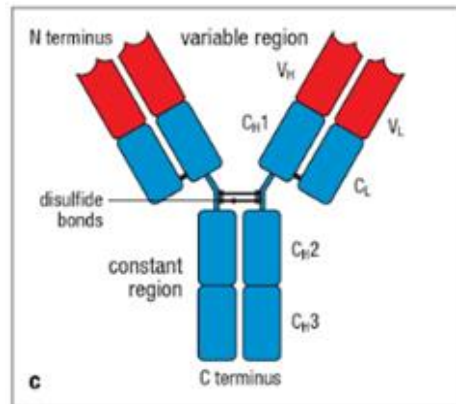
Figure-1: Diagram of a Typical Antibody Structure. Antibodies have a general structure that is similar to a "Y". It contains variable domains (red) that specifically bind to the foreign antigen, and constant domains (blue) that dictate which of the body's immune cells are engaged by the antibody (Murphy and Weaver, 2016).

The antibody variable region that binds the antigen is unique to each antibody clone (the group of antibody molecules secreted from a mature plasma cell and all its derivatives). It has been estimated that the human immune system can produce over 5×10^{13} different types of antibodies (Murphy and Weaver, 2016). The constant regions of the antibody molecules dictate which class the antibody belongs to. There are five main classes of antibodies: IgA, IgD, IgE, IgG, and IgM. The constant regions remain fairly conserved within within each class (Vidarsson et al., 2014). The constant region allows the antibody molecule to interact with effector molecules and phagocytic cells that internalize the antibody-antigen complex.

Once an antibody binds its antigen, based on its type of constant region, it signals for a specific effector function to help destroy the pathogen. There are three main paths for destruction: 1) neutralization (an antibody blocks a binding site keeping the pathogen from entering a cell, 2) opsonization (the antibody-antigen complex is taken inside a macrophage cell for destruction), or 3) complement system activation by the constant region (this system signals plasma proteins to bind to and puncture the pathogen's membrane, leading to cell lysis, and coats the pathogen's membrane to attract phagocytic cells.

B-Lymphocytes (B-Cells)

B-lymphocytes (B-cells) are produced in the bone marrow, the tissue from which they derive their name. Pre-B-cells recognize a foreign antigen presented by an antigen-presenting cell (Immunobiology, 2001). This interaction initiates a maturation process that commits the B-cell to producing antibodies against the antigen. Cytokine hormones and helper T-cells aid the maturation process, which results in a plasma cell that secretes antibodies. The committed plasma or B-cells are then clonally expanded to increase their numbers.

Dendritic Cells (DCs)

Dendritic cells (DCs) derive their name from their *branched* appearance at specific stages of their development. They are potent "professional" antigen-presenting cells whose main function is to recognize foreign antigens (usually small epitope domains of proteins) on the surface of invading pathogens (and sometimes cancer cells), process the antigen within the cell, and then present it on its surface to other cells of the immune system, such as T-cells and B-cells, so they can help eliminate the tumor. The B-cells and T-cells interact with the presented antigen to commit to it. Half of the 2011 Nobel Prize in Physiology or Medicine went to Ralph M. Steinman for "his discovery of the dendritic cell and its role in adaptive immunity" (The Nobel Prize, 2011). Because of their ability to present antigens to the immune system, DCs are used in some types of cancer vaccines to induce a patient's immune response against an antigen on the surface of a patient's tumor cell.

T-Cells

T-lymphocytes (T-cells) are a type of nucleated white blood cell that functions in adaptive cellular immunity. T-cells are distinguished from other lymphocytes, such as B-cells and natural killer cells (NK cells), by the presence of a T-cell receptor (TCR) on their cell surface which recognizes a presented antigen and commits the cell against that antigen (Immunobiology, 2001). They are called T-cells because they mature in the thymus (although some T-cells also mature in the tonsils). Several types of T-cells exist, each with a different function: helper (CD4+), cytotoxic (CD8+), memory, suppressor, mucosal, and gamma delta T-cells.

With respect to the topic of cancer vaccines, **tumor infiltrating lymphocytes (TILs)** are a type of T-cell found in tumors that help kill it. High levels of TILs in tumors are often associated with a better clinical outcome for the patient. TILs isolated from tumors usually include both CD4+ (helper T-cells) and CD8+ (cytotoxic killer T-cells, CTLs). TILs circulate through the bloodstream, recognize the tumor and infiltrate it. The CD4+ helper T-cells secrete cytokines to boost the immune system. CTLs directly lyse the tumor cell.

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Background Section-3:
Introduction to Cancer Vaccines

The subject of this IQP is a form of targeted cancer therapy, **cancer vaccines**. This type of therapy uses the patient's own immune system, or components of the immune system (such as antibodies, T-cells, dendritic cells, etc.), to fight the patient's tumor. Several types of cancer vaccines have been developed, and each will be investigated in this IQP:

    1) injecting **cancer neo-antigens** (peptides specific to the cancer cell surface) to help the immune system make antibodies and T-cells against the tumor,

    2) injecting **therapeutic antibodies** against proteins on the surface of tumor cells (mono-specific antibodies, bi-specific antibodies, and antibody-drug conjugates),

    3) injecting **dendritic cell vaccines**,

    4) injecting **T-cell vaccines** (TILs and CARs), and

    5) injecting **immune system modulators** (such as immune checkpoint inhibitors).

As is the case for all experimental therapies, cancer vaccines are approved for use in cancer patients only if their tumors do not respond to traditional therapies such as chemotherapy or radiation treatment. So, patients assigned to cancer vaccine trials tend to be those with very poor prognosis. Although the early cancer vaccines did not work well, the past few years have shown some spectacular successes, including some complete cancer remissions for highly refractive tumors. Thus, the topic of cancer vaccines has become one of the hottest topics in all of cancer research.

The past few years have seen unprecedented clinical successes, rapid drug developments, and "first-in-kind" treatment approvals from the FDA. In 2016, the American Society of Clinical Oncology (ASCO) announced "immunotherapy" as the year's top cancer advance, and in 2017 that society named immunotherapy as its "advance of the year" (Madden, 2018). The society emphasized the rapid pace of research in this field, emphasizing that "these agents have extended the lives of many patients with late-stage cancers for which there have been few treatment options" (Madden, 2018). In 2017, approximately 2,000 immuno-therapeutic agents were under development (Schmidt, 2017).

Researchers are racing to expand the use of immuno-therapies to benefit more types of cancer patients. Hundreds of clinical trials are now underway to see whether improved responses can be achieved by using a **combination of two immunotherapies**, each working with a different mechanism. The number of clinical trials is increasing at an exponential pace, as evidenced by the number of combination trials with checkpoint inhibitors and another treatment (**Figure-2**).
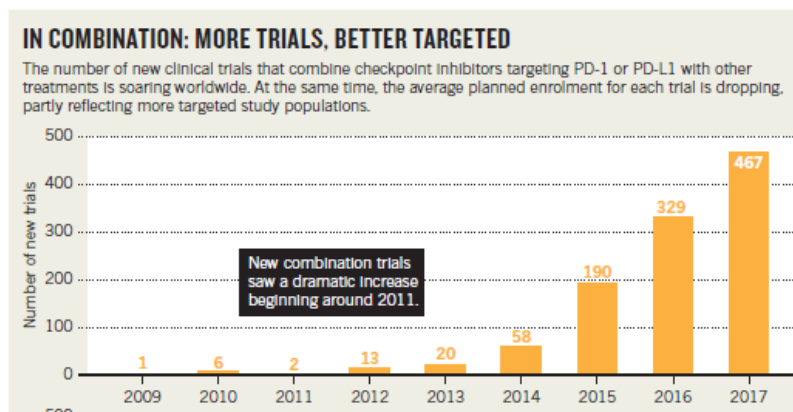
Figure-2: Increase in Cancer Vaccine Clinical Trials. Shown is an example of the exponential increase in clinical trials combining a checkpoint inhibitor vaccine with another treatment. Diagram is from Schmidt, 2017.

16

Advances in rapid and affordable DNA sequencing technologies have allowed the identification of a patient's own tumor neo-antigens as targets, creating a form of **personalized medicine**. In addition, scientists have found that the patient's **gut microbiome** (type and number of microbes present in the patient's GI tract) helps determine whether the patient will respond to therapy.

The breadth of cancers treatable with cancer vaccine combinations has increased in recent years (**Figure-3**). Lung cancer, melanoma, breast cancer, lymphoma, kidney cancer, and head and neck cancers are among the most researched cancers treated with immuno-vaccines.
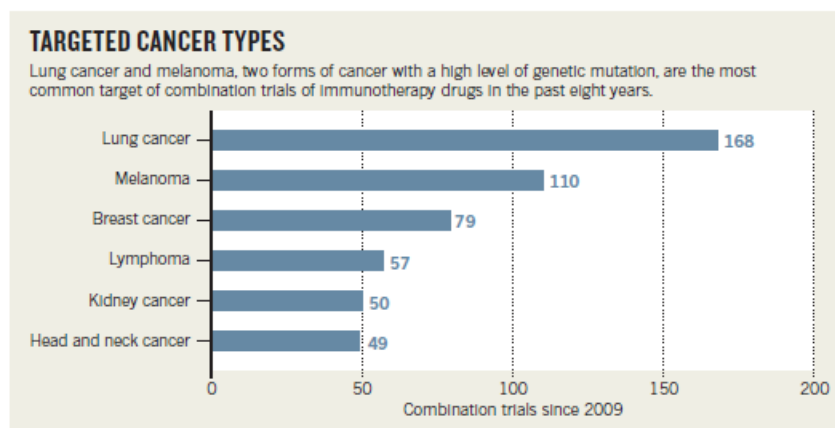
Figure-3: Main Types of Cancer Treated with Vaccines. Shown are the most common cancers treated with cancer vaccine combination trials. Diagram is from Schmidt, 2017.

Problems with Cancer Vaccines

While recent years have shown some spectacular successes with cancer vaccines, much still needs to be done, as these new cancer vaccines come with problems:

1) It remains unclear why only a subset of patients respond to a particular therapy. Does the tumor stop making the antigen targeted by the therapy, allowing re-growth? Are components of the patient's immune system blocking the success of the therapy?

2) Why are the immuno-therapies so expensive? A recent study indicates that the average checkpoint vaccine in the US costs $150,000, and the average CAR vaccine about $475,000 (Couzin-Frankel, 2018). Who should pay the price for such expensive medicines?

3) Why do most cancer vaccines induce side-effects? Are the side-effects transient and manageable?

The overall goal of this IQP is to document and evaluate the technology of cancer vaccines, to document technique problems and help prioritize future directions.

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LITERATURE REVIEW

Section-1: Therapeutic Antibody Vaccines
Johan Girgenrath

Cancer Antigens and Neo-Antigens as Antibody Targets

The success of all cancer vaccines depends on the existence of **antigens** (proteins or sugars, or portions thereof) on the surface of the cancer cells that can be specifically targeted by the vaccine. Tumors are caused by mutations in DNA. Some of these mutations alter the expression levels or types of antigens on the surface of the tumor (Parmiani et al., 2007). New antigens presented on the tumor surface that are lacking in normal cells are termed **neo-antigens (**Schumacher and Schreiber, 2015). Neo-antigens can vary from tumor to tumor, and from patient to patient, so they are the subject of much research in the personalized medicine field.

Tumor cells in the body are poor antigen-presenting cells. Tumor cells are derived from normal cells by DNA mutation, so the vast majority of the tumor cell DNA is identical to the patient's normal cells. Thus, the tumor surface antigens look mostly like "self" to the immune system, and are ignored, allowing the tumor to grow. Only a small portion of the cancer DNA mutations create neo-antigens unique to the patient's tumor, and these provide excellent candidates for cancer vaccine designs. One of the goals of cancer vaccine research is to develop rapid affordable methods for determining the exact neo-antigens present in a specific patient's tumor, and designing a personal vaccine for that patient.

Much research in the cancer vaccine field has focused on identifying specific antigens for targeting. These antigens should not be found in large quantities in normal cells, to prevent their damage by the vaccine. Examples include proteins **CD19, CD20, or CD22** on the surface of B-cells, which are targeted by cancer vaccines against B-cell tumors (such as leukemia), overactive B-cells (autoimmune disorders, transplant rejection), or for killing dysfunctional B-cells. Another well-known example is the protein **Her-2**, which is over-expressed on some types of breast cancer cells, and is targeted by the monovalent cancer vaccine antibody **Herceptin** (also known as **Trastuzumab** or Herclon).

Mono-Specific Antibody Vaccines

One type of cancer vaccine consists of injecting the patient with antibodies against the tumor cell. As mentioned in the Immunology Introduction section, antibodies are proteins secreted by mature B-cells (plasma cells) into the bloodstream that interact with foreign invaders to bring them to the attention of the immune system for elimination. Injecting antibodies into a patient is termed **passive immunity**. It does not activate the patient's own immune system to create the antibodies, but instead the antibodies are produced by a bio-engineering process. The antibodies bind to antigen to create antigen-antibody complexes, which are then recognized and cleared from body by other cells of the immune system, such as macrophages or T-cells. The antibodies used in a cancer vaccine can be **mono-specific, bi-specific, or antibody-drug conjugates**.

Mono-specific (or monovalent) antibodies recognize only one type of antigen. Most natural antibodies produced in the human body against an infection are of this type. The variable domains on both arms of the "Y" shaped antibody recognize the *same* antigen. For example, the first antibody

19

approved as a cancer vaccine in the U.S. was Rituximab, an antibody against surface protein CD20. This protein is found on the surface of B-cells, so the antibody is used to treat patients with high B-cell numbers (leukemia and lymphoma), patients with overactive B-cells (autoimmune disorders, transplant rejection), or patients with dysfunctional B-cells. Rituximab was initially approved by the FDA in 1997 to treat non-Hodgkin (Maloney et al., 1997). Several studies have used it in clinical trials with various success. Another example is Inotuzumab, a mono-specific antibody against CD22, used to treat patients with refractory acute lymphoblastic leukemia (ALL). Perhaps the best known antibody in this category is Herceptin (also known as Trastuzumab or Herclon), the second antibody approved by the FDA for cancer treatment in the U.S. Herceptin binds to the HER2/neu receptor, which is over-expressed on some types of cancer cells (Bange et al., 2001). Herceptin was approved by the FDA in September 1998 for treating HER2-positive breast cancers, and is now used to treat colorectal and pancreatic cancers (Perez et al., 2002).

Example of Mono-Specific Antibody Clinical Trials

With respect to clinical trials using mono-specific antibodies, although the treatments in this category sometimes prolonged a patient's life, a review of the literature showed that full cancer remissions have not been that common. **Table-I** below shows examples of clinical trials done with mono-specific antibodies.

Table-I: Example Clinical Trials With Mono-Specific Antibody Vaccines

Target Antigen	Cancer	Notes	Side-Effects	Reference
CD20	Low-grade non-Hodgkin's lymphoma	Phase-III trial on 116 patients, 48% achieved a measurable tumor response, 6% achieved complete tumor responses, 76% achieved ≥20% reduction in tumor volume.		Berinstein et al., 1998
	Indolent B-cell lymphoma	Combination trial of Rituximab with chemotherapy, 40 patient group, 55% complete remission and 40% partial remission.		Czuczman, 1999
	Stage II-IV low-grade non-Hodgkin's lymphoma	39 patients, 54% of the patients showed objective responses, at 1 year 77% showed progression-free survival.	The treatment was well tolerated.	Hainsworth, 2000
	Aggressive non-Hodgkin's lymphoma (NHL)	Rituxan plus chemotherapy combination, 33 patients, 61% of the patients experienced a "complete response". 29 of 31 responding patients remained in remission during a 26 month follow-up period.	The most frequent adverse events attributed to the Rituxan antibody were fever and chills.	Vose et al., 2001
	Non-Hodgkin lymphoma (NHL)	Rituximab, 50 patients, the response rate after 50 days was 73%, with 10 patients (20%) in complete remission, 3 patients in complete remission/unconfirmed, and 23 patients in partial remission.		Colombat et al., 2001

	Indolent non-Hodgkin's lymphoma	62 patients, Following a second Rituximab treatment, the major response rate increased from 47% to 65%, and the complete response rate increased from 7% to 27%.	There was no observable toxicity with repeat courses of rituximab.	Hainsworth, 2002
HER-2	Metastatic breast cancer	222 women, blinded, independent response evaluation committee identified 8 complete, and 26 partial responses, providing an overall response rate of 15%.	The most common adverse events were infusion-associated fever and/or chills (40% of the patients).	Cobleigh et al., 1999
	Metastatic breast cancer	234 patients were randomly assigned to receive standard chemotherapy, and 234 patients were assigned to receive standard chemotherapy plus trastuzumab. The results indicated that the addition of trastuzumab antibody to the chemotherapy regime provided a longer time to disease progression (median 7.4 vs 4.6 months; $p<0.001$) and a lower rate of death at 1 year (22% vs 33%, $p=0.008$).	The most important adverse event observed was cardiac dysfunction (27% in chemo group versus 13% in the combination group). The symptoms were manageable.	Slamon et al., 2001
	Advanced breast cancer	An international, multicenter, randomized trial, 1,694 received trastuzumab and 1,693 were controls. At one year, recurring breast cancer or death was observed in 127 patients in the trastuzumab group versus 220 in the control group.	Severe cardiotoxicity developed in 0.5% of the women treated with trastuzumab.	Piccart-Gebhart et al., 2005
	Breast cancer patients	27 breast cancer patients were treated with trastuzumab and chemotherapy. Anti-HER-2/neu antibodies were detectable in 29% of the patients before treatment, and in 56% of the patients during treatment. Of the twenty-two individuals treated for metastatic disease, those showing improved clinical responses had higher levels of HER-2 antibodies.		Taylor et al., 2007
CD22	Acute lymphocytic leukemia (ALL)	Phase-II clinical trial. The antibody was conjugated to the toxin calecheamicin to kill the CD22+ cells. Of the 49 treated patients: 9 (18%) had a complete response, 19 (39%) had resistant disease, and only 2 (4%) died.	The most frequent side effects were fever (20 patients), hypotension (12 patients), and liver toxicity (12 patients).	Kantarjian et al., 2012

	Acute lymphocytic leukemia (ALL)	Follow-up study, Inotuzumab (a CD22 mAb bound to toxin calicheamicin), 90 patients, 49 received a single-dose 3-4 weeks, while 41 patients received inotuzumab weekly every three to four weeks. The overall response rate was 58%, 19% achieved a complete response, 30% had a complete response with no platelet recover (CRp), and 9% had a bone marrow complete recovery. The response rates were similar between the two groups.	Some of the adverse side effects observed were reversible bilibrubin elevation, fever, and hypotension.	Kantarjian et al., 2013
EGFR	Non-small-cell lung cancer (NSCLC)	Phase III clinical trial. Treatment with mAb Cetuximab (binds EGFR) + chemotherapy (n = 557) vs treatment with chemotherapy (n = 568). Patients treated with both cetuximab and chemotherapy survived several months longer than patients treated solely with chemotherapy.	10% of patients that were administered cetuximab observed an acne-like rash.	Pirker et al., 2009

Problems with Monovalent Vaccines

Because tumor cells are mutated patient cells, there are still many similarities between normal patient cells and tumor cells. As a result, monovalent antibodies that are designed against antigens present on a tumor cell may also recognize these *same antigens* on healthy cells, disrupting normal function and causing off-target effects. In addition, some tumors downregulate expression of the target antigen, or don't express it at all. For example, in B-cell cancers, CD22 is present on about 60-90% of B-cell malignancies, but not in 10-40% of leukemic patients (Hoelzer, 2013). In spite of this, some success has been achieved with CD19 antibodies for B-cell tumors and leukemia (Naddafi et al., 2015), likely because CD19 is restricted to the B-cell lineage, and lost B-cells can be replaced in the patient post-cancer treatment. To be effective, monovalent antibody vaccines need a healthy patient's immune system to help clear the cancer cells tagged by the vaccine; binding of the antibody by itself does not kill the cancer cell. So, monovalent vaccines might not work well in immuno-compromised patients.

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Bivalent Antibody Vaccines

Bi-specific (or **bivalent**) antibody molecules recognize two different epitopes, one for each variable region on the antibody molecule. The rationale behind this class of cancer vaccine is that two functions are better than one (reviewed in: Sedykh et al., 2018; Krishnamurthy and Jimeno, 2018). For example, a bivalent antibody could recognize and bind a tumor antigen with one arm, while its other arm recognizes and binds to an antigen on a cytotoxic T-cell. This double-binding brings the cancer cell in close proximity to the T-cell that kills it. The binding to the T-cell antigen not only physically tethers the cancer cell and T-cell together, in some cases (depending on the T-cell antigen) it activates the T-cell. A study directly comparing the activities of mono-specific and bi-specific antibody treatments showed the latter group generally has higher potency against tumor cells at a lower dosing amount and with lower costs of production (Molhoj et al., 2007).

A convenient code for referring to bi-specific antibodies uses an "x" to separate the two binding functions. For example, the best characterized bi-specific antibody is Blinatumomab, where one antibody arm recognizes CD19 on the surface of B-cells (such as in leukemia), and the other antibody arm recognizes CD3 (a T-cell activator present on the surface of T-cells). This antibody is conveniently coded as **CD19 x CD3**. Other examples of bi-specific antibodies include: CD3 x glioma marker (Nitta et al., 1990), CD3 x folate receptor (ovarian cancer cells) (Canevari et al., 1995), CD16 x CD30 (Hodgkin's disease) (Hartmann et al., 1997), CD319 x CD28 (B-cell lymphomas) (Daniel et al., 1998), CD64 x Fc-Receptor (B-cell lymphomas) (Honeychurch et al., 2000), CD30 x CD64 (Hodgkin's lymphoma) (Borchmann et al., 2002).

Blinatumomab (CD19 x CD3) (also known as AMG-103) was the first bi-specific antibody approved for use in the U.S. (in 2014), is (Haagen et al., 1992; Bohlen et al., 1993; Haagen et al., 1994; DeGast et al., 1995; Weiner and DeGast, 1995). An example of Blinatumomab's spectacular success is seen in a 2014 clinical trial performed on 9 patients with acute lymphoblastic leukemia (ALL) (Schlegel et al., 2014). Of the 9 patients, 4 showed complete cancer remission after one cycle of treatment, and 2 more showed complete remission after the second cycle (6 of 9 complete remissions, 67%) (Schlegel et al., 2014). Targeting CD19 with antibodies is one of the best success stories for cancer vaccines. CD19 is present on early-stage B-cells, but it is lost when B-cells mature to plasma cells, and it is not present on stem cells or other normal cells in the body, so these latter cells are not targeted. CD19 is an excellent target for leukemia, because the patient produces large amounts of early-stage B-cells (hopefully eliminated by the treatment), while the treatment would leave the stem cells and mature plasma cells

(lacking CD19) alone to produce needed antibodies to fight infections (Scheuermann and Racila, 1995). On December 3, 2014, the bi-specific antibody was approved by the FDA for treating acute lymphoblastic leukemia (ALL) (FDA, 2014).

In non-small cell lung cancers (NSCLCs), mutations in the epidermal growth factor receptor (EGFR) are often observed and are thought to play a role in promoting tumor growth (Harari, 2004; Midha et al., 2015). A monoclonal antibody has been developed that targets the EGFR (**Cetuximab**), though tumors can acquire resistance to these treatments through several mechanisms (Pao et al., 2005). One mechanism identified is the over-expression of the EGFR mutant receptor c-Met, which when bound by its ligand confers resistance (Turke et al., 2010). To overcome this limitation, scientists at Biologics Research in Pennsylvania have created a bi-specific antibody that antagonistically binds to EGFR and mutant c-Met, which when administered to human lung cells showed up to 80% tumor growth inhibition (Grugan et al., 2016). Clinical trials for this bispecific antibody have not yet been reported.

Another example of a bi-specific antibody is **Catumaxomab** (also called Trion or Removab), the first bi-specific and tri-functional antibody approved for use in Europe (in 2009). This antibody binds EpCAM (present on the surface of ascites tumors) and CD3 (present on T-cells), while the Fc fragment binds macrophages and dendritic cells (**Figure-4**). Catumaxomab has several methods of killing the tumor cells, including T-cell mediated lysis, phagocytosis, and cytokine activity (secreted by the macrophage and dendritic cells.
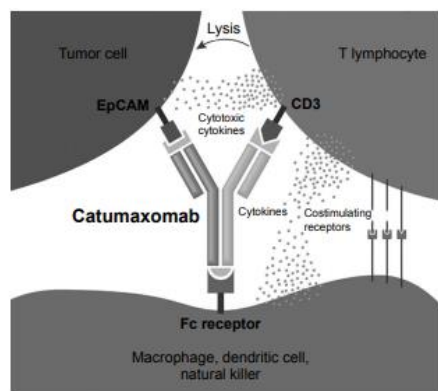
Figure-4: Diagram of Bispecific Antibody Catumaxomab. This antibody binds EpCAM (upper left) present on ascites tumors, and CD3 (upper right) present on T-cells, while the Fc fragment binds macrophage and dendritic cells (lower center). The mode of killing includes T-cell activation, phagocytosis, and cytokine release. Figure is from Sedykh et al., 2018.

Example Clinical Trials with Bi-specific Antibodies

Bispecific antibodies have been used since the early 1990's in a variety of clinical trials against several types of cancer (**Table-II**).

Table-II: Example Clinical Trials with Bispecific Antibodies

Targets	Cancer	Notes	Side-Effects	Reference
CD3 X CD19	B-cell non-Hodgkin's lymphoma	3 human patients. The results showed evidence of successful T-cell activation.	Relatively safe toxicity.	De Gast et al., 1995

B-cell chronic lymphocytic leukemia (B-CLL)	Pre-clinical testing showed the tumor lysis occurs mostly via CD8+ cytotoxic T-cells, and that CD19-negative cells (non-leukemic cells) were not harmed.	CD19-negative cells (non-leukemic cells) were not harmed by the CD8+ T-cells induced by the antibody vaccine.	Dreier et al., 2002
B-cell chronic lymphocytic leukemia (B-CLL)	Their data showed a depletion of lymphoma cells in 22 out of 25 (88%) patient cases, and the depletion did not require IL-2 supplement.		Löffler et al., 2003
CD19-positive B-cell chronic lymphocytic leukemia (B-CLL)	Used video-assisted microscopy to show that each activated T-cell eliminated multiple CD19 tumor cell targets within a 9 hour time period, and the tumor cell targets were completely eliminated within 24 hours using ratios as low as 1:5.		Hoffmann et al., 2005
Variety of cancers	Under identical experimental conditions, the bispecific CD19/CD3 format has far superior activity compared to the monospecific formats.		Molhol et al., 2007
Non-Hodgkin's lymphoma	Doses of antibody as low as 0.005 milligrams per square meter body per day led to an elimination of all target cells in the blood, and partial and complete tumor regressions were observed at 0.015 milligram doses. **At 0.06 milligram doses, 100% of the patients experienced tumor regression!**		Bargou et al., 2008
Acute lymphoblastic leukemia (ALL)	Phase-II. 16 of 21 patients showed a successful minimal residual disease (MRD). 12 of the 16 responders had been refractory to previous cancer treatments, so any improvement in their condition is a significant event.	The most frequently observed side-effects were grade-3 and 4 lymphopenia, but were completely reversible.	Topp et al., 2011

	Relapsing acute lymphoblastic leukemia (ALL)	Phase-II long-term follow-up of the above study. 61% of the 20 patients had a hematologic relapse-free survival rate. In a subgroup of 9 patients who progressed well enough to also receive an allogeneic hematopoietic stem cell transplant, 65% showed hematologic relapse-free survival.		Topp et al., 2012
	B-precursor acute lymphoblastic leukemia (ALL)	Of the 9 patients, 4 achieved complete remission after their first cycle of treatment, 2 showed a complete remission after the second cycle, and the remaining 3 patients did not respond to the treatment.	1 patient experienced grade-3 seizures, and 2 patients experienced grade-3 cytokine release syndrome, but those events were treatable.	Schlegel et al., 2014
	B-precursor acute lymphoblastic leukemia (B-ALL)	Multicenter Phase-II study. After two treatments with CD19 x CD3 antibody, 81 of 189 patients **(43%) showed complete cancer remission.**	The most frequent adverse events were febrile neutropenia (25%), neutropenia (16%), and anemia (14%). 2% of the patients showed grade-3 cytokine release syndrome. 3 deaths due to sepsis from E. coli and Candida were thought to result from the treatment by hindering antibody formation against the pathogen.	Topp et al., 2015
CD19 x CD3	Refractory/ relapsed (r/r) acute lymphoblastic leukemia (ALL)	65 patients. The complete remission (CR) rate was 33/65 (51%). Low responses correlated with initial high leukemia burden (p = .02), history of prior extra-medullary disease (EM) (p = .005), and active EM at the time of treatment (p = .05). Of the refractory cases, 41% had evidence of EM-ALL progression, and CD19 expression was negative (18%) or low (23%).		Aldoss et al., 2017

CD3 x Glioma marker	Malignant glioma	10 patients treated with lymphokine-activated killer (LAK) cells treated in vitro with bispecific antibody against CD3 (T-cell activator) x anti-glioma marker, compared to 10 patients treated with LAKs alone. In the control group, 9 patients relapsed (and 8 died within 4 years). In the antibody-treated group no patients relapsed (in 18 months), 4 showed tumor regression, and 4 showed tumor eradication.		Nitta et al., 1990
CD3 x Folate Receptor	Ovarian carcinoma	Of the 19 patients evaluated, 3 showed complete responses (lasting an average of 22 months), 1 showed complete intraperitoneal response with progressive disease in the lymph nodes, 3 showed partial responses, 7 had stable disease, and 5 showed progressive disease.		Canevari et al., 1995
CD16 x tumor marker CD30	Refractory Hodgkin's disease	1 patient experienced complete remission lasting 16 months, one patient experienced partial remission lasting 3 months, 3 had minor responses, and 1 mixed response.	Side effects were rare, and consisted of fever, lymph node pain, and a rash.	Hartmann et al., 1997
CD30 x immune activator CD64	Refractory Hodgkin lymphoma	Phase 1 trial. Of 10 treated patients, 1 showed complete remission, 3 partial remissions, and 4 had stable disease.	The observed side effects were transient and mild. The most serious were hypotension (4 of 10), tachycardia (6 of 10), fatigue (10 of 10), and fever.	Borchmann et al., 2002
Transferrin receptor (TfR) x β-secretase (BACE1)		The team developed two humanized bispecific antibodies against the transferrin receptor (TfR) (to facilitate transcytosis across the blood brain barrier, BBB), and against β-secretase (BACE1) (to lower amyloid-beta production in the brain). Dosing primates with anti-TfR/BACE1 allowed the antibodies to cross the BBB and reduce brain Aβ.		Wu et al., 2014
EpCAM (epithelial	EpCAM positive	Antibody Catumaxomab. 16 patients, several groups of increasing antibody doses. The	The most common adverse events were chills (93.8 %), fever (87.5 %), and grade ≥3	Mau-Sørensen et al., 2015

cancer cells) x CD3 (T cells)	epithelial cancer	maximum tolerated dose (MTD) appears to be 7 µg.	increases in liver enzymes(56.3 %). One patient at the highest dose of 10 µg died of hepatic failure related to the treatment, leading to termination of the study.	
CD30 x CD16A (to recruit NKs)	Heavily pretreated relapsed or refractory Hodgkin lymphoma	Antibody AFM13, Phase-I dose-escalation study, 28 patients. Doses of 0.01 to 7 mg/kg body weight. The maximum tolerated dose was not reached. Three of 26 evaluable patients (11.5%) achieved partial remission, and 13 patients (50%) achieved stable disease. AFM13 was also active in brentuximab vedotin-refractory patients (CD30). Phase-II currently underway.	Adverse events were generally mild to moderate.	Rothe et al., 2015
CD20 x CD3	CD20-positive human tumors	This study addressed two of the problems associated with bispecific antibodies: high cost and inconvenient administration. They used a non-viral DNA vector mini-circle (MC) to produce a bispecific antibody CD20 x CD3. The procedure produced T-cell mediated killing of multiple CD20-positive tumor lines in vitro, and delivery of the DNA to mouse liver produced an effective anti-cancer effect in mouse xenograft models.		Pang et al., 2017
EGFR x HER3	KRAS-positive MAPK-positive tumors	KRAS-mutant tumors possess abnormal MAPK pathway signaling and cell proliferation. This was a Phase-IB dose-escalation study of a combination of Cobimetinib (which blocks MAPK signaling) and the bi-specific antibody **duligotuzumab** (which inhibits ligand binding to two types of receptors: EGFR and human epidermal growth factor receptor 3 (HER3). 23 patients KRAS-mutant tumors were enrolled. The best response was limited to 9 patients (39%) with stable disease.	The cobimetinib and duligotuzumab combination was associated with increased toxicity, and limited efficacy, so the study did not proceed to expansion stage and closed for enrollment.	Lieu et al., 2017

The studies summarized above in the table illustrate that using bi-specific antibodies is better than earlier studies with mono-specific antibodies. While the mono-specific therapies showed few complete

cancer remissions, the bi-specific therapies frequently showed complete remissions, including: 22 of 25 patients (88%) (Löffler et al., 2003), 3/3 (100%) (Bargou et al., 2008), 12/20 (60%) (Topp et al., 2012), 6/9 (67%) (Schlegel et al., 2014), 81/189 (43%) (Topp et al., 2015), and 33/65 (51%) (Aldoss et al., 2017). Thus, bispecific antibody treatments are an improvement over earlier antibody versions.

Problems with Bi-Specific Antibodies

Although bi-specific antibodies have shown strong results against cancer that are generally better than mono-specific antibodies, they are not perfect. Bi-specific antibodies have many of the same problems associated with mono-specific antibodies: 1) in some cases the treatment causes patient death, 2) some patient tumors become resistant to the treatment, and 3) the vaccines almost always cause side-effects. The worst adverse effect observed was patient death. For example, one patient died from hepatic failure caused by administration of an EpCAM x CD3 bispecific antibody treatment, leading to termination of the entire clinical study (Mau-Sørensen et al., 2015). And in another study, 3 patients died from *E. coli* and *Candida* sepsis caused by treatment with a CD19 x CD3 bispecific antibody that eliminated B-cells from the patients hindering their ability to make antibodies against the pathogens (Topp et al., 2015). But patient death was rare, and most patients treated with bispecific antibodies showed side-effects that were generally mild, transient, and treatable (Borchmann et al., 2002; Topp et al., 2011). With respect to patient tumors resistant to the treatments, the reason given most often in the studies was a loss of target antigen expression by the tumor. This antigen loss was quantitated in one study targeting CD19 in B-cell lineage tumors whose results showed that of the 41% of the non-responder patients, CD19 expression was absent (18% of the patients) or low (23% of the patients) (Aldoss et al., 2017). Two other problems encountered with bispecific antibodies include their high cost and their short half-life in the body. The high cost typically results from the method of antibody production using expensive cell culture, and the short half-life results from the rapid clearance and degradation of antibodies passively administered to the body. Both of these latter problems have recently been addressed using DNAs encoding the antibodies. For example, one study used a non-viral mini-circle DNA to produce a bispecific antibody against CD20 x CD3. The procedure allowed long-term production of an antibody that had strong anti-tumor effects *in vitro* against human cancer cell lines and *in vivo* in humanized mouse xenograft models (Pang et al., 2017).

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Antibody-Drug Conjugate Vaccines (ADCs)

In addition to monovalent and bivalent antibody vaccines, the third type of antibody vaccine is the **antibody-drug conjugate (ADC)**. Simply targeting and attaching an antibody by itself to a cancer cell antigen does not always kill the cell (Thomas et al., 2016). The attachment does not always attract sufficient attention from the immune system to remove the tagged tumor. So, scientists have designed new types of antibody drugs, ADCs, that *combine* the power of antibodies (to recognize and bind specific antigens) with cytotoxic drugs (new highly potent drugs that kill cells in very small quantities).

An ADC drug (**Figure-5**) typically contains an antibody directed against a tumor antigen connected by a linker to a cytotoxic drug (that kills the cancer cell) (Casi and Neri, 2012; Flygare et al.,

2012; Bouchard et al., 2014; Perez et al., 2014; Chiu and Gilliland, 2016; Kraynov et al., 2016). For a well-designed ADC, the antibody should strongly bind a tumor-specific antigen, the antigen should not be expressed strongly by normal cells, the linker should not release the cytotoxic drug (cargo) prematurely into the circulation, and the toxic cargo should be potent enough to kill a cell with only a few molecules.
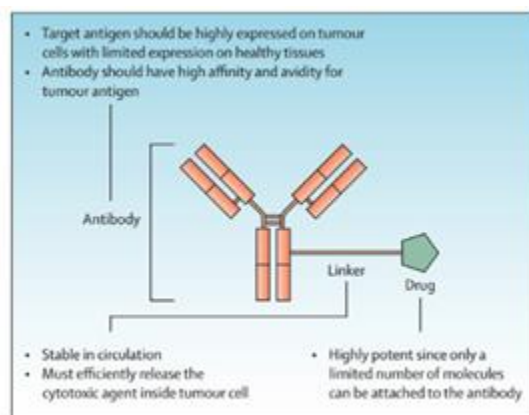
Figure-5: General Structure of an Antibody-Drug Conjugate. Shown is the general structure of an ADC, consisting of a "Y"-shaped antibody (brown), bound to a highly potent cytotoxic drug (green, diagram right) using a linker (diagram center). Shown in text are the required properties for each component (Thomas et al., 2016).

Most ADCs are designed to be internalized in the cancer cell. Once the ADC binds to its target antigen, the cell engulfs the ADC by receptor-mediated endocytosis, and the ADC enters a membrane-enclosed endocytic vesicle (endosome). This vesicle becomes acidified, which in some cases releases the cytotoxic drug into the cytoplasm where it kills the cell (Pastan et al., 2006). To make a good ADC, the antibody should strongly bind the antigen, the antigen should be highly expressed on the tumor cell (not normal cells), the linker should be stable in the patient's circulation to avoid releasing the toxin too soon, and the cargo drug should be highly potent since only a few molecules will enter the cell.

Several types of **cytotoxic drugs** are used in ADCs, the most commonly used include drugs that: 1) bind DNA (leading to DNA degradation or alkylation) (i.e. calicheamicin and nemorubicin), 2) block tubulin (inhibiting cancer cell division) (i.e. DM1 and MMAE), or 3) inhibit RNA polymerases (blocking ancer cell RNA synthesis and gene expression) (Thomas et al., 2016). Usually 2-4 drug molecules are attached to each antibody at locations that do not hinder interaction with the antigen (Hughes, 2010).

ADCs also have several types of **linkers**. The antibody can be linked to drug by one of four methods: 1) disulfide bond formation, 2) glycol-conjugation, 3) protein tags, or 4) amino acid incorporation (Pastan et al., 2006; Agarwal and Bertozzi, 2015; Thomas et al., 2016). Linkers can be cleavable (degraded by proteases inside the endocytic vesicle), or non-cleavable (degraded along with the antibody inside the vesicle) (Doronina et al., 2006; Jain et al., 2015). Cleavable linkers are usually more stable in the bloodstream (Thomas et al., 2016). Linkers can be placed at antibody variable regions, hinge regions, constant regions, or any combination (Agarwal and Bertozzi, 2015).

ADC Examples

Two ADCs are currently approved by the FDA: Trastuzumab emtansine (**Kadcyla®**) and Brentuximab vedotin (**Adcetris®**) (Thomas et al., 2016). A third ADC, Gemtuzumab ozogamicin (**Mylotarg®**), was initially approved but was later withdrawn (Nelson, 2010; Richwine, 2010). Kadcyla®

was developed by Genentech to treat HER2-positive metastatic breast cancer in patients resistant to other treatments (Niculescu-Duvaz, 2010; LoRusso et al., 2011; Lopus, 2011; Verma et al., 2012; Drugs.com, 2013; About Kadcyla, 2017). Kadcyla is composed of an antibody (Trastuzumab, Herceptin) against the HER2 receptor on the surface of some types of breast cancer cells conjugated to the cytotoxic drug DM1 (Barok et al., 2014; Jain et al., 2015).

Adcetris® is used to treat CD30-positive lympho-proliferative disorders, including Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL) (Van de Donk and Dhimolea, 2012; Brentuximab vedotin, 2016). CD30 often occurs on the surface of cells of these tumor types, but rarely on normal cells (Küppers and Hansmann, 2005). Adcetris (**Figure-6**) contains an antibody (Brentuximab or cAC10) against CD30 conjugated to 3-5 molecules of the drug monomethyl auristatin E (MMAE) using a cathepsin-cleavable linker (Van de Donk and Dhimolea, 2012).
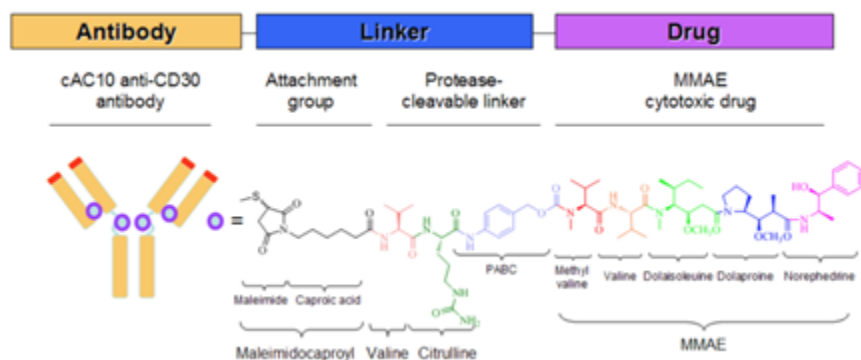
Figure-6: Structure of the ADC Drug Adcetris. This antibody-drug conjugate consists of a mouse-human chimeric monoclonal antibody against CD30 (yellow) linked via a cathepsin-cleavable linker (blue) to 3-5 units of the cytotoxic drug monomethyl auristatin-E (MMAE) (purple) that strongly binds tubulin and blocks cell division (Francisco et al., 2003).

More than 40 ADCs are in the clinical trial stages of development (Thomas et al., 2016), and the future of ADCs seems bright. However, ADCs are not perfect. Sometimes an ADC drug shows strong pre-clinical data in mice, but this strong performance does not always carry over to the human clinical trials. In addition, all ADCs produce adverse side-effects (although they appear to be mostly manageable, and the side-effects pale in comparison to the poor patient prognosis for untreatable cancer). And ADCs show varying levels of effectiveness. Therefore, it is necessary to continue developing improved ADCs that are more effective with fewer side-effects.

First-Generation Versus Second-Generation ADCs

Over the years, the design of ADC drugs has improved significantly (Thomas et al., 2016). Early ADCs contained mouse monoclonal antibodies (mAbs) against the target antigen, but injecting a mouse antibody into a human patient often stimulated immune rejection (or lowered half-life) of the drug (Teicher and Chari, 2011). Early ADCs also had short half-lives in the blood, releasing their toxic payload into the bloodstream instead of the cancer cell. And the cytotoxic payloads (such as doxorubicin, vinblastine, or methotrexate) were not very potent, requiring the internalization of multiple ADCs per cell.

Second-generation ADCs contain mouse-human chimera antibodies or fully humanized antibodies that produce less of an immune rejection in the patients. An example of an ADC with a fully

human antibody is CDX-011 (Keir and Vahdat, 2012). In addition, ADC linkers have been designed to be specifically cleaved by proteases or the acidic environment inside the endocytic vesicle, releasing the toxic cargo only inside the cancer cell. Moreover, newer ADCs use highly potent payloads (such as Calicheamicin, Maytansine derivatives like DM1, or Auristatins like MMAE) that are far more toxic (Lopus, 2011). These highly potent drugs have IC_{50} values in the nano-molar range compared to the micro-molar range for first-generation drugs, so they have the same cell-killing effectiveness at 1000-fold lower concentrations.

ADC Clinical Trials

ADC clinical trials have been performed on a variety of target antigens, including some of the examples shown in **Table-III**.

Table-III: Example ADC Clinical Trials

Target Antigen	ADC Drug	Trial Type	References
CD19	ADC SAR-3419	Phase-I	Younes et al., 2009 Coiffier et al., 2011
CD22	Inotuzumab ozogamicin CAT-8015 Moxetumomab pasudotox	Phase-I	Advani et al., 2010 Kreitman et al., 2012
		Phase-II	Kantarjian et al., 2012 Wagner-Johnson et al., 2015
CD30	Brentuximab vedotin Adcetris®	Phase-I	Seattle Genetics, 2010 Younes et al., 2010 Younes et al., 2013
		Phase-II	Younes et al., 2012 Pro et al., 2012
CD33	Gemtuzumab ozogamicin, Mylotarg®	Phase-II	Daver et al., 2016
		Phase-III	Castaigne et al., 2012 Petersdorf et al., 2013 Hills et al., 2014
Her2	Kadcyla® Trastuzumab emtansine T-DM1	Phase-I	Krop et al., 2010
		Phase-II	Burris et al., 2011 Perez et al., 2014 Phillips et al., 2014
		Phase-III	Verma et al., 2012 Krop et al., 2014
gpNMB	Glembatumumab vedotin CDX-011	Phase-I	Hamid et al., 2010 Bendell et al., 2014

	(formerly CR011-vcMMAE)	Phase-II	Yardley et al., 2015
Trop-2	Sacituzumab govitecan IMMU-132	Phase-I	Starodub et al., 2015 Faltas et al., 2016
EGFR	Depatuxizumab Mafodotin (ABT-414)	Phase-I	Reardon et al., 2017 Gan et al., 2018
DLL3	Rovalpituzumab	Phase-I	Rudin et al., 2017

The ADC clinical trials performed to date show a wide range of results, from complete remissions to no response (Sassoon and Blanc, 2013). But some of the trials can be called spectacular successes, including the 46% complete remissions seen with a CD22-targeting ADC (Kantarjian et al., 2012), and the 95% complete remission (21 of 22 patients) seen for CD30-targeting Adcetris (Pro et al., 2012). Side-effects occurred in most trials, but they were relatively mild and treatable, and should be considered a necessary risk for these recurring untreatable cancers. Neutropenia (low neutrophil count) was almost always observed, but it was not fatal. Other common side-effects (as with Adcetris) were: Grade 3 or 4 (serious) adverse events of neutropenia (21%), thrombocytopenia (14%), and peripheral sensory neuropathy (12%) (Pro et al., 2012).

ADC Side-Effects

Most of the clinical trials performed with ADC drugs showed some side-effects caused by the treatments. Examples of the side-effects are shown in **Table-IV**:

Table-IV: Example ADC Side-Effects

Trial Type	Reference	Summary of Reported Side-Effects
Phase-I	Younes et al., 2009	Occular problems (such as blurred vision), but no other clinically significant toxicities.
Phase-I	Coiffier et al., 2011	Ocular toxicity, but the incidence (2%) and severity were low. The hematological toxicity was insignificant.
Phase-I	Advani et al., 2010	Common adverse effects were thrombocytopenia (decrease in platelets) (90% of patients), asthenia (weakness) (67%), nausea (51%), and neutropenia (decrease in neutrophils) (51%).
Phase-I	Kreitman et al., 2012	At the doses used, no dose-limiting toxicity was observed. Minor side-effects (seen in 25-64% of the patients) included: hypo-albuminemia (low serum albumin), aminotransferase elevations (mild liver damage), edema, headache, hypotension, nausea, and fatigue.
Phase-II	Kantarjian et al., 2012	The most frequent adverse effects were: fever (41%), hypotension (26%), and grade 1-2 liver problems (24%). Two patients died within 4 weeks of starting treatment, but it was not clear whether the deaths resulted from the treatment or the cancer.

Phase-II	Wagner-Johnson et al., 2015	Common grade 3 or 4 side-effects during the R-INO portion of the treatment included: thrombocytopenia, lymphopenia, and neutropenia.
Phase-I	Younes et al., 2010	The most common side-effects were fatigue, pyrexia, diarrhea, nausea, neutropenia and peripheral neuropathy.
Phase-II	Younes et al., 2012	The most common treatment-related adverse effects were peripheral neuropathy, nausea, fatigue, neutropenia, and diarrhea.
Phase-II	Pro et al., 2012	Grade 3 or 4 adverse events included neutropenia (21%), thrombocytopenia (14%), and peripheral sensory neuropathy (12%)
Phase-I	Younes et al., 2013	Adverse events were generally grade 1 or 2, but occurred in 41% of all patients.
Phase-III	Castaigne et al., 2012	Persistent thrombocytopenia (16%).
Phase-III	Petersdorf et al., 2013	None reported.
Phase-III	Hills et al., 2014	Doses of Mylotarg at 3 mg/m^2 were associated with fewer early deaths than the higher dose of 6 mg/m^2.
Phase-II	Daver et al., 2016	The most frequent side-effects observed were nausea, mucositis, and hemorrhage.
Phase-I	Krop et al., 2010	The most common drug-related adverse events were thrombocytopenia, elevated transaminases, fatigue, nausea, and anemia. No serious cardiac events that would have required drug lowering were observed.
Phase-II	Burris et al., 2011	The drug appeared to be well tolerated; the most frequent side-effects were only at grade-1 or -2 (mild). Observed grade-3 (serious) problems included hypokalemia (lowered serum potassium levels) (8.9%), thrombocytopenia (8.0%), and fatigue (4.5%), although these were observed only in a small minority of patients.
Phase-III	Verma et al., 2012	Grade-3 (serious) adverse events decreased from 57% to 41%. Thrombocytopenia and liver damage were higher with Kadcyla, while diarrhea, nausea, vomiting, and erythrodysthesia were higher with the chemotherapy.
Phase-III	Krop et al., 2014	The Kadcyla group showed higher incidence of thrombocytopenia (5% versus 2%), but had lower incidence of neutropenia and diarrhea.
Phase-II	Perez et al., 2014	No major events reported.
Phase-II	Phillips et al., 2014	Tested a combined treatment which caused only mild grade-1 and -2 adverse events which were treatable.
Phase-I	Hamid et al., 2010	No major events reported.

Phase-I	Bendell et al., 2014	Initially, the maximum tolerated dose (MTD) was determined to be 1.34 mg/kg (limited by the patient's worsening neuropathy), but the MTD was increased to 1.88 mg/kg (their formal Phase-II dose) after eliminating patients with baseline neuropathy.
Phase-II	Yardley et al., 2015	The ADC drug showed less hematologic toxicity than the chemotherapy, but produced more rashes, pruritus (itching), neuropathy, and alopecia. The authors concluded that the ADC was well tolerated.
Phase-I	Starodub et al., 2015	The MTD was determined to be 12 mg/kg for one cycle of treatment, but that dose could not be continued for additional cycles due to the formation of neutropenia. After extended treatments at a lower dose of 10 mg/kg, no level-4 (serious) adverse events were observed, and grade-3 toxicities were fatigue, neutropenia, diarrhea, and leukopenia.
Phase-I	Faltas et al., 2016	The drug was well tolerated.
Phase-I	Rudin et al., 2016	At a treatment amount of 0.8 mg/kg administered every three weeks, grade 4 thrombocytopenia was observed in two patients. Grade 3 adverse effects that were noted include thrombocytopenia (11% of individuals), pleural effusion (8%), and increased lipase (7%). The MTD noted was 0.4 mg/kg every 3 weeks.
Phase-I	Reardon et al., 2017	The most common adverse effects noted were blurred vision, dry eyes, keratitis, photophobia, and eye pain. MTD was determined to be 2.4 mg/kg.
Phase-I	Gan et al., 2018	The most common adverse effects noted were ocular related, occurring in 92% of patients. Keratitis was the most observed adverse effect. The MTD was determined to be 1.25 mg/kg.

ADC Problems and Future Directions

Although ADC drugs show great promise, they are not perfect. The continued approval of more ADC drugs by the FDA will likely require continued improvements in their targeting and efficacy (Panowski et al., 2014). Thus, there is always room for ADC improvement.

ADC drugs are complex, requiring a number of key steps to be effective. The disruption of any of these steps can lower the effectiveness of the drug (Loganzo et al., 2016). The ADC must travel through the circulatory system without losing its toxic cargo. It must bind the tumor cell without targeting normal cells. It must be internalized into the cell using the correct vesicle which either degrades the linker or degrades the entire complex, releasing the drug into the cytoplasm. The cytotoxic drug must correctly localize to the proper cellular compartment (nucleus for DNA damaging agents, microtubules for tubulin-binding drugs). A response by a tumor cell to alter any of these key steps can lower drug effectiveness. Thus, there is room for improvement in each of these processes.

For example, in the situation of a tumor cell that has down-regulated the expression of the target antigen, thus evading the ADC, the treatment strategy could be altered to include a different ADC that targets a different antigen on the same tumor cell (Loganzo et al., 2015). Or alternatively, if a tumor cell over time mutates its DNA to where the gene encoding tubulin expresses a product that no longer binds DM1 or MMAE cytotoxic drugs (Kavallaris, 2010; Gillet and Gottesman, 2010; Holohan et al., 2013), then perhaps switching to an ADC that kills by a different mechanism would help. And with respect to

drug movement from the vesicle into the cytoplasm, in some cases the drug is carried outside the cell by drug transporter molecules such as MDR1 and MRP1, decreasing drug effectiveness (Chen et al., 2015; Yu et al., 2015). Perhaps this problem could be overcome by co-treating with a drug to lower MDR1 or MRP1 expression.

The following topics were identified in our review of the ADC literature as potential future directions:

1) Cargo Switching: In some cases, a patient's tumor can become resistant to the cytotoxic drug used in a therapy, so perhaps switching to a different ADC that targets the same antigen but contains a different cytotoxic cargo that works by a different mechanism might improve effectiveness. This switching approach has successfully been used in a mouse model of non-Hodgkin lymphoma (NHL), where altering the CD22-targeting ADC payload from MMAE (which blocks tubulin polymerization, preventing cell division) to nemorubicin (which targets DNA) overcame the resistance (Yu et al., 2015).

2) Closely Monitoring Target Antigen Expression: One of the best practices observed in the clinical trials was the constant monitoring of target antigen expression by the patient's tumor cells. In some cases, the tumor down-regulates antigen expression, so any targeted therapy (such as an ADC) no longer targets those cells. The best clinical response rates were observed for patients still expressing the target antigen. Antigen expression can be monitored by IHC (immuno-histochemistry), or RT-PCR (reverse transcriptase polymerase chain reaction).

3) Dual-Targeted Therapies: This approach uses two different ADCs targeting different antigens on the same tumor. This strategy would allow continued targeting, even in those cases where the tumor cells no longer express one of the target antigens.

4) New ADC Conjugation Reactions: Chemical conjugation reactions are used to link the antibody to the drug cargo. Early-generation ADCs used conjugation reactions that could add drug molecules onto any site on the antibody containing a reactive amino acid (Panowski et al., 2014; Agarwal et al., 2015). But these reactions added the drugs randomly onto the antibody, producing a heterogeneous mixture of ADC molecules, each with their own activities. And each production batch varied in composition, making it difficult to compare clinical trial data (Panowski et al., 2014; Agarwal et al., 2015). Newer methods of conjugation allow the drug to be added to specific sites, helping eliminate heterogeneity. The newer methods include the use of engineered cysteine residues, and the use of non-natural amino acids.

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Lit Review Section-2:
Dendritic Cell Vaccines
Leila Camplese

Half of the 2011 Nobel Prize in Physiology or Medicine went to Ralph M. Steinman for his "discovery of the **dendritic cell** and its role in adaptive immunity" (Nobel Prize, 2011). As mentioned previously in the Introduction to Immunology section, dendritic cells (DCs) are "professional" antigen-presenting cells that reside in tissues that contact the external environment (skin, lining of the respiratory tract, nasal epithelium, etc.). Their main function is to recognize foreign antigens on the surface of invading pathogens, process the antigen within the DC, present the antigen on the cell surface, then migrate to the lymph nodes to present the antigen to other cells of the immune system (T-cells and B-cells) (Steinman and Cohn, 1973; Banchereau and Steinman, 1998; Sallusto and Lanzavecchia, 2002; Trombetta and Mellman, 2005). These latter immune cells then differentiate and commit to that antigen to help eliminate the threat.

In the case of cancer, the tumor cells themselves are poor antigen-presenting cells, so DCs help facilitate the tumor removal by presenting their antigens to the immune system to induce a response to the tumor. Animal experiments have shown that DCs are a required component of the body's immune attack against cancer. When a tumor forms in the body, DCs help process the tumor's neo-antigens and present them to the immune system to generate active B-cell and T-cell responses against the tumor (Palucka and Banchereau, 2012).

With respect to tumor vaccines, the antigen-presenting properties of DCs are sometimes used to "prime" a patient's DCs against a single antigen, or mixture of antigens (**Figure-7**). In a common *ex vivo* approach, a patient's DC cells are isolated from peripheral blood mononuclear cells (PBMCs) using various techniques, and are cultured to expand their numbers. The isolated DCs are then "pulsed" or "primed" (mixed) with foreign tumor antigen (purified antigen or entire tumor cells themselves) (dagram upper left), and the pulsed DCs are injected back into the patient. Hopefully, these primed DCs migrate to the lymph nodes to engage B-cells and T-cells (diagram right) to commit them against the tumor antigen (Davis et al., 2003; Steinman and Banchereau, 2007; Koski et al., 2008; Schuler, 2010; Ueno et al., 2010). In a less used *in vivo* approach, DCs in the patient's body are induced to take up tumor-specific antigens, and the antigen-presentation is done naturally to stimulate the patient's T-cells. This latter process does not involve purification of the DCs nor their *ex vivo* expansion.
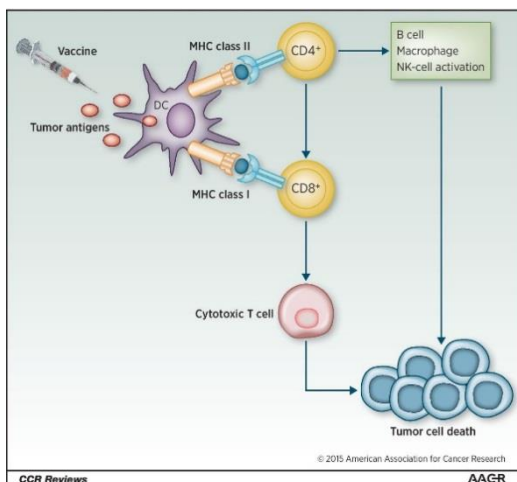
Figure-7: Diagram of a Typical DC Vaccine. In this approach, dendritic cells (DCs) (purple) are primed with tumor antigens (diagram upper left). The priming can be performed *in vitro* or *in vivo*. The activated DC cells migrate to nearby lymph nodes and present the antigens bound to MHCs (light brown). MHC-I presents to T-cell receptors (blue) on CD8+ cells (yellow), while MHC-II presents to T-cell receptors on CD4+ cells (also yellow). CD4+ helper T-cells produce cytokines that promote CD8+ T-cell maturation. CD8+ cytotoxic T-cells leave the lymph node into the circulation where they recognize the tumor cells expressing the antigen, killing the cell. Figure from Anagnostou and Brahmer, 2015.

Example Studies with DC Vaccines

The first DC vaccine approved by the FDA was **Provenge** (Ledford, 2015). This vaccine is also called Sipuleucel-T or APC8015. Provenge is directed against prostate cancer, the second leading cause of cancer death in men following skin cancer. Researchers chose prostate cancer, because so many men have it, and also because men can live without a prostate so if the vaccine accidently targeted normal prostate cells it would not be fatal (Ledford, 2015).

Much research went into the development of this vaccine. A patient's peripheral blood mononuclear cells (PBMCs) (including DCs) are isolated by leukapheresis, and cultured to increase their numbers. They are then mixed *in vitro* with a patented recombinant fusion protein (PA-2024). PA-2024 used to prime the DCs contains the target antigen prostatic acid phosphatase (PAP) fused with granulocyte macrophage colony stimulating factor (GM-CSF) which helps activate the immune system. The primed DCs are then injected back into the same patient.

The clinical results of Provenge (**Table-V**) are mixed, showing some successes, followed by an underwhelming response. Early experiments demonstrated that CTLs had been formed against the tumor cells, and that PSA levels dropped, but the survival statistics were relatively unimpressive compared to controls, for example increasing from 4.9 months survival before treatment to 7.9 months after treatment (Beinart et al., 2005), but few of the clinical trials showed complete remissions. Despite Provenge's FDA approval, the company who developed it (Dendreon Corp.) went bankrupt. Dendreon was hurt by the long wait for FDA approval for an early cancer vaccine (18 years), by confusion over Medicare reimbursements, and by lukewarm results (Ledford, 2015). In 2015, the rights to Provenge were purchased by Valeant Pharmaceuticals.

As with any clinical treatment, there are several risks associated with Provenge. Many of them are clearly listed on their website as acute infusion reactions, including (but not limited to): fever, headache, nausea, and joint pain (Dendreon Pharmaceuticals, 2017). More rarely the patients can experience more severe side-effects, such as chest pain, vomiting, stroke, and thrombosis. 1.5% of the participating patients backed out of the clinical trial upon experiencing these side effects, however this number is so low that it would not have a significant impact on the study results (Dendreon Pharmaceuticals, 2017).

Melanoma is another cancer treated with DC vaccines. In these cases, the patient's DC cells were either pulsed with tumor lysate or were pulsed with a mixture of melanoma peptides identified from animal studies. Some of the melanoma trials produced stronger data than obtained with Provenge. DC vaccines have also been used to treat glioblastomas (GBMs), a particularly devastating cancer with a median survival time of less than 2 years (Johnson and O'Neill, 2012). **Table-V** below shows examples of experiments with DC vaccines.

Table-V: Example Experiments with DC Vaccines

Cancer	Notes	Side-Effects	Reference
Prostate Cancer	Phase-I study. Autologous DCs pulsed with HLA-A0201-specific prostate-specific membrane antigen (PSMA) peptides. A decrease in PSA was observed only in the group receiving DCs pulsed with peptide P2.	No significant toxicity was observed for any of the treatment groups.	Murphy et al., 1996

Melanoma	Phase-I. Regression of metastases was observed in 5 of the 16 patients.	The vaccinations appeared to be well tolerated, and did not generate any visible autoimmune responses.	Nestle et al., 1998
Prostate Cancer	Phase-I/II. 100% of the patients showed an immune response against the priming PA-2024.	The most common side-effect observed was fever (14.7% of the patients).	Small et al., 2000
Prostate Cancer	Phase-I. Circulating levels of PSA dropped in 3 of the 13 treated patients.	The patients experienced mild grade-1 and -2 side effects, such as fever, chills, myalgia, pain, and fatigue.	Burch et al., 2000
Melanoma	Phase-I. 2 of the 14 patients showed anti-tumor responses, including regression of metastasis.	1 patient developed vitiligo, but that skin discoloration was minor and treatable.	Mackensen et al., 2000
Melanoma	Phase-I. 9 of 12 patients developed CTLs capable of destroying melanoma cells.	The vaccine seemed to be well tolerated, except 2 patients showed progressive vitiligo (skin discoloring)	Banchereau et al., 2001
Glioblastoma	Phase-I. 4 out of 7 evaluated patients showed sustained anti-tumor CTL responses. Median survival time of the DC group was 455 days compared to 257 days for the control group.	No serious side effects were seen.	Yu et al., 2001
Prostate Cancer	Phase-II. 2 of the 21 patients showed a 25-50% drop in PSA levels. One patient dropped PSA to undetectable levels and resolved his cancer.	Most of the side-effects were grade-1 and -2, with only 4 of the 21 patients showing grade-3 or 4 side-effects.	Burch et al., 2004
Glioblastoma	Phase-I. The DCs were primed with tumor lysates from the same patient. 6 of 10 evaluated patients showed robust T-cell responses against the tumors.	The vaccines appeared to be well-tolerated, and no evidence of autoimmune disease was seen.	Yu et al., 2004
Glioblastoma	Phase-I. 1 out of 12 patients showed a clinical response (improved MRI). 6 had measurable anti-tumor CTL responses, but those did not translate into clinical responses or prolong patient survival.		Liau, et al., 2005
Prostate Cancer	Phase-II. 13 of the 18 patients slowed the rate of increase of their serum PSA levels.	None noted.	Beinart et al., 2005
Melanoma	Phase-I. 67% of the patients showed CD8+ cells reactive against the priming peptide G280, and 9 of 9 patients tested had T-cells that were able to lyse tumor cells in vitro. 3 of the 9 patients tested showed stable disease, and 2 showed partially stable disease.	None reported.	Linette et al., 2005

Melanoma	Phase-I. 11 of the 15 showed enhancement of immune responses against the target antigens. 2 out of 9 evaluable patients showed clinical responses, one showed complete cancer regression, the other one showed disease stabilization.		Salcedo et al., 2006
Prostate Cancer	Phase-III. 147 patients treated with Provenge, and 78 placebo. Found an average 33% reduction in death for the patients receiving Provenge versus the placebo (p=0.011).	The most common Provenge-induced side-effects were all grade-1 or 2 (mild), lasting only 1-2 days. They included: chills, pyrexia (fever), headache, asthenia (weakness), dyspnea (labored breathing), vomiting, and tremor.	Higano et al., 2009
Melanoma	Review. 626 patients. Improved clinical responses correlated with the use of peptide antigens to pulse the DC cells (p=0.03), the use of adjuvant (p=0.002), and the induction of antigen-specific T-cells (p=0.0004)		Engell-Noerregaard et al., 2009
Prostate Cancer	Phase-III. 341 patients treated with Provenge, and 171 placebo. Double-blind, placebo controlled, multicenter trial. Provenge patients had an average 22% reduction of death, with survival extending from 21.7 months to 25.8 months.	Adverse effects included chills, fever, and headache.	Kantoff et al., 2010
Prostate Cancer	Summary of 17 Provenge trials. Stable disease was observed in 54% of the Provenge patients. High DC doses significantly correlated with clinical benefit.		Draube et al., 2011
Melanoma	Phase-I. Compared intra-nodal and intra-dermal vaccinations. All of the intradermal vaccinated patients showed beneficial DC migration to the nodes, compared to no migration for 7 of 24 intra-nodal patients.		Lesterhuis et al., 2011
Glioblastoma	Phase-I. 23 patients with grade-4 GBM were vaccinated with tumor lysate-pulsed DCs accompanied by adjuvant. The vaccines produced a median survival time of 31.4 months, compared to less than 24 in controls.	The vaccines appeared to be well tolerated.	Prins et al., 2011
Glioblastoma	Phase-I. This was the first study to treat recurrent human malignant gliomas with a combination of αDC1 cells and adjuvant. Of 19 evaluable patients, 58% had positive immune responses against the target antigen. 9 patients had no sign of tumor progression for at least 12 months, and 1 patient showed sustained complete remission.	The vaccines were well-tolerated.	Okada et al., 2011

Melanoma	Phase-I. 6 of the 7 patients showed sustained anti-tumor T-cell responses. 1 patient showed complete remission, and 2 showed partial remission. Strong improvements correlated with with DC cells producing IL-12, and for patients with strong T-cell responses.		Carreno et al., 2013
Glioblastoma	Pre-Clinical. Pulsing DC cells with glioma *stem cell* lysate (these cells often re-seed the tumor) better forms anti-tumor T-cell responses than non-stem cell primed DCs.		Ji et al., 2013
Melanoma	Phase-I. Used new exome sequencing technologies to identify neo-antigens present in the tumor cells, not normal cells. Bio-informatics was used to identify missense mutations likely to produce neoantigens. They selected about 7 neo-antigens per patient, synthesized them chemically, then charged the DC cells. T-cell responses were generated against some, but not all 7 of the neoantigens, indicating they are not equal in their ability to induce an immune response.		Carreno et al., 2015
Glioblastoma	Phase-I. Patients were vaccinated with DCs primed against cytomegalovirus phosphoprotein-65. Patients also receiving an adjuvant of tetanus toxoid Td (to generally boost the immune system) showed a greater DC migration to the desired location (vascular draining lymph nodes) than patients without adjuvant.		Mitchell et al., 2015
Metastatic Melanoma	Melanoma patients sometimes develop resistance to immunotherapies via an immune suppressive tumor microenvironment. Here, the authors demonstrated in mouse models that macrophages (MOs) and dendritic cells (DCs) are suppressed in metastatic melanoma, and that peptide C36L1 can restore MO and DC function, including inhibiting metastatic growth in lungs. The C36L1 treatment activates MOs, increase the immunogenic DCs, increase activated cytotoxic T-cells, and reduce the number of regulatory T-cells in metastatic lungs. The C36L1 peptide directly binds receptor CD74 on MOs and DCs, inhibiting MIF signaling.		Figueiredo et al., 2018
Colon Carcionma	In this study, the authors investigated the use of stereotactic body radiation therapy (SBRT) as a method for facilitating the presentation of tumor-associated antigens (TAA) to immature dendritic cells (iDCs). They tested their method on mouse xenograft models of CT-26 colon carcinoma with 3 groups of mice: 1) radiation therapy alone (RT), 2) intra-tumor		Choi et al., 2018

	injection of DCs electroporated with tumor antigens (DC), or 3) the combination (RT/iDC). The data showed that the radiation method achieved the best DC priming and T-cell activation compared to other priming methods, and that mouse survival was highest in the combination treatment group. The authors conclude that clinical trials are warranted.		
Glioblastoma	Phase-III trial of 331 patients with glioblastoma. The standard therapy for glioblastoma includes surgery, radiotherapy, and oral chemotherapy temozolomide. This study evaluated the addition of an autologous tumor lysate-pulsed dendritic cell vaccine (DCVax®-L) to standard therapy for newly diagnosed glioblastoma patients. After surgery and chemo-radiotherapy, patients were randomly assigned to two groups: 1) chemo + DC vaccine (232 patients), or 2) chemo + placebo (99 patients). But if the cancer recurred, the patients were allowed to receive the DC vaccine. Due to this cross-over design, nearly 90% of the patients received the DC vaccine, so patient survival relative to a placebo could not be determined. However, median overall survival 34.7 months, with a 3-year survival of 46.4%.	Only 2.1% (n = 7) of the patients had a grade 3 or 4 adverse event that was deemed at least possibly related to the vaccine. Overall adverse events with DC vaccine were comparable to standard therapy alone.	Liau et al., 2018
Lewis lung carcinoma and breast cancer cells	In some cases, the tumor microenvironment can inhibit the activation of the immune system to fight a tumor, including antigen-pulsed DC cells. The authors developed a system to determine whether exosomes (membrane vesicles) produced by LLC Lewis lung carcinoma or 4T1 breast cancer cells contribute to DC immune suppression. They found that exosomes from these tumors blocked the differentiation of myeloid precursor cells into DC cells, and inhibited the migration and maturation of DCs. The inhibitory response was partially blocked by treating with anti-PD-L1 antibody, suggesting this checkpoint inhibition was important to the inhibition.		Ning et al., 2018
Diffuse Instinsic Pontine Glioma	Phase-Ib clinical trial of 9 patients with diffuse intrinsic pontine glioma (DIPG), a lethal brainstem tumor in children. They tested autologous dendritic cell vaccines (ADCVs) pulsed with an allogeneic tumor cell-line lysates in newly diagnosed patients following radiation therapy. The DCs were prepared from monocytes obtained by leukapheresis. The authors found that their procedure boosted non-specific (KLH) (9/9 patients) and specific glioma immune anti-tumor responses (8 of 9	The DC vaccine administration was safe in all treated patients.	Benitez-Ribas et al., 2018

	patients) in PBMCs and in T-lymphocytes isolated from CSF.		
Glioblastoma	Glioblastoma (GBM) tumors strongly suppress the immune system. Checkpoint blockage vaccines (such as antibodies against PD-1) might be useful for overcoming the blockade, but some experiments suggest the checkpoint approach may not be sufficient. This team investigated the activation of DC cells as a supplement to anti-PD-1 therapy in mice. Their data shows that activating DCs (by stimulating the TLR3 receptor with poly(I:C) enhances the PD-1 anti-tumor response and increases survival in mouse models of glioblastoma. DC depletion experiments showed that DCs are required for the anti-tumor response. The authors conclude that increasing DC antigen presentation is important to the anti-tumor response, and that multi-modal immunotherapy strategies are important.		Garzon-Muvdi et al., 2018
Metastatic breast cancer	The authors investigated the use of chemotherapy agent Dasatinib as a supplement for a DC vaccine against metastatic breast cancer in mice. Their data showed that tumor volume deceased in the group receiving the combined treatment, but not in groups receiving single vaccines. Mouse survival was longest in the combined treatment group.		Song et al., 2018

DC Vaccine Future Directions

Overall, while some scientists argue that DC clinical trials have produced somewhat modest results so far, the data suggests some potential ways for improving efficacy:

1) Adjuvants: Some trials showed improved outcomes combining DCs with adjuvants such as poly(I:C) (Okada et al., 2011; Ammi et al., 2015), or with IL-12 hormone to boost the immune response (Carreno et al., 2013), or tetanus toxoid Td (Mitchell et al., 2015). For example, in the latter study, glioblastoma patients were pretreated at the site of the injection with a recall antigen (in this case, tetanus/diphtheria (Td) toxoid) with the intention of increasing the efficacy of tumor antigen-specific dendritic cells. Out of thirteen patients, all of them showed greater accumulation of DCs when given Td than those who were not pretreated. Three of these patients' glioblastomas halted progression and had extended survival time. The overall results of this study concluded that patients given a recall antigen pretreatment had generally increased survival times compared to patients treated with DCs alone (Mitchell et al., 2015). With further research, application of adjuvants under different types of circumstances may lead to significantly improved results.

2) Tumor Heterogeneity: Some experiments have shown that tumors are not homogenous, but instead are composed of different types of cells each expressing different neo-antigens. Each type of tumor cell is genetically distinct from its neighbors, meaning they are different regarding growth rates, metabolic pathways, and overall aggression, among other characteristics. Perhaps most importantly, certain types of cells may develop immunity to treatments while others do not. This situation would require several levels of treatment and decrease the chance that growth will be suppressed or that the tumor will regress on its own. In these cases, targeting only one antigen would not kill all the tumor cells, so perhaps targeting multiple antigens would improve efficacy.

3) Which DCs: DC cells are not all alike. Isolating DCs on the basis of surface antigen CD14 selects for immature cells, but it is not clear whether priming mature or immature DCs is most efficient. Expanding DCs with hormone Flt3L appears to enrich for a more mature population. Other investigators have had success using αDC1 cells (Andrews et al., 2008; Okada et al., 2011).

4) Patient Selection: Some patients respond better to DC vaccines than others, but it is not clear why. Experiments should be done on the tumor micro-environments from various patients to determine if differences there block immune responses in some patients. For example, the amount of TGF-beta present in the tumor can strongly affect the immune response (Derynck et al., 2001).

5) Combination Vaccines: To further improve vaccine efficacy, perhaps combinations of cancer vaccines could be tested, such as combining DC vaccines plus an immune checkpoint vaccine against CTLA. A clinical study was done to test this mechanism in 16 patients suffering from metastatic melanoma. The patients were administered the DC vaccine along with a dose of cytotoxic T lymphocyte-associated antigen 4 (CTLA4)-blocking antibodies. The purpose of adding the antibodies is that they provide a negative-feedback response to the body's immune system to promote the activation of T lymphocytes. The results of this study showed that four patients were entirely tumor-free within two to four years after the start of the treatment, with no observable relapse. One patient also had total lung metastasis regression after 4 months, as well as significant (55%) regression of a spinal mass. This suggests that the combination of both treatments has a better overall result than that of each treatment individually (Ribas et al., 2009). Other example combination vaccines are Garzon-Muvd et al., 2018 and Song et al., 2018.

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Lit Review Section-3:
TIL Cancer Vaccines

Michaela Hunter

In addition to cancer vaccines that use therapeutic antibodies or dendritic cells (the topics of previous sections), T-cells are also used as cancer vaccines. **T-cells** are a type of nucleated white blood cell that matures in the thymus (thus their name) (although some T-cells also mature in the tonsils) (Zhang and Bevan, 2011). There are several types of T-cells, including: helper (CD4+), cytotoxic (CD8+), memory, suppressor, mucosal associated, and gamma delta T-cells.

With respect to cancer vaccines, **tumor infiltrating lymphocytes (TILs)** are a mixture of T-cells that locate and infiltrate a tumor to help kill it. TILs isolated from patient tumors include CD4+ (helper T-cells) and CD8+ cells (cytotoxic T-lymphocytes, CTLs). The CD4+ cells secrete cytokines to activate the immune system, while the CTLs directly lyse the tumor cell. High levels of TILs in tumors are often associated with a better clinical outcome (Vanky et al., 1986). T-cell therapy is sometimes referred to as **adoptive cell therapy (ACT)** because T-cells are isolated from a patient, expanded *in vitro*, selected for particular T-cells targeting a specific antigen, and perfused back (adopted) into the same patient (reviewed in: Rosenberg and Restifo, 2015; Mayor et al., 2018; Saint-Jean et al., 2018). So, TIL or ACT therapy is a form of personalized medicine.

So far, TIL therapy can only be performed in a few large medical centers due to the highly specialized patient care required and the complexity of TIL culture. After isolation of a patient's TILs, and before their re-perfusion, the patient is often treated with high-doses of chemotherapy to deplete any remaining lymphocytes that could block the therapy. And sometimes interleukin-2 (IL-2) injections are given to increase survival of the perfused T-cells. One advantage of using TILs to fight cancer is that the *in vitro* expansion process can sometimes avoid the negative regulation T-cells encounter near the tumor site by checkpoint inhibition via PD-1, PD-L1, or CTLA-4 (discussed in a later section). So, the ability to grow and expand T-cells *in vitro* has been a major advance in the field of cancer therapy.

Examples of TILs and Cancer

Dr. Steven Rosenberg at the National Cancer Institute pioneered the use of TILs to fight cancer, especially melanomas (for a review, see Rosenberg and Dudley, 2009). His lab helped develop the procedures for isolating a patient's TILs and amplifying them *in vitro*. He also helped develop the procedure of chemoablation, the use of chemotherapy to deplete a patient's *in vivo* lymphocytes that can suppress TIL function, and then afterwards re-perfusing the therapeutic TILs into the patient. The TIL studies published so far on melanomas show an impressive clinical response rate of up to 50% with no side-effects, including a significant proportion of patients with durable complete response (Mayor et al., 2018). In addition to melanomas, TILs have also been used to fight epithelial and ovarian cancers. **Table-VI** below shows some example studies of treating cancer with TILs.

Table-VI: Example Studies Treating Cancer with TILs

Cancer Type	Notes	Side-Effects	Reference
Metastatic melanoma	9 of the 41 patients (22%) achieved complete or partial cancer remissions. Positive outcomes correlated with high TIL numbers.		Schwartzentruber et al., 1994 Rosenberg's team
Metastatic melanoma	Phase-I study, 10 patients. TILs were isolated against antigens MART1 and gp100 targets. The T-cells persisted in vivo at least 21 months (using supplement IL-2) and localized to tumor sites.	No serious toxicity was observed.	Yee et al., 2002
Metastatic melanoma	13 patients. All received chemoablation in advance and high dose IL2 therapy. 6 of 13 patients (46%) showed significant tumor regression.		Dudley et al., 2002 Rosenberg's team
Metastatic melanoma	2 of the 15 patients showed high levels of circulating TILs one year after injection, and observable tumor regression.		Morgan et al., 2006 Rosenberg's team
Metastatic Melanoma	Patients received pre-treatment chemoablation and/or total body irradiation, followed by TILs. 49% of the patients receiving the TILs and no irradiation had an objective response. Adding 2 Gy irradiation increased the response to 52%, and adding 12 Gy increased the response rate to 72%.		Dudley et al., 2008 Rosenberg's team
Metastatic melanoma	20 patients received chemoablation and TILs. 50% of the patients achieved an objective clinical response: 2 complete remissions, and 8 partial remissions.	Manageable toxicity.	Besser et al., 2010
Metastatic melanoma	93 patients, pre-treated with chemoablation and radiation. 20 of the 93 patients (22%) achieved complete tumor regression, and 19 of the 20 regressions remained negative for at least 3 years! The 3 and 5-year survival rates for the 20 remission patients were 100% and 93%, respectively.		Rosenberg et al., 2011
Metastatic melanoma	31 patients. Each received chemoablation and IL-2. 15 of the 31 (48.3%) showed an objective clinical response.		Radvanyi et al., 2012
Metastatic melanoma	19 patients, but only 13 completed the treatments. Each received chemoablation and IL2. 2 of 13 had complete responses, and 3 had partial responses. In addition, 4 patients had stable disease.		Pilon-Thomas et al., 2012
Metastatic melanoma	69 patients, 35 receiving TILs with no selection and 35 enriched for CD8+ CTLs. 12 patients receiving the unselected TILs responded to the therapy, while only 7 responded to the CD8+-enriched TILs, so the CD8 enrichment may not be worth the effort.		Dudley et al., 2013 Rosenberg's team

Metastatic melanoma	Used whole-exome DNA sequencing of tumor DNA followed by bioinformatics to identify potential neoantigens. They synthesized the neo-antigens synthetically, and tested their recognition by patient TILs. They identified neoantigens present in about 40% of long-term (5-years) survival patients.		Robbins et al., 2013 Rosenberg's team
Ovarian cancer	Designed a new digital DNA-based assay (termed QuanTILfy) to count TIL cells and assess their clonality (percent activity against various antigens). They demonstrated an association between higher patient TIL counts and improved patient survival		Robins et al., 2013 Bielas' team at the Fred Hutchinson
Metastatic melanoma	Used deep sequencing techniques to determine which cancer antigens the TIL cells recognized and whether any TILs expressed inhibitory receptors. Their data indicated that 6 of 6 analyzed tumors contained TILs positive for mutated neo-antigens, and all 6 contained TILs positive for negative immune receptors PD-1, LAG-3, and TIM-3, indicating that the TILs in their in vivo state were functionally impaired by the tumor. Thus, antibody therapy designed against the negative regulators might improve vaccine effectiveness.		Gros et al., 2014 Rosenberg's team
Epithelial cancer	Performed whole exome sequencing on TILs isolated from epithelial tumors, and showed the TILs specifically reacted against erbb2-interacting protein (erbb2ip). When treated with a TIL cell population where 25% were specific for erbb2ip, the patient showed a decrease in lesions and disease stabilization.		Tran et al., 2014 Rosenberg's team
Inflammatory breast cancer (IBC)	The authors measured the levels of proteins PD-L1 (checkpoint inhibitor) and CD20 (tumor marker) in 221 biopsies as potential biomarkers of patient outcomes. The presence of high levels of CD20+ TILs plus high levels of PD-L1+ TILs was an independent prognostic factor for patient disease free survival. The authors suggest pursuing the use of anti-PD-1 or anti-PD-L1 therapies in these patients.		Arias-Pulido et al., 2018
Advanced Melanoma	TILs were expanded from excised cutaneous or subcutaneous metastases and then infused into the patients who also received subcutaneous IL-2. 9 patients were treated (4 had stage-IIIC melanoma, and 6 had stage-IV melanoma). All but 1 patient had previously received at least 2 other treatments. The results showed 1 complete remission, 1 partial remission, 2 stabilizations, and 6 cancer progressions.	No serious adverse effects were reported.	Saint-Jean et al., 2018
Colorectal Cancer (CRC)	The purpose of the study was to help standardize the method of evaluating TILs in colorectal cancer (CRC) clinical trials, as the methods currently differ in each study. They analyzed 160 patients with Stage II or III CRC using a new method proposed by the International TILs Working Group in breast cancer that measured		Iseki et al., 2018

	the area occupied by mononuclear cells over the stromal area on H&E stained sections. They classified patients into high-TIL density and low-TIL density groups. Their results showed that the rates of relapse-free survival (RFS) and overall survival (OS) in the high-TILs group were significantly higher than those in the low-TILs group.		
Advanced Breast Cancer	The authors investigated whether the TIL scores taken from core needle biopsies (CNBs) represent those taken from resected specimens. They analyzed 220 matched pairs of CNBs and resected specimens, scoring stromal TILs on slides stained with H&E. The authors concluded that more than five CNB cores may accurately predict the TIL score of the entire tumor.		Cha et al., 2018
Metastatic Urothelial Carcinoma	The authors investigated the prognostic role of TIL levels on survival in patients with metastatic urothelial carcinoma (mUC) receiving platinum based chemotherapy. They analyzed 259 mUC patients, of which 179 (69%) had intense TILs, and 80 (31%) had non-intense TILs. The median overall survival was 15.7 months for the intense TIL group versus 6.7 months for the non-intense group (p < 0.001). The authors conclude that assaying TIL staining intensity (numbers) for mUC patients is clinically useful for patient risk stratification and counseling.		Huang et al., 2018

TIL Problems and Future Directions

The data with TIL therapy shows great promise. In some cases, the teams observed 50% tumor reduction in about half the patients (Dudley et al., 2008; Besser et al., 2010; Radvanyi et al., 2012; Pilon-Thomas et al., 2012), and in another study, 22% of the melanoma patients showed complete cancer remission even 3 years post-treatment (Rosenberg et al., 2011). Some studies noted a direct correlation between high TIL load and positive patient outcomes (Iseki et al., 2018; Huang et al., 2018), so future research should focus on methods for further amplifying the cells. And due to poor patient prognosis, the amplification process needs to be done quickly, so methods to speed amplification are important. In the case of ovarian tumors, they presented a large variety of neo-antigens, so future experiments should determine whether amplifying TILs targeting one neo-antigen is sufficient in these cases. In some cases, the patient's tumor is found to have very low or no detectable TILs, so future research should also focus on devising new methods for detecting rare TILs. Immune checkpoint inhibitors can sometimes be a problem with TILs. One study noted a high expression of negative regulator receptors PD-1, LAG-3, and TIM-3 on the isolated TILs, so some tumors may be expressing ligands that engage these inhibitory receptors inactivating the TILs. Future tests should be done with combination treatments of TIL cells plus an antibody against one of these inhibitory receptors.

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Lit Review Section-4:
CAR Cancer Vaccines

Talal Hamza

In addition to therapeutic antibody vaccines, DC vaccines, and TIL vaccines, are **chimeric antigen receptor vaccines** (CARs). CARs, also known as chimeric T-cell receptors, or chimeric immune-receptors, are a different type of T-cell vaccine than TILs in that the T-cells contain a *genetically engineered T-cell receptor* that confers at least two key properties to the T-cells: 1) binding affinity for the tumor, and 2) signaling properties to activate the T-cells to destroy the tumor (reviewed in: Pule et al., 2003; Lipowska-Bhalla et al., 2012; Curran et al., 2012; Lim and June, 2017; June et al., 2018; June and Sadelain, 2018). In this approach, an engineered CAR gene is delivered inside the patient's own T-cells *in vitro* using retroviral vectors, the CAR is expressed on the cell surface, and the engineered T-cells are delivered back into the same patient. CARs are typically engineered to have a monoclonal antibody-like affinity for a specific tumor antigen, so they do not rely on formal antigen presentation to recognize the antigen.

CAR structures have evolved over the years, and are based on the T-cell receptor (TCR) (**Figure-8**). The TCR (left panel) consists of extracellular alpha and beta domains associated with CD3 subunits. The T-cell becomes activated when the external TCR domains bind peptides presented by MHC on the surface of antigen presenting cells or tumor cells. The binding activates signalling via an intracellular CD3-zeta domain (red). Early CARs (second panel) consisted of antibody-like antigen-binding domains (antibody variable domains, turquoise), a hydrophobic transmembrane domain, and one intracellular CD3-zeta signaling domain (red) that becomes activated once the receptor engages the target antigen. Later generation CARs (third and fourth panels) added additional "co-stimulatory domains" (such as CD28 or 4-1BB) to help the T-cell divide *in vivo*. Thus, structurally, CARs combine the powerful properties of highly specific target antigen recognition (the antibody-like portion), co-stimulation to increase T-cell survival, and T-cell signaling activation to kill the cancer cell, all combined in a single engineered receptor molecule (Sadelain et al., 2009).
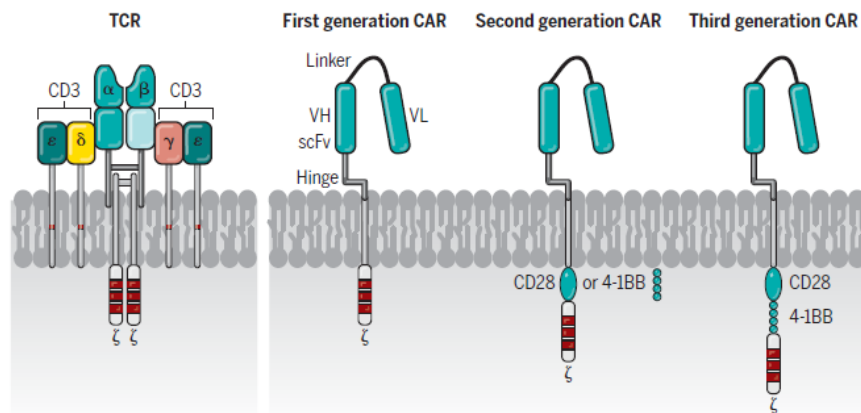
Figure-8: The Evolution of CAR Structures. Shown are the structures of a typical T-cell receptor (TCR) (left panel), first-generation (second panel), second-generation (third panel), and third-generation (fourth panel) CAR cells. Gray denotes the engineered T-cell. Turquoise represents the extracellular monoclonal antibody variable fragments that recognize the target antigen, gray represents the hydrophobic transmembrane domain, red denotes the cytoplasmic CD3-zeta stimulation domain, and turquoise ovals represent the cytoplasmic co-stimulatory domains such as CD28 and 4-1BB (that help the CAR cells divide and survive *in vivo*) (June et al., 2018).

The foundation of the CAR field was laid in the 1980's by Israeli immunologist Zelig Eshhar (reviewed in: Eshhar, 2014), and the technique was further refined by other big-name cancer researchers such as Steven Rosenberg (National Cancer Institute), Carl June (University of Pennsylvania), and Michel Sadelain (Memorial Sloan-Kettering Cancer Center). By far, the most successful application of CAR vaccines to date are those against CD19 for leukemia (reviewed in: Kochenderfer and Rosenberg, 2013; Jena et al., 2013; June et al., 2018). CARs against CD19 have provided some of the most striking successes in the entire cancer vaccine field because: 1) CD19 is universally expressed on the surface of all leukemic B-cells, 2) killing normal B-cells if they happen to express CD19 is not problematic (antibodies can be provided to the patient passively to compensate for the loss), and 3) CD19 is not expressed outside the B-cell lineage (so there is little off target killing with the vaccine). Early CAR experiments were disappointing, and focused on improving CAR design after realizing the injected T-cells quickly die unless they are co-stimulated *in vivo*. **Table-VII** below shows examples of clinical trials using CAR therapies.

Table-VII: Example Clinical Trial Experiments With CAR Therapies

Target Antigen	Cancer Type	Notes	Reference
	Metastatic melanoma	15 patients. The CAR cells survived in the peripheral blood for at least 2 months, and survived for at least one year in two patients who showed significant tumor regression.	Morgan et al., 2006 Rosenberg's team
α-Folate Receptor	Ovarian cancer	Phase-I, 14 patients, but none showed tumor reduction. PCR analysis showed that the engineered T-cells were present in the circulation within the first 2 days, but then quickly declined after one month. Perhaps the T-cells need a co-stimulator.	Kershaw et al., 2006 Rosenberg's team
G-250	Metastatic renal cell carcinoma	3 patients. Each patient developed liver toxicity so the treatment had to be discontinued. The patients continued to show progressive disease. Perhaps the target antigen is too widespread.	Lamers et al., 2006
CD20	Lymphoma	3 patients received CARs plus chemoablation to prevent inhibition of the CARs in vivo. The treatment was relatively well tolerated. Two patients became progression-free, with no measurable disease at 12 and 24 months. The third patient initially had a measurable remission, but relapsed at 12 months.	Till et al., 2008
CD19	Lymphoblastic leukemia	Mouse experiments. Designed a new type of CAR containing activation domains CD28, CD137 (previously unknown), and/or TCR-zeta. More than 85% of the treated T-cells expressed the engineered receptor, and the cells survived at least 6 months.	Milone et al., 2009 Carl June's team

CD19	Advanced follicular lymphoma	1 patient. **The first successful clinical treatment of leukemia using CD19 CARs**. Also used a CD28 co-stimulation receptor. The lymphoma underwent a dramatic regression, but was this due to the chemo or the CAR?	Kochenderfer et al., 2010 Rosenberg's team
HER-2 (Erbb2)	Colon cancer metastasized to the lungs and liver	Tested the vaccine on 1 patient, but he died 5 days after treatment from a "cytokine storm" (overly elevated cytokines). The authors speculated that a large number of perfused CARs localized to the lung cells (with low but sufficient HER-2) triggering cytokine release. The study also reminded the researchers that CARs are not cleared as fast as antibody treatments, so should be closely monitored.	Morgan et al., 2010 Rosenberg's team
CD19	Advanced chronic lymphocytic leukemia (CLL)	**Breakout year for CARs**. CD137 and TCR-zeta co-stimulators. The CAR cells expanded at least 1000-fold, migrated to the bone marrow, and continued to produce functional CARs for at least 6 months, well beyond the survival of earlier CARs. Each CAR cell was calculated to destroy about 1,000 cancer cells. **One CLL patient showed complete remission.**	Porter et al., 2011 Carl June's group
CD19	Advanced leukemia	3 CLL patients. **Two of the 3 patients showed complete remission, even 2 years later.** Normal B-cells expressing CD19 were also destroyed causing grade-3 and 4 B-cell aplasia, but the authors say this was treatable. These two 2011 studies were later attributed by Carl June as breaking open the funding for the entire CAR field.	Kalos et al., 2011 Carl June's group
CD19	Chronic lymphocytic leukemia (CLL)	Also received chemoablation. 8 of the 9 CAR patients tolerated the treatment well, and **3 of 4 evaluable patients showed a significant tumor reduction**.	Brentjens et al., 2011 Michel Sadelian's team
GD-2	Neuroblastoma	**Three of 11 patients with active disease achieved complete remission.**	Louis et al., 2011
CD19	Relapsed and refractory acute lymphoblastic leukemia (ALL)	2 children with very poor prognosis. The CARs expanded 1000-fold in vivo, and colonized the bone marrow and CSF. **1 of the 2 showed complete remission.** The other patient's tumor lost CD19 expression, so no response. The patients developed grade-3 and -4 adverse events, including a cytokine release syndrome, but those events were fully treatable with cytokine blockade antibodies.	Grupp et al., 2013 Carl June and Stephan Grupp's teams
CD19	Relapsed B-cell acute lymphoblastic leukemia (B-ALL)	5 patients. CD28 and CD3-zeta co-stimulatory receptors. **All 5 patients showed a rapid tumor eradication**, and have no residual disease as assayed by deep sequencing PCR, although one patient eventually relapsed. With respect to side-effects, some patients showed significant cytokine elevations, but those incidences were treatable with steroid therapy.	Brentjens et al., 2013 Michel Saedlain's team at Sloan Kettering

CD19	Chronic lymphocytic leukemia (CLL)	None of the patients received prior chemoablation. PCR showed CD19-CARs in the blood of 8 of the 10 patients. **3 of the 10 treated patients showed regressions** of their previously untreatable malignancies. One showed complete remission, while another showed tumor lysis syndrome as his leukemia regressed.	Kochenderfer et al., 2013 Steven Rosenberg's team
CD19	Refractory B-ALL	16 patients. 88% of the patients responded well enough to the therapy to later receive a "routine" allogenic stem cell transplant. They also identified C-reactive protein (CRP) as a predictor of which patients will develop severe cytokine release syndrome (sCRS), and showed they could be treated with corticosteroids and IL-6-receptor antibodies.	Davila et al., 2014 Michel Saedlain's team
CD19	Relapsed acute lymphoblastic leukemia (ALL)	**One of the most spectacular successes in all of cancer vaccine research. 27 of the 30 patients (90%) showed complete remission**, and 67% were event-free at 6 months. All 30 developed cytokine-release syndrome (CRS), but it was effectively treated with anti-interleukin-6 receptor antibody (tocilizumab), and the patients remained in remission.	Maude et al., 2014 Dr. Grupp's team, Children's Hospital, Philadelphia
CD19	Refractory B-cell cancers	Phase-I. 21 patients. CD28 and TCR-zeta co-stimulatory receptors. Pre-treatment chemoablation. The maximum tolerable dose (MTD) was determined to be 1 x 106 cells per kg, all the side-effects were reversible at that dose. The most severe side-effects were a grade-4 cytokine release syndrome observed in 3 of the 21 patients (14%).	Lee et al., 2015
HER-2	Refractory HER-2 Positive Sarcomas	Phase-I/II trial, escalating doses of HER2-CARs with a CD28 signaling domain. 19 patients. The cell infusions were well tolerated with no dose-limiting toxicity. The CARs persisted for at least 6 weeks in 7 of 9 patients who received a dose of greater than $1 \times 10E6$ cells per m^2. Of 17 evaluable patients, 4 had stable disease for 12 weeks to 14 months; 3 of these patients had their tumor removed, with one showing $\geq 90\%$ necrosis.	Ahmed et al., 2015
CD19	Refractory Multiple Myeloma	1 patient vaccinated after myeloablative chemotherapy (melphalan). CAR therapy lead to a complete response, with no evidence of cancer progression 12 months post-treatment.	Garfall et al., 2015
BCMA (B-cell maturation antigen)	Multiple Myeloma	First in-humans clinical trial of CARs against BCMA. 12 patients, various doses. Observed 1 partial remission at the 3rd dose level, and at the fourth dose level (9x10E6 CARs/kg body weight) for two patients, 1 showed undetectable cancer for 17 weeks then relapse, the other patient showed ongoing partial remission. The highest dose level-4 caused cytokine release syndrome in both patients, with fever, hypotension, dyspnea, and cytopenia.	Ali et al., 2016

CD22	Pre-B-Cell Acute Lymphoblastic Leukemia (B-ALL)	Phase-I trial, in 21 children and adults, including 17 who previously treated with CD19-directed immunotherapy. Complete remission was observed in 11 of 15 (73%) patients receiving more than 1 x 10E6 CD22-CARs per kg body weight. Median remission duration was 6 months. Relapses were associated with decreased CD22 expression.	Fry et al., 2018
CEACAM5	Advanced CEACAM5+ malignancies	The authors tested a first-generation CAR T-cell therapy. Patients were treated with Fludarabine pre-conditioning, followed by CEACAM5-CAR T-cells (various doses), followed by systemic IL2 support. But no objective clinical responses were observed. T-cell engraftment showed a rapid decline within 14 days.	Thistlethwaite et al., 2017
GD2	Diffuse Midline Gliomas (DMGs)	DMGs are aggressive and universally fatal pediatric brain cancers. The authors showed that patient-derived DMGs uniformly express high levels of GD2. Treatment of these cells with GD2-CARs *in vitro* showed strong GD2-dependent cytokine generation and cell killing. The treatment also cleared tumors from 5 patient-derived xenograft mouse models. The treatment was generally tolerated in mice, but neuro-inflammation occurred during the acute phase of anti-tumor activity resulting in hydrocephalus that was lethal in some animals. They predict that human DMG patients, given the neuro-anatomical location of the midline gliomas, will require careful monitoring and aggressive care management.	Mount et al., 2018
GD2 ganglioside	Neuroblastoma	The authors generated variant CARs to improve the stability and the affinity for the target. One variant (GD2-E101K) showed enhanced antitumor activity in GD2+ human neuroblastoma xenograft models, but it also caused lethal CNS toxicity, including brain CAR T-cell infiltration and proliferation, and neuronal destruction. The results highlight the challenges associated with target antigens also expressed on normal tissues. While GD2-targeted antibody therapies have shown some success against neuroblastoma, the fatal neurotoxicity of GD2-CARs suggests additional strategies are needed for controlling T-cell function in the brain.	Richman et al., 2018
CD19	Advanced Lymphoma	Clinical trial with 22 patients receiving a single dose of CAR-19 T-cells 2 days after a low-dose chemotherapy. The overall remission rate was 73% with 55% complete remissions and 18% partial remissions. Remission patients had a median peak blood CAR+ cell level of 98/μL compared to 15/μL without remission, and the high CAR levels associated with high serum IL-15 levels (P = .001) and remissions(P < .001).	Kochenderfer et al., 2017

CD19	Diffuse Large B Cell Lymphoma	KTE-C19 is an autologous CAR T-cell therapy against CD19 using CD3-zeta and CD28 co-stimulators. It was was used to treat 7 patients with refractory DLBCL. 1/7 patients 14%) experienced dose-limiting toxicity of grade-4 cytokine release syndrome (CRS), 1/7 patients (14%) showed grade >3 CRS, and 4/7 patients (57%) showed neurotoxicity. All >grade-3 events resolved within 1 month. 3/7 patients showed clinical responses (stabilizations) at 12 months. The regimen appears to be safe for phase 2 studies.	Locke et al., 2017

An assessment of the number of CAR clinical trials as of January of 2018 (June et al., 2018) identified 253 CAR trials worldwide (**Figure-9**). The vast majority of CAR trials are being conducted in China and the U.S. (left panel). Since China ranks 3rd in the world (behind North America and Europe) for total clinical trials (right panel), their #1 ranking for CAR trials is quite impressive in this area.
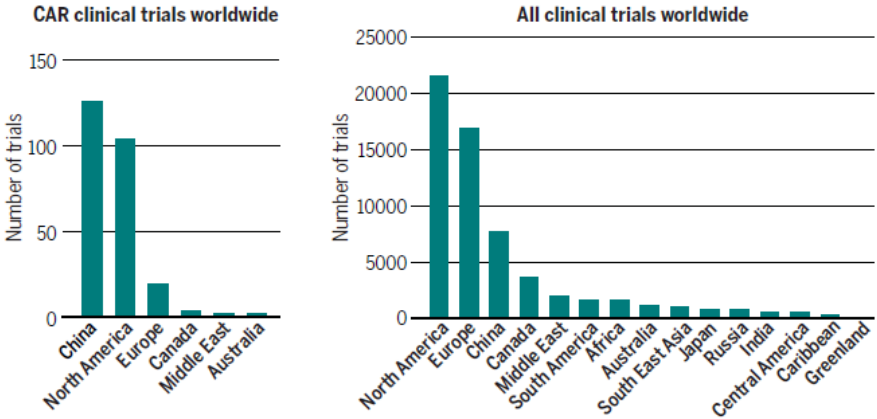
Figure-9: Assessment of CAR Clinical Trials Worldwide. The left panel shows the number of CAR clinical trials assessed as of January of 2018 (total of 253) compared to the total clinical trials in various countries (right panel) (June et al., 2018).

CAR Problems and Future Directions

Overall, CAR vaccines have shown some of the most spectacular successes in the entire field of cancer vaccine research. With so many advances and clinical trial successes, it's hard not to become excited. As stated in a review on the topic: "although some scientists are urging caution, it is hard not to be swept up in this moment. No cell therapy has proliferated in the body as well, endured so well, and slain cancer, quite like this therapy (Couzin-Frankel, 2013). The overall low number of patients treated so far likely will improve shortly with the exponential increase in the number of clinical trials. The use of second and third-generation CARs using a variety of activating and co-stimulating domains appears to have overcome the earlier problem of CAR cell death in vivo. And pre-treating the patients with chemotherapy to ablate the endogenous B and T-cells appears to have removed the inhibition against CAR expansion.

CRISPR-CAR Combination Therapy

One of the most promising and recent innovative uses of CAR cells combines CAR receptor engineering with the use of CRISPR cas9 to eliminate a host gene. One example showed that using Lentiviruses and CRISPR greatly increased the efficiency of CAR gene delivery to T-cells. The results showed CAR expression in human peripheral blood T cells and enhanced effectiveness (Eyquem et al., 2017), showing better results than typical CAR cells. And in another example, the CRISPR system has been used to eliminate the gene TRAC which is associated with allo-recognition, and its elimination might result in CAR cells that could be used universally in any patient (Georgiadis et al., 2018). CD-19 specific CARs were treated with Lentiviruses encoding guide RNAs and Cas9 against TRAC. The technique produced CAR cells that are far more homogenous than CARs produced using standard methods (Georgiadis et al., 2018). Furthermore, this combination method produced CAR T-cells with anti-leukemic effects that are longer lasting than conventionally produced CAR T-cells (Georgiadis et al., 2018). Ultimately, the combined use of CAR transfections and CRISPR Cas9 transfections is one of the next steps in CAR T cell therapy.

However, CARs also have problems, including causing patient death in some studies. This is especially true for CAR treatment of patients with acute leukemia. The following areas are worth pursuing in the future to make more effective CARs:

1) Side-Effects: The most serious adverse effect observed with CAR trials is patient death (Couzin-Frankel, 2016; Ledford, 2016). In 2016, 7 trial patients died over about a year due to the CAR therapy causing fatal brain swelling, and 5 of the 7 patients died in a single clinical trial. Most of the deaths occurred in adult patients with acute leukemia, and these patients tend to have the worst side-effects due to rapid expansion of their T-cells (Couzin-Frankel, 2016). The second most serious side-effect is cytokine release syndrome (CRS), a potentially deadly condition that can cause organ failure (Ledford, 2016). In a large trial of patients with aggressive non-Hodgkin's lymphoma, about 18% developed CRS (Ledford, 2016), however this syndrome was often treatable in the patients by blocking the effects of hormone IL-6 to rapidly reverse the fevers, hypotension, and hypoxia (June et al., 2018).

2) Loss of Target Antigen by the Tumor: The major mode of resistance of some tumors to CAR therapy is the loss of target antigen by the tumor. This is especially the case for patients with acute leukemia (June et al., 2018). This underscores the importance of constantly monitoring the patient's tumors throughout the entire procedure for target antigen expression, and if a down-regulation occurs, perhaps the patient could be treated with a CAR targeting a *different antigen* on the same tumor (if available). For example, for B-ALL leukemia patients whose tumors no longer expressed CD19, using a CAR targeting CD22 allowed remissions for at least 6 months (Fry et al., 2018).

3) Failure of the CAR cells to proliferate: The second most common mode of tumor resistance to CARs is their failure to proliferate in vivo. This is especially the case for patients with chronic cancer (June et al., 2018). Perhaps these patients would benefit from a combination therapy of CARs plus checkpoint inhibitor vaccine to activate the immune system. Or alternatively, a CAR using different co-stimulatory domains would allow CAR proliferation.

4) Correlates of Protection: Some of the studies done with TIL vaccines showed a positive correlation of a high number of TILs with the best patient prognosis. Is that also true for CARs? Is location important: Does the location of CARs in the bone marrow affect long-term survival and improve patient prognosis? Are TILs directed against a patient's neo-antigens (isolated from the patient's tumor) better than CARs directed against a single antigen, such as CD19?

5) Combination Therapies: TILs isolated from a patient's tumor can target multiple target antigens naturally, while artificially engineered CARs target one antigen. Perhaps it would be worth testing both CARs and TILs in one patient. Or perhaps a combination of a CAR therapy plus a checkpoint inhibitor treatment could be tested to help block the shutdown of T-cell activity.

6) Personalized Medicine: Neo-antigens are newly expressed proteins or sugars on the surface of tumor cells that form following DNA mutations in exons (coding DNA). Because neo-antigens are not expressed during development, they are viewed as foreign once expressed, and make good target antigens (Delamarre et al., 2015). The use of new rapid DNA sequencing methods allows a patient's tumor cells to be sequenced to analyze for neo-antigen formation (Robbins et al., 2013; Rajasagi et al., 2014; Schumacher and Schreiber, 2015), but the process needs to be *fast*, as some patients have a very poor prognosis (Kalos and June, 2013). For example, patients diagnosed with malignant melanoma in stage-IV can die within weeks.

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Lit Review Section-5:
Immune Checkpoint Vaccines

Cole Royer

Although some very exciting remissions have been achieved with cancer vaccines, as more patients have been tested over the years in clinical trials, scientists have come to realize that not all patients respond to the vaccines. So, recent research has focused on why some tumors are killed and others are not. One of the most exciting advances in this area is the discovery that T-cells that have migrated into a patient's tumor can sometimes become inactivated by immune checkpoint inhibitors (Hirano et al., 2005; Peggs et al., 2006; Topalian et al., 2012; Sznol and Chen, 2013; Cha et al., 2014; Herbst et al., 2014). Immune checkpoint inhibitors are receptors, such as **PD-1** and **CTLA-4**, on the surface of T-cells that bind to inhibitory ligands on other immune cells to inactivate them (reviewed in: Toplian et al., 2012). Under normal situations, this "checking response" is important for preventing immune hyper-activation, such as autoimmunity.

In the case of cancer patients, sometimes their tumors present the inhibitory ligands on their surface, blocking T-cell activation. So, in this case the immune checking response works against the patient by allowing the tumor to block anti-tumor responses (both native and vaccine) that can kill the tumor cell. Thus, much vaccine research has focused on using antibodies to block the checkpoint inhibitors (block the blockers), for example by using antibodies against PD-1 or CTLA-4 to remove the inhibition against the T-cells that have infiltrated the tumor. This **checkpoint inhibitor approach** provides a new and exciting approach for cancer vaccines that is different than other approaches, and is often used in combination with other therapies. A recent review article on checkpoint vaccines called the checkpoint therapy approach "arguably one of the most important advances in the history of cancer treatment" (Ribas and Wolchok, 2018).

PD-1 is inhibitory receptor "Programmed Cell Death-1" located on the surface of activated T-cells (Freeman et al., 2000; Ribas and Wolchok, 2018). Its ligand is "Programmed Cell Death-Ligand-1 (**PD-L1**), present on the surface of some tumors and on normal cells exposed to pro-inflammatory cytokines. When the PD-L1 ligand binds PD-1 receptor, a series of signal transduction events are activated that decrease T-cell function, such as decreasing T-cell migration and proliferation, restricting tumor cell killing, and increasing T-cell death (Herbst et al., 2014). **Figure-10** below shows how the PD-1 pathway works to the advantage of tumors. The **left panel** shows a cancer cell over-expressing PD-L1 protein (blue) on its surface bound to PD-1 receptor (turquoise) on a T-cell. This binding occurs as the T-cell receptor (dark brown) engages a tumor-specific antigen (yellow) bringing the two cells into close proximity (McCune, 2018). The right panel shows therapy using antibodies against PD-1 (red) or PD-L1 (yellow) preventing activation of the PD-1 checkpoint pathway, allowing the T-cell to remain active to kill the tumor cell (McCune, 2018; Abdin et al., 2018).
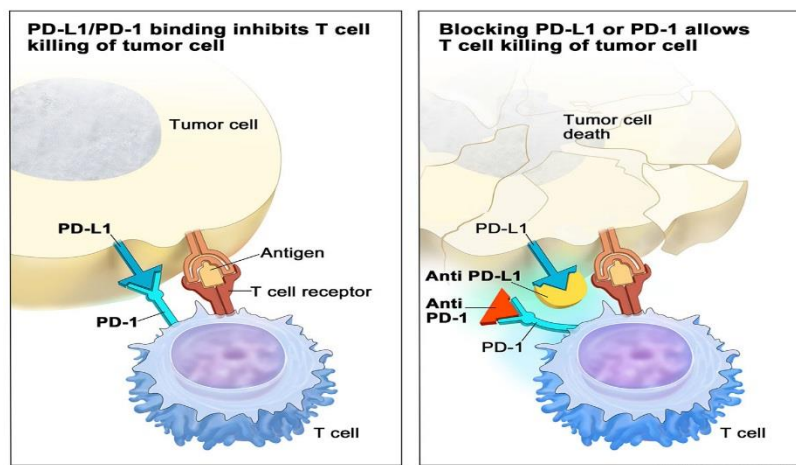
Figure-10: Biology of the PD-1 Receptor. The **left panel** illustrates T-cell inactivation when the T-cell is brought into close proximity to a tumor cell, the ligand PD-L1 (blue) on the surface of the tumor cell engages receptor PD-1 (turquoise). The **right panel** denotes therapy with antibodies against PD-L1 (yellow) or PD-1 (red) binding their respective receptors to prevent activation of the PD-1 inhibitory signalling pathway. From National Cancer Institute, 2016.

CTLA-4 is the "Cytotoxic T-Lymphocyte-Associated Protein-4" receptor present on the surface of T-cells. It was discovered in 1987 (Brunet et al., 1987), and when it is bound to its ligands CD80 or CD86 lowers T-cell activation (Walunas et al., 1994). Activation of the CTLA-4 pathway is often used by cancer cells to inactivate tumor-infiltrating lymphocytes (TILs) (reviewed in: Peggs et al., 2006; Cha et al., 2014). CTLA-4 has been demonstrated to have a potent inhibitory role in regulating T-cell responses. As opposed to inducing cell death like the PD-1 protein, CTLA-4 inhibits T-cell proliferation and activation upon binding its ligand (usually protein B7) by outcompeting co-stimulatory molecules like CD28 that are also trying to bind B7 (**Figure-11**) (Ribas and Wolchok, 2018). Working with a similar mechanism as PD-1, under normal conditions during immune activation, B7 on an antigen presenting cell (yellow) engages co-stimulatory receptor CD28 on a T-cell to help activate it. CTLA-4 (purple) is a break to this activation, moving to the cell surface during activation to outcompete CD28 for binding B7. Unfortunately, this T-cell inhibition pathway is sometimes used by cancer cells presenting B7 on their surface, leading to a silencing of the T-cell and evasion of the cancer from the immune system. Checkpoint therapy drugs for CTLA-4 (i.e. Ipilimumab antibody, red) bind CTLA-4 to block activation of the inhibitory pathway, preventing the tumor from silencing T-cell signaling. This allows the T-cells to act at their full capacity and attack the tumor (Sharma and Allison, 2015).

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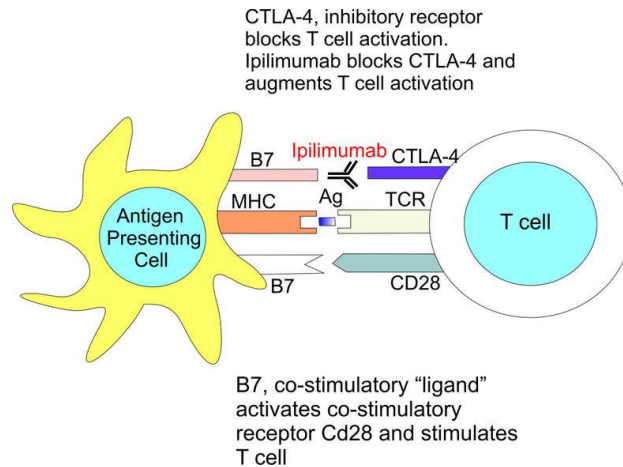
Figure-11: Biology of the CTLA-4 Pathway. During immune activation, an antigen presenting cell (yellow) binds a T-cell (blue) to induce its differentiation and activation. In addition to the T-cell receptor engaging the presented antigen (diagram center), a co-stimulatory pathway involving CD28 and B7 interaction also occurs to further increase the activation. The CTLA-4 pathway is a break to this activation, being upregulated in the T-cell to outcompete CD28 for B7. Cancer cells sometimes over-express B7 to engage the CTLA-4 pathway to inactivate the T-cell, and therapeutic antibodies against CTLA-4 (red) prevent this inactivation. From Lee et al., 2012.

The incredible success of checkpoint vaccines is illustrated by the exponential increase in the number of human clinical trials using these inhibitors (**Figure-12**). From 2009 to 2017, the number of clinical trials using checkpoint inhibitors increased from 1 trial (with 136 patients) to 469 trials (with 52,539 patients). The checkpoint treatments are often used in combination with other therapies (lower row in the diagram), the most frequent being other immune therapies.
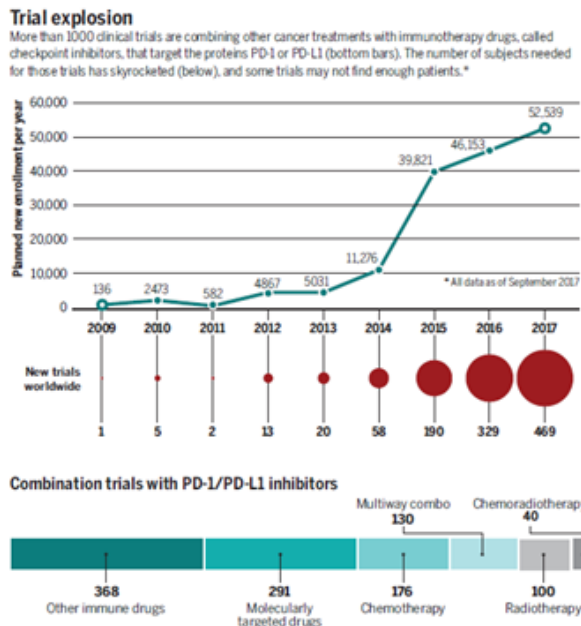
Figure-12: Exponential Increase of Clinical Trials Using Checkpoint Inhibitors. The graph shows the exponential increase in the patients enrolled in clinical trials from 2009 to 2017 using checkpoint inhibitors (upper graph), and the number of clinical trials (middle row). The lower row denotes the combination drug used with the checkpoint drug, with the largest cohort being another immune drug (lower left). Figure is from Kaiser, 2018.

Checkpoint Vaccine Examples

Figure-13 below shows the time-line of FDA approval for 6 checkpoint antibodies and one combination therapy. The green dots show the date of first patient treatment with the drug, and the red dots indicate the date of FDA approval for using that particular antibody to treat a specific form of cancer. The first checkpoint antibody used in a patient was **Ipilimumab**, an antibody against CTLA-4. It was first used in a patient in June of 2000, and in 2011 was FDA-approved for melanoma patients. The second checkpoint antibody used was **Nivolumab** (marketed as Opdivo), an antibody against PD-1. It was first used on a patient in 2006, and from 2014 to the present has been FDA-approved for treating melanoma (Topalian et al., 2014), non-small cell lung carcinoma, renal cell carcinoma, Hodgkin's lymphoma (Ansell et al., 2015), head and neck cancer, urothelial cancer, high microsatellite instability, and hepatocellular carcinoma (Gettinger et al., 2015; Rizvi et al., 2015; Tanner, 2015).
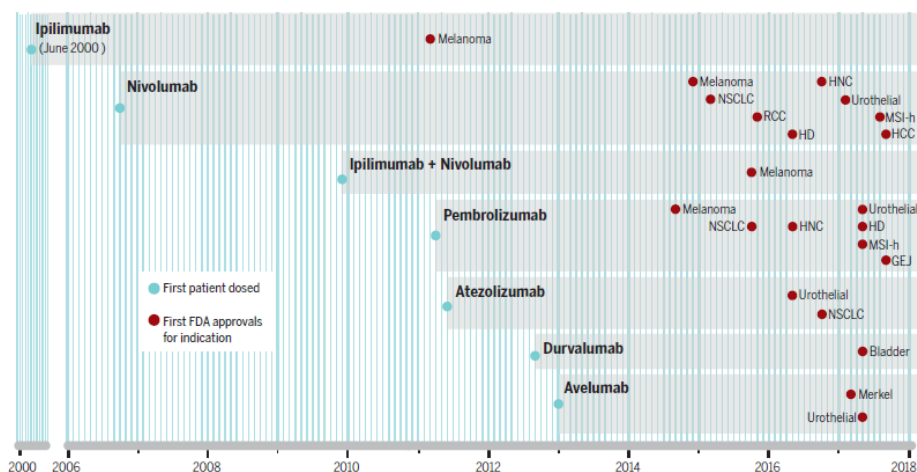
Figure-13: Time-Line of FDA Approval for Several Checkpoint Vaccines. The green dots denote the date of first patient treatment with the drug, and the red dots indicate the date of FDA approval for using that particular antibody to treat a specific form of cancer. Figure is from Ribas and Wolchok, 2018.

A recent trend with checkpoint vaccines is their combination with antibodies directed against tumors, and these studies have produced some spectacular successes. **Table-VIII** below shows example studies with checkpoint vaccines.

Table-VIII: Example Studies Using Checkpoint Vaccines

Target	Notes	Side-Effects	Reference
	Phase-I study of 39 patients with advanced metastatic melanoma, colorectal cancer (CRC), castrate-resistant prostate cancer, non-small-cell lung cancer (NSCLC), or renal cell carcinoma (RCC). Anti-PD-1 (MDX-1106) treatment. The results showed one durable complete response, and two partial responses. The serum half-life of anti-PD-1 was 12 to 20 days.	The anti-PD-1 was well tolerated, with one serious adverse event of inflammatory colitis.	Brahmer et al., 2010

PD-1	Phase-I multi-center Phase-I trial of anti-PD-L1 therapy, 207 patients with non-small-cell lung cancer, melanoma, colorectal cancer, renal-cell cancer, ovarian cancer, pancreatic cancer, gastric cancer, and breast cancer. Among patients with a response that could be evaluated, an objective response (a complete or partial response) was observed in 9 of 52 patients with melanoma, 2 of 17 with renal-cell cancer, 5 of 49 with non-small-cell lung cancer, and 1 of 17 with ovarian cancer.	Grade-3 or 4 toxic effects related to treatment occurred in 9% of patients.	Bramer et al., 2012
	International Phase-II study, anti-PD-1 monoclonal antibody, in 36 patients with diffuse large B-cell lymphoma (DLBCL). Among the 35 patients with measurable disease after stem cell transplant, the overall response rate after antibody treatment was 51%.	Toxicity was mild.	Armand et al., 2013
	135 patients with advanced melanoma, anti-PD-1 antibody treatment in patients previously receiving CTLA-4 antibody and those who had not. The confirmed response rate across all dose cohorts was 38% The response rate did not differ significantly between patients who had received prior anti-CTLA-4 treatment and those who had not.	Common adverse events attributed to treatment were grade-1 or 2, including fatigue, rash, pruritus, and diarrhea.	Hamid et al., 2013
	International, multi-center Phase-I trial of 173 patients with advanced melanoma previously treated with anti-CTLA-4 antibody. The overall response rate was 26%.	The treatment was well tolerated. There were no drug-related deaths. The most common drug-related adverse events in the 10 mg/kg group were fatigue (37%), pruritus (19%), and rash (18%). Grade-3 fatigue was reported in five (3%) patients.	Robert et al., 2014
	The treated 107 melanoma patients showed a mean overall survival of 16.8 months, with 1- year and 2-year survival rates of 62% and 43%, respectively.	The antibody safety was "acceptable", with toxicity rates similar to previous reports.	Topalian et al., 2014
	Phase-III controlled study, Nivolumab anti-PD-1 therapy versus dacarbazine chemotherapy, 418 patients with metastatic melanoma. At 1 year, the overall rate of survival was 72.9% in the antibody group compared to 42.1% in the chemotherapy group.	Common adverse events associated with the antibody treatment included fatigue, pruritus, and nausea, with grade-3 or 4 events in 11.7% of the patients treated with antibody and 17.6% treated with chemotherapy.	Robert et al., 2015

23 patients with relapsed or refractory Hodgkin's lymphoma received Nivolumab anti-PD-1 treatment. A high percent of the patients had received previous treatments with stem-cell transplantation or Brentuximab vedotin (antibody-drug conjugate targeting CD30). An objective response was reported in 87% of the patients, including 17% with a complete response and 70% with a partial response.	Drug-related adverse events of any grade occurred in 78% of the patients, grade-3 events in 22%. and of grade 3 occurred in 78% and 22% of patients, respectively. Discontinuation of the study due to the drug occurred in 2 patients.	Ansell et al., 2015
Phase-II study of 22 patients with non-small cell lung cancer (NSCLC). the drug produced "durable immune responses and encouraging survival rates	14% of the patients showed grade-3 or -4 treatment-related adverse events.	Gettinger et al., 2015
Non-small cell lung cancer patients. Analyzed non-synonymous mutations to show that higher neo-antigen formation (more targets) correlated with better clinical benefits and progression-free patient survivals.		Rizvi et al., 2015
600 patients with non-small cell lung cancer. The median survival rate increased from 9 months (chemotherapy alone) to 12 months (for the immunotherapy group), and the tumors shrank in 12% of the chemotherapy patients versus 20% of the immunotherapy patients.		Tanner, 2015
Phase-I trial for 655 patients with advanced metastatic melanoma using humanized anti-PD-1 monoclonal antibody Pembrolizumab, performed in academic medical centers in Australia, Canada, France, and the United States. An objective response was reported in 194 of 581 patients (33%). 44% (90/205) had response duration of at least 1 year.		Ribas et al., 2016
Multicenter Phase-II trial of 315 patients with metastatic urothelial carcinoma that have failed standard platinum chemotherapy using humanized antibody against PD-L1. The antibody-treated group showed an overall response rate of 15%, compared to a historical response rate of 10%.	Grade 3-4 treatment-related adverse events occurred in 50 (16%) of 310 treated patients. Fatigue was the most common (5 patients, 2%). No treatment-related deaths occurred during the study.	Rosenberg et al., 2016
112 melanoma patients treated with anti-PD-1 therapy. Identified oral and gut microbiome differences in patient responders versus non-responders. Responder gut microbiomes showed greater microbial diversity, higher abundance of *Ruminococcaceae*, and enrichment of anabolic pathways. Mice receiving patient		Gopalakrishnan et al., 2018

	responder fecal transplants showed better vaccine responses.		
	Determined that a primary mode of epithelial cancer patient resistance to anti-PD-1 therapy is the gut microbiome composition. Antibiotics tended to lower the clinical benefit the therapy, while fecal microbiota transplantation (FMT) from responder patients into mice improved the treatment. Responders tended to have high levels of Akkermansia muciniphila, and supplementation with this microbe tended to improve outcomes in mouse models.		Routy et al., 2018
	The team developed mouse models of colorectal cancer with increased levels of TGFβ in the tumor microenvironment to mimic patients with T-cell exclusion and resistance to anti-PD-1 therapy. The mice showed limited response to anti-PD-1 therapy. Blocking the TGFβ signaling pathway unleashed a potent and enduring cytoxoxic T-cell response against the tumors and improved the anti-PD-1 response. Thus, TGFβ in the tumor microenvironment is a primary mechanism of tumor immune evasion.		Tauriello et al., 2018
	Performed a risk analysis for anti-PD-1 Nivolumab for hepatotoxicity. Analyzed all Phase-I through Phase-III trials through December 2016 for elevated levels of liver enzymes in the blood: aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Overall hepatotoxicity was only 5.4%, and high-grade toxicity was 1.6%.		Zarrabi and Wu, 2018
CTLA-4	9 patients with various cancers. CTLA-4 antibody treatment caused extensive tumor necrosis in three of three (100%) metastatic melanoma patients, and the reduction or stabilization of cancer in two of two (100%) metastatic ovarian carcinoma patients, but caused no reduction in 4 of 4 metastatic melanoma patients previously immunized with defined melanoma antigens.	No serious toxicities directly attributable to the antibody therapy were observed.	Hodi et al., 2003
	Phase-I trial. 39 patients with solid malignancies (34 melanoma, 4 renal cell, and 1 colon, n = 1). The maximum tolerated dose (MTD) was determined to be 15 mg/kg. 2 patients experienced complete responses, and two experienced partial responses.	Dose-limiting toxicities and autoimmune phenomena included diarrhea, dermatitis, vitiligo, pan hypo-pituitarism and hyper-thyroidism.	Ribas et al., 2005

CTLA-4	Metastatic melanoma patients. The median overall survival of patients receiving the CTLA-4 antibody + gp100 peptide vaccine was 10 months compared to 6.4 months for those receiving only the gp100.	The side effects were severe in only 10-15% of the patients receiving both treatments, and in only 3% of the patients receiving gp100. Most were manageable. 2.1% of the patients died from the study, 7 related to immune adverse events.	Hodi et al., 2010
		The article discusses the different kinds of immune-related adverse events associated with CTLA-4 treatment, and the necessary treatments to manage them. They conclude that one of the most important aspects of side-effect treatment is early recognition.	Weber et al., 2012
	11 melanoma patients. Performed whole exome sequencing was performed to correlate which neo-antigens correlate with best improvements using CTLA-4 antibody treatments.		Snyder et al., 2014
	Tested advanced melanoma patients with CTLA-4 antibody if they did not respond to PD-1 antibody. 31.7% showed positive responses compared to 10.6% with chemotherapy.	Noted grade-3 to 4 (serious) adverse events in 5% of antibody-treated patients versus 9% for chemotherapy. There were no treatment-related deaths.	Weber et al., 2015
	Metastatic osteosarcoma. The tumor burden decreased, and the survival rate increased.		Lussier et al., 2015
	A pooled analysis of overall survival data from 1,861 patients from 10 prospective and two retrospective studies of ipilimumab, including 2 phase-III trials. Patients were previously treated (n = 1,257) or treatment naive (n = 604), and the majority of patients received ipilimumab 3 mg/kg (n = 965) or 10 mg/kg (n = 706). Of the 1,861 patients, the median overall survival was 11.4 months, including 254 with at least 3 years of survival.		Schadendorf et al., 2015
	A review of the data of 4 Phase-I and II trials at 2 sites for 143 patients with advanced melanoma. The median overall survival was 13 months, with a 5 year survival rate of 20%, and a 12.5 year survival rate 16%.		Eroglu et al., 2015

	Analyzed 536 metastatic cancer patients treated with anti-PD-1, PD-L1, or CTLA-4 immunotherapies for liver related side-effects. Only 19 patients (3.5%) needed referral to the liver unit for grade ≥3 hepatitis. No patients developed hepatic failure. 6 patients improved spontaneously; the remainder received various doses of oral corticosteroids. Liver biopsy was helpful for the diagnosis and evaluation of the severity of liver injury. The severity of liver injury was helpful for tailoring patient management, which does not require systemic corticosteroid administration.		De Martin et al., 2018
Combination Treatments	PD-1 + CTLA-4. Melanoma patients. 53% of the combination patients showed a tumor reduction of 80% or more, compared to only 20% for single antibody treatments.	Grade-3 and 4 side-effects occurred in 53% of the combination patients compared to 18% in the single antibody patients. The side-effects were generally reversible.	Wolchok et al. 2013
	PD-1 + CTLA-4. Various cancers. Inverse treatments, used the opposite antibody against patients showing resistance to the first antibody.	Found that in most cases, the second antibody was well received.	Weber et al., 2013
	PD-1 + CTLA-4. Studied metastatic osteosarcoma in mice. The combination treatment prevented tumor escape and allowed complete control of the cancer.		Lussier et al., 2015
	Phase-III trial of 1096 patients with advanced clear-cell renal-cell carcinoma with antibodies to PD-1 + CTLA-4. At 25.2 months, the 18-month survival rate was 75% for the combination versus 60% for treatment with a tyrosine kinase inhibitor.	Treatment-related adverse events occurred in 509 of 547 patients (93%) in the combination group versus 521 of 535 patients (97%) in the kinase inhibitor group. Treatment-related adverse events causing termination from the trial occurred in 22% and 12% of the patients, respectively.	Motzer et al., 2018
	Cancer cells sometimes express TGF-β which activates regulatory T-cells to inhibit CD8+ cytotoxic T-cells. The authors developed a bi-specific antibody, with one arm recognizing CTLA-4 or PD-1, and the other arm representing the ectodomain of the TGFβ receptor II (to bind and sequester TGFβ). The bi-specific therapy was more effective at inducing tumor regression (and reducing regulatory T-cells) than the mono-specific antibody.		Ravi et al., 2018

Checkpoint Vaccine Problems and Future Directions

Some of the data using checkpoint inhibitor vaccines is striking, including some complete remissions from very difficult cancers. The data also shows that using a *combination* of antibodies (for example against PD-1 and CTLA-4, or combining PD-1 with a bi-specific antibody) may produce better data than using either antibody alone. But as groundbreaking and promising as immune checkpoint vaccines have been, several factors are drawbacks to their use. Blocking the immune blockers can sometimes activate the immune system too far, producing autoimmunity or inflammation (Melero et al., 2007; Frankel, 2017), so inflammation should be constantly monitored during the treatments. Other studies showed the treatments caused grade-3 and -4 (serious) side-effects, some even resulting in death (Eggermont et al., 2016). Although most of the side-effects appeared to be transient and treatable. The side-effects were especially treatable if they were detected early, so these events should be constantly monitored throughout the entire treatment. In other cases, the patient simply did not respond to the treatment. Even in studies with the best objective response rates, roughly 30% of patients would see no response. Finally, as with many aspects of our healthcare system, the cost of these drugs can put them out of reach for many patients, with one course costing up to $150,000 (McCune, 2018). These problems should be addressed with future experiments.

High Incidence of Complications/Side-Effects

In nearly all the clinical trials examined in this section of the Literature Review, regardless of drug, dosage, or disease, a large percentage of patients suffered severe (grade 3 or 4) side-effects caused by the treatment. One study saw nearly half of the patients with side-effects, with 1 in 5 patients being grade-3 or 4, while another study saw 68% with adverse effects, with 1 patient in 10 being serious (Topalian et al., 2012; Weber et al., 2015). Another study noted that while most of the side-effects were manageable, 2.1% of the patients died from the study, 7 related to immune adverse events (Hodi et al., 2010).

When combining the individual drugs Ipilimumab and Nivolumab into one therapy, the rate of grade-3 and 4 adverse effects was 53% compared to 18% in the single antibody patients (Wolchok et al., 2013). Another combination trial observed adverse events in 93% of 547 patients, with those events causing termination from the trial in 22% of the patients (Motzer et al., 2018). While these adverse incidences are troubling, they are not out of the norm for cancer therapies. Indeed, some of the trials reported less frequent occurrence of severe adverse effects than standard chemotherapy (Abdin et al., 2018). And in most cases, the side-effects were transient and treatable, with a prognosis far better than the terminal cancer being treated. However, any treatment where a large portion of patients have high grade complications is not ideal, and this keeps the checkpoint vaccines from being a first-line treatment.

Autoimmune Disorders

Most of the adverse side-effects described above were autoimmune related. The most commonly observed were thyroid disease, type-1 diabetes, colitis, and liver damage. Checkpoint inhibitors dial up the immune system beyond physiologically normal levels to overcome the mechanisms that tumors deploy to hide from the immune cells, so it is natural to see how autoimmune disorders could rise from this strengthened immune system. The checkpoint inhibitor antibodies can act on normal cells as well the cancer, allowing the immune system to overcome an important block for the body (Frankel, 2017). Liver damage (hepatitis) was a particularly well reported side effect, with one study seeing 3.5% of all checkpoint patients with hepatitis severe enough to require hospitalization (De Martin et al., 2018). Another study with a 20% rate of grade-3 or 4 complications showed 10.3% with liver toxicity (Zarrabi and Wu, 2018). One of the more serious autoimmune diseases seen was autoimmune myocarditis, an immune attack on the heart which can be fatal (Frankel, 2017). Fortunately, this was rare and most of these autoimmune side-effects were treatable and preferable to progression of

cancer. However, their burden and potential fatality must be taken into consideration before checkpoint-based treatment can be initiated (Zarrabi and Wu, 2018).

Low Response Rate

The clinical trials examined here show that even in the best scenarios, up to one in three patients had no response to the checkpoint therapy. And some types of cancer simply do not appear to respond to checkpoint inhibitors (Ravi et al., 2018). New research is focusing on how the specific genetic makeup and personal microenvironment of the patient's tumor and gut can determine how effective the checkpoint therapy might be, hopefully identifying a variety of molecular and microbial biomarkers that can be used to personalize therapy and determine which patients will best respond.

A recent fascinating finding relates to the hormone transforming growth factor-beta (TGF-β) and its potential role in determining the receptibility of a tumor to a checkpoint therapy. TGF-β is a multi-functional cytokine with a critical role in regulating the adaptive immune system. When secreted into the bloodstream during an immune response, TGF-β restricts the differentiation of helper T-cells, stops the development of memory T-cells, and induces the production of regulatory T-cells during an immune response. Like PD-1 and CTLA-4, TGF-β protects healthy somatic cells from an overactive immune system and prevents an autoimmune response. However, many tumors also over-express TGF-β to evade the immune system. In tumors that do this, checkpoint therapy targeting PD-1 has not been effective; although the PD-1/PD-L1 system was inactivated by the therapy, the related cytokine response was still keeping the immune system in check in the tumor microenvironment. The best results for these tumors came when an antibody targeting TGF-β was used in conjunction with a checkpoint inhibitor to block both mechanisms of immune system evasion (Ravi et al., 2018). Another study supporting these results showed that a tumor microenvironment with high levels of TGF-β had almost no response to checkpoint therapy, but showed a potent response after inhibition of TGF-β (Tauriello et al., 2018). These results point to the potential use of TGF-β levels as a biomarker for identifying which specific patients have a cancer microenvironment that would respond to checkpoint therapy alone and those that would require a combination treatment.

Other recent work has shown that the bacterial composition of the patient's gut microbiome can modulate the effectiveness of checkpoint inhibitors. Bacterial mediated interactions with the immune system are essential for its normal function, and by extension optimal checkpoint inhibitor therapy outcomes (Jobin, 2018). The gut provides an environment that houses many immune cells. Beneficial gut bacteria can cause these immune cells to secrete antibodies. Patients who responded to checkpoint therapy were shown to have functional differences in the types of bacteria that predominated their gut relative to patients that did not respond (Gopalakrishnan et al., 2018). One study found that patients who responded to Nivolumab had an abundance of bacteria of the genus *Faecalibacterium*, while non-responders had a much lower abundance of this microbe. This team also showed that targeted microbial enrichment of non-responding patients with the *Faecalibacterium* correlated with a greater response to PD-1 therapy and an increase of T-cells in the tumor (Jobin, 2018). Another study showed that resistance to checkpoint therapy can be attributed to an abnormal gut microbiome. When antibiotics were prescribed to return the gut microbiome to its optimal state, the effectiveness of checkpoint inhibitors increased (Routy et al., 2018). The same study correlated the abundance of the species *Akkermansia muciniphila* to checkpoint efficacy, as oral supplementation of this microbe increased therapy results (Routy et al., 2018). These studies point to the composition of a patient's gut fauna as a promising indicator of whether they will respond to checkpoint therapy, and also provide hope that those who do not have an optimal gut microbiome can be given supplemental treatment that will increase their chances of a positive response.

A third variable that has received increased attention for its role in improving checkpoint therapy effectiveness is the human leukocyte antigen (HLA) class of molecules. The HLAs are a set of cellular surface proteins that immune cells use to distinguish the body's cells from foreign cells. Every person has

a slightly different set of HLAs that their immune system uses as markers, and the exact makeup of these proteins is determined by the genotype of the HLA gene complex (American Cancer Society, 2017). One study demonstrated that melanoma patients with increased heterozygosity in their HLA genes had increased survival rates compared to patients with a high level of HLA homozygosity (Chowell et al., 2018). A meta-analysis of the outcomes of over 1,500 cancer patients treated with checkpoint therapy supported these conclusions, finding that better outcomes often occurred in patients with HLA subtype heterozygosity. These results make sense because checkpoint therapy depends on the ability of the immune system to recognize a tumor as an invading cell, and this is easier if the somatic cells have a more complex and varied identification system (from heterozygosity of the HLA genes). It is easier for a tumor to avoid detection when HLA is homozygous because the identification proteins on somatic cells will be less complex and easier to mimic. The same study also showed that a specific subtype of HLA (HLA-B44) gave the best outcomes for the checkpoint patients, and the HLA-B62 subtype gave a poor outcome. It is not well understood why the subtypes affected checkpoint therapy this way, but this work showed that a patient's HLA genetic makeup might be used as a biomarker to predict effectiveness of the checkpoint therapy (Chowell et al., 2018).

As this last section has shown, numerous factors beyond the type and stage of the cancer affect the success of checkpoint therapy. Individual differences in the tumor microenvironment (TGF-β levels), the gut microbiome, and patient HLA immunogenetics can explain why two people with the same disease respond so differently to therapy. These individualized determinants have led to the rise of personalized therapy using these individual differences as biomarkers to indicate whether a patient will respond well to the checkpoint inhibitor therapy. However, the use of biomarkers is not perfect. Ledford 2018 showed that biomarker tests can be inconclusive or give false negatives. Nevertheless, individual differences in patients and their tumors have been clearly demonstrated to determine the effectiveness of immunotherapy, even though such experiments remain in their infancy. More work to solidify the identification of biomarkers and the patients best fit for checkpoint therapy is needed for providing more positive data for the FDA approval of more immune treatments (Leford, 2018).

Recent Failed Clinical Trial

The checkpoint vaccine field also recently suffered a setback with the failure of one of its Phase-III clinical trials (Garber, 2018). The Phase-III trial was being conducted by the biotech company Incyte using a combination treatment of Opdivo (PD-1 antibody) and Epacadostat. Epacadostat blocks the enzyme indoleamine 2,3-dioxygenbase (IDO) which is thought to become activated in T-cells that have been treated with a checkpoint inhibitor creating a "negative feedback loop" to put a brake on the activated T-cells. But this feedback loop works against the cancer treatment, so scientists thought that inhibiting IDO might allow a more prolonged activation of the T-cells. In earlier Phase-I and Phase-II trials, the combination treatment worked well, but in the recent Phase-III trial the combination was no better than Opdivo alone. So, maybe the IDO enzyme doesn't really block the activated T-cells as scientists believed? Or maybe the wrong patients were treated? Unfortunately, the Incyte failure caused three other companies to cancel, suspend, or downsize their Phase-III trials with Epacadostat (Garber, 2018).

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METHODS

To accomplish project objective-1, we performed an extensive review of the available research literature, including reputable academic journal articles, relevant books, scholarly websites, newspaper articles, and other pertinent materials.

To accomplish objective-2, we conducted a set of interviews with various biomedical researchers who have performed cancer vaccine research on either animals or humans. We also interviewed scientists who have used traditional cancer fighting technologies, to determine their range of opinions on newer forms of treatment.

Who: The "stakeholders" (interviewees) included academic biomedical experts on cancer vaccines and traditional cancer therapies, and general health experts on cancer. These experts helped answer questions resulting from our Lit Review search, and helped us prioritize any remaining problems. Some of the stakeholders interviewed were initially identified by referral from the project advisor, Prof. David Adams. Other interviewees were identified from the published literature as authors on key scientific papers, or were referred to us from the initial interviewees.

Where and When: Once contact was made with a potential interviewee (see below), a time and place was set up for the interview to be performed at the interviewee's workplace. Whenever possible, interviews were conducted in person, although most were conducted by email, phone, or Skype.

How: Our first round of prospective interviewees was contacted by email and/or phone. If no response was received, we used follow-up emails and phone calls. We developed our interview questions (see preliminary questions in the Appendix) based on our review of the literature, and tailored the questions to the interviewee's expertise. Based on the interviewee's response to our first questions, in some cases we asked follow-up questions to clarify the information provided, or to press the interviewee for more information. The preliminary list of questions in the Appendix covers the full range of topics needed to cover our project.

With respect to the method of the interview, whenever possible each interview involved two team members, so that one member could ask questions while the other member wrote detailed notes, and vice versa. We asked whether the interviewee consented to be digitally recorded, and if not, we used written notes or emails as the main method of recording the conversation.

At the start of the interview, we informed the interviewee about the purpose of our project, and asked for permission to quote them (see draft interview preamble in the Appendix). We explained how we will protect their confidentiality, if necessary, by giving them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that they may stop the interview at any time or refuse to answer any question.

After the interview, we asked each interviewee for permission to follow-up with them at a later date if needed to fill in any gaps in the information. And, as mentioned above, we asked the interviewee to recommend other potential stakeholders we might interview, to further increase the number of interviews with key individuals.

With respect to the total number of interviews needed for our project, we stopped interviewing additional subjects when we obtained a sufficient amount of information to represent all sides of the cancer vaccine story, good and bad, and when all unclear points had been clarified.

To accomplish objectives-3 and 4, the group synthesized all of the information collected in the literature research, interviews, and follow-up interviews, to ascertain the strength of the evidence, and to create recommendations for further research.

RESULTS / FINDINGS

Our review of the cancer vaccine literature identified several problems that needed further clarification in interviews. We performed interviews with a variety of scientists, including those who helped develop cancer vaccines, doctors who performed cancer vaccine clinical trials, and scientists actively engaged in developing new generations of cancer vaccine drugs. We chose to focus on two main problems and directions: 1) What causes vaccine side-effects, and what new approaches might minimize them? 2) Why are some patients resistant to vaccines, and what new approaches might improve this?

Results for Therapeutic Antibody Vaccines

A major problem with cancer vaccines is that a substantial proportion of the patient's tumors do not respond to them. To understand this problem further, we interviewed **Dr. Sergey E. Sedykh** of the Laboratory of Repair Enzymes, Siberian Branch of the Russian Academy of Sciences Institute of Chemical Biology and Fundamental Medicine, Novosibirsk State University, Novosibirsk, Russia. Dr. Sedykh was corresponding author on a 2018 review article on bi-specific antibodies: Sedykh et al., 2018, Bispecific antibodies: design, therapy, perspectives. *Drug Design, Development, and Therapy*, 2018 Jan 22; 12: 195-208. When asked his opinion on the leading cause of resistance to bi-specific antibody therapies, he replied: "My opinion is that bispecific antibodies (like blinatumomab and catumaxomab) act by attracting a T-cell (via the CD3 receptor) to a tumor cell, but if there are few T-cells remaining in the patient [for example due to a prior chemotherapy], the patient cannot fight the cancer. The down-regulation of surface antigens on cancer cells may be another tumor-evolution route of cancer cell resistance. In some cases, therapeutic BsAbs are considered as the auxiliary treatment after inductional and consolidation therapy". So, Dr. Sedykh indicated that tumor resistance to bi-specific antibody therapy can result from a general low number of T-cells to attract to the tumor (with the anti-CD3 portion), and from the down-regulation of target antigen expression.

Our Lit Review indicated that one approach for overcoming resistance is to change the type of vaccine used. A very good example of this is the paper: Rothe et al., 2015, A phase-I study of the bispecific anti-CD30/CD16A antibody construct AFM13 in patients with relapsed or refractory Hodgkin lymphoma. *Blood*, 2015 Jun 25; 125(26): 4024-4031. The authors used bispecific antibody AFM13, which has affinities for CD30 (present in lymphomas) and CD16A (which recruits natural killer cells to the tumor instead of T-cells). This was a Phase-I dose-escalation study (doses of 0.01 to 7 mg/kg body weight) of 28 patients with heavily pretreated relapsed or refractory Hodgkin lymphoma. Three of 26 evaluable patients (11.5%) achieved partial remission, and 13 patients (50%) achieved stable disease. Importantly, AFM13 was active in brentuximab vedotin (CD30)-refractory patients. A phase 2 study is currently planned. Adverse events were generally mild to moderate. So, attracting NK cells to a tumor might be an alternative approach for treating patients resistant to T-cell attracting therapies. To shed more light on this topic, we interviewed the corresponding author of the article: **Dr. Andreas Engert**, Professor of Internal Medicine, Hematology & Oncology, and Chairman of the German Hodgkin Study Group, Department of Internal Medicine, University Hospital of Cologne, Germany. When asked whether their NK-recruiting strategy to switch the method of cell killing might work for other types of cancers besides Kodgkin lymphoma, he stated: "We have only used this approach in Hodgkin lymphoma, but it might certainly be possible that this also works in other malignancies, we have not pursued this yet". Thus, Dr. Engert indicated that this new approach of switching the method of cell killing might indeed work with other types of cancers, but they have not yet tested it.

Another possible mechanism for tumor resistance is a mutation occurs that switches the tumor from one growth signaling pathway to another. An example of this is the article: Lieu et al., 2017, A

Phase Ib Dose-Escalation Study of the Safety, Tolerability, and Pharmacokinetics of Cobimetinib and Duligotuzumab in Patients with Previously Treated Locally Advanced or Metastatic Cancers with Mutant KRAS. *Oncologist*, 2017 Sep; 22(9): 1024-e89. The authors investigated patients with KRAS-mutant tumors that possess abnormal MAPK pathway signaling and cell proliferation. They did a Phase-IB dose-escalation study of a combination of Cobimetinib (which blocks MAPK signaling) and the bi-specific antibody duligotuzumab (which inhibits ligand binding to two types of receptors: EGFR and human epidermal growth factor receptor 3 (HER3). They enrolled 23 patients KRAS-mutant tumors. Nine of the 23 patients (39%) showed stable disease, but 14 were non-responders. To get at the basis of the tumor resistance, we interviewed the corresponding author on the article: **Dr. Christopher H. Lieu, MD**, Director of GI Medical Oncology, and Deputy Associate Director for Clinical Research, University of Colorado Anschutz Medical Campus, Division of Medical Oncology, Aurora, Colorado. When asked his opinion about why the patients were non-reponders, he replied: "I think if a patient's tumor is not addicted to the MAPK pathway, they may be using another growth pathway to grow and metastasize. In our case, the non-responders may not have needed the MAPK pathway (which both of our drugs inhibit), but may have used another growth pathway that we were not blocking". Thus, according to Dr. Lieu, when using therapies that work by binding to receptors to block a specific cell growth pathway, to determine whether a particular tumor will respond to the antibody, the tumor needs to be verified as being dependent on that specific pathway, otherwise the treatment won't work.

Reduction of target antigen expression in a portion of the tumor is another mechanism of resistance. This situation was seen in a recent 2018 paper: Bosco et al., 2018, Preclinical evaluation of a GFRA1-targeted antibody-drug conjugate in breast cancer. *Oncotarget*, 2018 May 1; 9(33): 22960-22975. The authors used a genomics approach to identify membrane-localized tumor-associated antigens (TAAs) in breast cancer cells that might serve as a target for antibody-drug conjugate (ADC) therapy. They identified glial cell line-derived neurotrophic factor (GDNF) family receptor-α1 (GFRA1) as a breast cancer tumor-associated antigen. They determined that GFRA1 shows limited expression in normal cells, over-expression in some breast tumor subtypes, and rapid internalization, making it a good ADC target. Their GFRA-targeting ADC showed strong anti-tumor activity against GFRA1-positive tumor cell lines *in vitro*, and *in vivo* against patient-derived human cancers in mouse xenograft models. The safety profile showed only transient problems in the bone marrow and peripheral blood, consistent with well known off-target effects of their chosen cytotoxic cargo. To determine the proportion of breast cancer patients that might benefit from anti-GFRA therapy, we interviewed the corresponding author of the paper: **Dr. Emily E. Bosco, PhD**, Scientist-II, Oncology Research, MedImmune, Gaithersburg, Maryland. When asked her opinion of what percent of breast cancer patients might express GFRA and benefit from their ADC, she replied: "we would first treat the moderate and strong GFRA-expressing breast cancer patients, and based on immunohistochemistry (IHC) these patients represent about 20% of estrogen receptor-positive breast cancer patients, and about 9% of triple negative breast cancer patients". So, Dr. Bosco indicates that based on their own immunohistochemical staining of breast cancer tumors, about 29% of breast cancer patients might benefit from the GFRA-targeting ADC drug. Presumably, the other breast cancer patients would need to be treated with a drug targeting a different surface antigen, such as HER2 or HER3.

A problem frequently encountered with new therapies is a lack of negative controls in the clinical trials. In the case of cancer vaccines, the patients are not allowed to receive the new therapy unless the cancer relapses from the patient's previous treatments. And the prognosis is very poor for relapsed patients, so almost any increase in patient survival is significant for these populations. An example of this problem is the article: Trněný et al., 2018, A Phase 2 multicenter study of the anti-CD19 antibody drug conjugate coltuximab ravtansine (SAR3419) in patients with relapsed or refractory diffuse large B-cell lymphoma previously treated with rituximab-based immunotherapy. *Haematologica*, 2018 May 10. The authors performed a Phase-II multi-center clinical trial on the safety and efficacy of their anti-CD19-targeting antibody-drug conjugate (ADC) Coltuximab ravtansine. They analyzed patients with relapsed or refractory diffuse large B-cell lymphoma who had previously received Rituximab therapy (antibody

targeting CD20). 41 patients were included in the treatment population. The overall response rate was 18/41 (43.9%). The median duration of response, progression-free survival, and overall survival were: 4.7 months, 4.4 months, and 9.2 months, respectively. Common non-hematologic adverse events included asthenia/fatigue (30%), nausea (23%), and diarrhea (20%). Grade 3-4 adverse events were reported in 23 patients (38%), the most frequent being hepatotoxicity (3%) and abdominal pain (3%). In this trial, there was no patient cohort treated only with traditional chemotherapy (negative control) because all patients were resistant to traditional treatments and received the ADC treatment. We interviewed the corresponding author of the paper **Dr. Marek Trněný, MD, CSc**, Professor and Chairman, 1st Dept Medicine, Charles University, General Hospital, Praha, Czech Republic. When asked his opinion about the median overall survival rate for the non-ADC-treated patients, he responded: "Generally speaking, their median overall survival is about one year, but some patients could reach long-term survival". So, Dr. Trněný indicates that for the patients they used for the Phase-II tests (with relapsed or refractory diffuse large B-cell lymphoma who had previously received Rituximab therapy), the median overall survival is about 1 year. This is slightly more than the 9.2 months seen for their patients treated with their ADC drug, so the ADC did not appear to lengthen overall survival in this particular study. This example shows the difficulty of determining whether a drug benefits a population whose prognosis is extremely poor from the beginning of the study.

Results for Dendritic Cell Vaccines

In the cancer vaccine field, the experiments typically move from pre-clinical testing (which includes testing the drug against cancer cell lines *in vitro*, and testing xenograft mice *in vivo*) into human clinical trials. But it is not clear exactly how much information is needed from the pre-clinical testing before moving into clinical trials. For example, we identified the following paper: Choi et al., 2018, Combination Treatment of Stereotactic Body Radiation Therapy and Immature Dendritic Cell Vaccination for Augmentation of Local and Systemic Effects. *Cancer Research and Treatment*, 2018 Jun 6. doi: 10.4143/crt.2018.186. The authors investigated dendritic cell (DC) vaccines and methods for improving the efficiency of priming the DCs with tumor antigens. DCs can be primed against tumor antigens *in vivo* in the patient, or *ex-vivo* by mixing isolated DCs with tumor antigens outside the body. The *ex-vivo* approach is used most often in clinical trials, but the *in vivo* method has the advantage of being available even when no tumor biopsy tissue is available to do the ex-vivo priming. Ionizing radiation has recently been tested as a method for facilitating *in vivo* DC antigen priming, but exactly how the radiation works to prime the DC cells is not clear. The radiation might help release tumor associated antigens from the tumor, or it might increase the release of damage-associated molecular patterns (DAMPs) (such as heat shock proteins) from dying tumor cells. In the Choi et al., 2018 study, the authors investigated the use of stereotactic body radiation therapy (SBRT) as a method for facilitating the presentation of tumor-associated antigens (TAA) to immature dendritic cells (iDCs). They tested their method on mouse xenograft models of CT-26 colon carcinoma with 3 groups of mice: 1) radiation therapy alone, 2) intra-tumor injection of DCs electroporated with tumor antigens, or 3) the combination treatment. The data showed that the radiation method achieved the best DC priming and T-cell activation compared to other priming methods, and that mouse survival was highest when using the combination treatment. The authors conclude that clinical trials are warranted. We interviewed the corresponding author on the paper: **Dr. Chul Won Choi, MD**, Department of Radiation Oncology, Dongnam Institute of Radiological & Medical Sciences, Busan, Korea. When asked his opinion about whether it will be necessary to more deeply understand the mechanism of *how* the radiation is facilitating the DC antigen presentation process before proceeding to clinical trials, he responded: "Actually, I don't think so. There are already many suggested mechanisms for how radiation enhances immune reactions. However, doing a study on the ratio of apoptosis [programmed cell death] to necrosis [generalized cell death] after irradiation would be interesting. Knowing this ratio would be helpful for determining the dose of radiation, or for enhancing efficacy". When asked whether his team was moving forward with clinical

trials, he responded: "No. There are many limitations to moving to clinical trials including financial, administrative, and legal issues. So, we won't be going to the next step". So, Dr. Choi thinks that it likely is not necessary to obtain a full understanding of exactly how their radiation method works to prime DC cells before proceeding to clinical trials, but that his lab is not moving forward due to financial, administrative, legal, and scientific reasons.

As mentioned previously, for some of the clinical trials published in the literature, it was not clear how the patient prognoses for patients receiving the cancer vaccine fared relative to untreated patients, or to patients treated with traditional chemotherapy, because all patients receiving cancer vaccines have received previous therapies and have relapsed/recurring cancers. For example, we identified the following paper: Liau et al., 2018, First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma. *Journal of Translational Medicine,* 2018 May 29; 16(1): 142. This paper showed the results of a Phase-III trial of 331 patients with glioblastoma. The standard therapy for glioblastomas is surgery, radiotherapy, and oral chemotherapy temozolomide. This study evaluated the addition of an autologous tumor lysate-pulsed dendritic cell vaccine (DCVax®-L) to standard therapy for newly diagnosed glioblastoma patients. After surgery and chemo-radiotherapy, patients were randomly assigned to two groups: 1) chemo + DC vaccine (232 patients), or 2) chemo + placebo (99 patients). However, if the patient's cancer recurred, the patient was allowed to receive the DC vaccine regardless of the initial group assignment. So, because of this "cross-over design", nearly 90% of the patients received the DC vaccine, and patient survival relative to a placebo could not be determined. The median overall survival was 34.7 months, with a 3-year survival of 46.4%. We interviewed the corresponding author on the paper: **Dr. Linda M. Liau, MD**, of the University of California Los Angeles (UCLA) David Geffen School of Medicine & Jonsson Comprehensive Cancer Center, Los Angeles, CA. When asked whether the median survival of 34.7 months was significantly better than the placebo group, she responded: "Yes, the median overall survival for the entire intent-to-treat (ITT) population does seem longer than the normal prognosis for glioblastoma patients based on historical controls" So, Dr. Liau believes the average survival for the patients receiving her dendritic cell vaccine is longer than patients not receiving it. Because most of the patients in her cross-over study received the DC vaccine, we must rely on "historical data" for the untreated patients.

One trend observed in the literature is the use of **combination cancer vaccines**. Research teams that had previously investigated single cancer vaccines were now testing combinations. And teams that had previously tested combinations were now expanding the combinations to other types of cancer. As an example, DC vaccines are being combined with checkpoint inhibitor vaccines. Checkpoint vaccines are used to over-ride the immuno-suppression caused by the tumor cell interacting with the immune system (typically DCs and T-cells). Three checkpoint targets came up repeatedly in our review: PD-1, PD-L1, and CTLA-4. But given their importance, it makes sense to identify new checkpoint receptors as vaccine targets. An interesting system was recently established using tumor "exosomes" (membrane vesicles) to identify checkpoint inhibitors: Ning et al., 2018, Tumor exosomes block dendritic cells maturation to decrease the T cell immune response. *Immunology Letters*, 2018 Jul; 199: 36-43. In this study, the authors developed a system for determining whether exosomes produced by two particular types of cancer (LLC Lewis lung carcinoma or 4T1 breast cancer) contribute to DC suppression. They found that exosomes from these tumors indeed blocked the differentiation of myeloid precursor cells into DC cells, and inhibited the migration and maturation of DCs. In addition, the inhibitory response was partially blocked by treating with anti-PD-L1 antibody, suggesting this PD-L1 checkpoint inhibition is important to the inhibition. We interviewed the corresponding author of the paper: **Dr. Chunjian Qi, MD, PhD**, Professor and Director, Medical Research Center, The Affiliated Changzhou No.2 People's Hospital of Nanjing Medical University, Changzhou, China. When asked now that he had set up the exosome system for studying the inhibition process, whether he intends to investigate other potential blockers of the DC activation besides PD-L1, he responded: "While PD-L1 antibody worked well, other blockers were not investigated in this exosome system". So, Dr. Qi indicated that they have not yet investigated any other

checkpoint blockers (such as CTLA-4), so the latter could in theory also be involved in the inhibition process.

An example of a new therapy that could in theory be combined with another to make a combination is the following paper: Benitez-Ribas et al., 2018, Immune Response Generated With the Administration of Autologous Dendritic Cells Pulsed With an Allogenic Tumoral Cell-Lines Lysate in Patients With Newly Diagnosed Diffuse Intrinsic Pontine Glioma. *Frontiers in Oncology*, 2018 Apr 26; 8: 127. This was a Phase-Ib clinical trial of 9 patients with diffuse intrinsic pontine glioma (DIPG), a lethal brainstem tumor in children. The authors tested autologous dendritic cell vaccines (ADCVs) pulsed with allogeneic tumor cell-line lysates in newly diagnosed patients following radiation therapy. The DCs were prepared from monocytes obtained by leukapheresis. The authors found that their pulsing procedure boosted non-specific immune responses (KLH) in 9 of 9 patients, and it boosted specific anti-tumor immune responses in 8 of 9 patients, in both PBMCs and T-lymphocytes isolated from CSF. We interviewed one of the two corresponding authors on the paper: **Dr. Andrés Morales La Madrid, MD**, Unidad de Neuro Oncología Pediátrica, Servicio de Oncología y Hematología Pediátrica, Hospital St Joan de Déu, Passeig St Joan de Déu, Barcelona, Spain. When asked his opinion whether his pulsed dendritic cell vaccine approach might be promising for use with other types of immunotherapies (especially combined with checkpoint vaccines), he responded: "Based on the unpublished data from our lab we are considering combining our DC vaccines with CTLA-4 inhibitors". So, indeed he agreed with our assessment that his DC vaccine approach for childhood glioma tumors might work better when combined with a checkpoint vaccine (in his case combined with anti-CTLA-4).

For DC vaccines, using the *ex vivo* priming method usually involves mixing immature DC cells with a tumor lysate. But when priming the DC cells *in vivo* in a patient, it is not clear how the DC cells are primed. One example of this is the article: Garzon-Muvdi et al., 2018, Dendritic cell activation enhances anti-PD-1 mediated immunotherapy against glioblastoma. *Oncotarget*, 2018 Apr 17; 9(29): 20681-20697. Glioblastoma (GBM) tumors strongly suppress the immune system. Checkpoint blockage vaccines (such as antibodies against PD-1) might be useful for overcoming the blockade, but some experiments suggest the checkpoint approach may not be sufficient by itself. The authors investigated the activation of DC cells as a supplement to anti-PD-1 therapy in mice. Their data shows that activating DCs by stimulating the TLR3 receptor with poly(I:C) enhances the PD-1 anti-tumor response, and increases survival in mouse glioblastoma models. DC-depletion experiments showed that DCs are required for the anti-tumor response. We interviewed the corresponding author for the paper: **Dr. Michael Lim, MD**, Professor of Neurosurgery, Oncology, Radiation Oncology, Otolaryngology, and Institute of NanoBiotechnology, Director of the Brain Tumor Immunotherapy Program, Director of the Metastatic Brain Tumor Center, Johns Hopkins University School of Medicine, Baltimore, MD. When asked about moving forward with clinical trials and whether he plans on using poly(I:C) to activate the patient's DC cells, he responded: "Yes, we are considering using polyIC". So, Dr. Lim believes that activating the toll-like receptor-3 TLR-3 using poly(I:C) should work in human patients, and can supplement his anti-PD-1 therapy.

Results for TIL Vaccines

As mentioned previously, our review of the literature indicated that using **combinations of vaccines** is a strong trend in the cancer vaccine field. An example of this using tumor-infiltrating lymphocyte (TIL) vaccines is: Arias-Pulido et al., 2018, The combined presence of CD20^{+} B cells and PD-L1^{+} tumor-infiltrating lymphocytes in inflammatory breast cancer is prognostic of improved patient outcome. *Breast Cancer Research and Treatment*, 2018 June 1. doi: 10.1007/s10549-018-4834-7. In this paper, the authors measured the levels of proteins PD-L1 (checkpoint inhibitor) and CD20 (tumor marker) in 221 biopsies of patients with inflammatory breast cancer (IBC) as potential biomarkers of patient outcomes. Their data showed that the combination of high levels of CD20-positive TILs plus high levels

of PD-L1-positive TILs correlates with patient disease-free survival. We interviewed the corresponding author for the paper **Anonymous**, a cancer vaccine researcher in the Departments of Microbiology and Immunology, Norris Cotton Cancer Center, Geisel School of Medicine at Dartmouth, Lebanon, NH. When asked his opinion whether there are mouse xenograft models for inflammatory breast cancer (IBC), and if so, whether he has tested checkpoint therapy in that model, he responded: "Yes, actually I have a few IBC [inflammatory breast cancer] cell line models that are being used to evaluate small molecule inhibitors. And I'm developing humanized PDX [patient-derived xenograft] models, but we have not used these [PDX] models yet, mainly due to money issues, and the issues of autologous vs. allogeneic immune cells used for those experiments". Thus, the cancer vaccine researcher agrees with our assessment that anti-PD-1 or anti-PD-L1 antibody checkpoint therapy might help supplement his TIL therapy for breast cancer, and he would like to test the therapy combination in mouse models for breast cancer once money is not an issue.

When using TIL vaccines, we found that some researchers isolated and expanded *in vitro* specific TILs that target a particular tumor antigen, while other researchers attempted to amplify all TILs isolated from a patient's tumor (which presumably can target multiple tumor antigens). But is was not clear to us which approach is best. To shed light on this issue, we interviewed **Dr. Emese Zsiros**, of the Department of Gynecologic Oncology, Roswell Park Comprehensive Cancer Center, Buffalo, NY, who is a corresponding author on the paper: Mayor et al., 2018, Adoptive cell transfer using autologous tumor infiltrating lymphocytes in gynecologic malignancies. *Gynecologic Oncology*, 2018 May 23. pii: S0090-8258(18)30920-X. The paper provides an overview of the current clinical results, risks, challenges, and future applications of TIL-based therapy in gynecologic malignancies. When asked her opinion about treating gynecologic tumors and whether the best results are obtained by selecting the TILs against a particular target antigen, or by attempting to amplify all TILs present in a tumor biopsy, she replied: "This is a tough question. It would be easier to just target one specific universal antigen and engineer cells against that antigen, but unfortunately we don't have such a universal antigen expressed by every ovarian cancer cell in every patient and not expressed by normal cells in the body. There are T cells engineered against target NY-ESO for example, but only about 20-30% of ovarian cancer patients express that. Using a diverse group of TILs isolated from tumor tissue is more attractive as they are polyclonal, however there are many patients where there are essentially no T cells in the tumor microenvironment, which makes this approach challenging as well. Also resecting tumor tissue from recurrent disease deep inside the pelvis and or other areas/organs is often challenging and risky in ovarian cancer patients. In an ideal world, finding a good neoantigen target that is specific would be the best, and just target that". So, Dr. Zsiros indicates that each of the two main TIL methods (isolating TILs that target one antigen, or isolating polyclonal TILs from a resected sample that target multiple antigens) have their advantages and problems. Using the first method fails to treat tumor cells that lack the target. A universal target does not exist for ovarian tumors, if it did that would be the way to go. Using the second method with polyclonal TILs is better as it kills a larger variety of tumor cells, but TILs are not always present and isolatable from a tumor, and resecting deep ovarian tumors has its problems.

For breast cancer tumors, obtaining tumor samples via core needle biopsy is increasingly common. But for cancer vaccines, it is unclear whether the biopsy sample is immunologically equal to the main tumor with respect to TIL cells. One paper that addresses this problem directly is: Cha et al., 2018, Comparison of tumor-infiltrating lymphocytes of breast cancer in core needle biopsies and resected specimens: a retrospective analysis. *Breast Cancer Research and Treatment*, 2018 June 5. doi: 10.1007/s10549-018-4842-7. The authors investigated whether the TIL scores in core needle biopsies (CNBs) taken from patients with advanced breast cancer are immunologically representative of those taken from resected specimens. They analyzed 220 matched pairs of CNBs versus their resected counterparts, scoring stromal TILs on slides stained with H&E. The authors concluded that more than five CNB cores accurately predicts the TIL score of the entire tumor. We interviewed the first author on the paper (whose name was forwarded to us from the corresponding author): **Dr. Yoon Jin Cha**, Department of Surgery, Gangnam Severance Hospital, Yonsei University College of Medicine, 211

Eonju-ro, Seoul, Republic of Korea. When asked whether she is aware of any study comparing the anti-tumor therapeutic activity of TILs amplified from core needle biopsies versus TILs prepared from resected specimens, she responded: "In general, high TILs in breast cancer are known to be a positive predictive marker of neoadjuvant chemotherapy in HER2 and TNBC tumors. Among the TILs, CD8+ T-cells may be the most important for the anti-tumor effect, and are known to be elevated in HER2 and TNBC tumors, which may have association with improved treatment response in those subtypes. With respect to your question about whether we are aware of any study comparing the anti-tumor therapeutic activity of TILs amplified from core needle biopsies versus TILs prepared from resected specimens, I am not aware of any. I think that to compare the anti-tumor activity of TILs in biopsies vs resections, each subpopulation of TILs (such as CD8, CD4, FOXP3) should be measured via IHC. And the regional heterogeneity of immune microenvironment should be considered". So, Dr. Cha indicates that, in general, high levels of TIL cells present in a breast cancer tumor predict a good prognosis. She also notes that among the various types of cells present in the TIL population, the presence of high levels of CD8-positive cells (cytotoxic T-cells) have the most important anti-tumor effect. She was not aware of any study attempting to determine whether TILs isolated from breast cancer core needle biopsies are as effective as those isolated from resected specimens, so that can be a future experiment.

Several studies in our review of the literature indicated that therapies with a **high number of TIL cells** provides a better prognosis for the patient. A paper related to this topic is: Huang et al., 2018, Prognostic impact of tumor infiltrating lymphocytes on patients with metastatic urothelial carcinoma receiving platinum based chemotherapy. *Scientific Reports*, 2018 May 10; 8(1): 7485. The authors investigated the prognostic role of TIL levels on patient survival for metastatic urothelial carcinoma (mUC). The patients also received standard platinum-based chemotherapy. They analyzed 259 mUC patients, of which 179 (69%) had intense (high) TILs, and 80 (31%) had non-intense (low) TILs. The median overall survival was 15.7 months for the intense TIL group versus 6.7 months for the non-intense group (p $<$ 0.001). The authors conclude that assaying TIL staining intensity (numbers) for mUC patients is clinically useful for patient risk stratification and counseling. We interviewed the corresponding author for the paper: **Anonymous**, Department of Hematology-Oncology, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung City, Taiwan. When asked whether their data showing that patients with metastatic urothelial cancer with intense TIL staining have a longer overall survival than those with low intense staining could be extended to hypothesize that treating mUC patients with high TIL numbers (perfusing a high number) should be superior than treating with low numbers, he replied: "You are right. The oncologic outcomes of treating mUC [metastatic urothelial carcinoma] patients with high TIL numbers are superior than those of treating with low numbers. The immune micro-environment is essential in the treatment". Thus, researchers have taken the general finding that high TIL numbers in a tumor correlate with good patient prognosis, and extended that finding to treat patients with a high number of TILs.

Another paper on the topic of **high TIL numbers** correlating with good patient prognosis is: Aghajani et al., 2018, Predictive relevance of programmed cell death protein 1 and tumor-infiltrating lymphocyte expression in papillary thyroid cancer. *Surgery*, 2018 Jan; 163(1): 130-136. In this paper, the authors studied the levels of TIL density and the levels of PD-1 which is produced by some tumor cells and binds to receptor PD-1 on T-cells to induce a strong inhibition of TILs that have migrated to the tumor site. The authors investigated the predictive value of assaying PD-1 expression and TIL density in 75 patients with papillary thyroid tumors. Their data showed that PD-1 expression significantly correlated with increased incidence of lymphovascular invasion (P = .038), extrathyroidal extension (P = .026), and concurrent lymphocytic thyroiditis (P = .003). In the TIL population, a low presence of CD8+ and CD3+ (cytotoxic T-cells) correlated with a significantly higher incidence of lymph node metastasis (P = .042) and extrathyroidal extension (P = .015). A high density of CD8+ TILs was significantly associated with favorable disease-free survival (P = .017), and the shortest patient survivals occurred in patients with high PD-1, and low CD8. We interviewed the corresponding author on the paper: **Anonymous**, Thyroid Cancer Group, Ingham Institute for Applied Medical Research, Liverpool, NSW, Australia; and the School of Medicine, Western Sydney University, Campbelltown, NSW,

Australia. When asked "based on the findings of your paper that patients with papillary thyroid cancer with high PD-1 expression and low CD8+ cells in the tumors have poor prognosis, we assume it could be hypothesized that treating such patients with a combination of a high number of TILs (containing CD8+ cells) and anti-PD-1 checkpoint therapy might benefit those patients?" She replied: "It is a possibility, and there have been some studies investigating the potential of combining PD-1/PD-L1 targeted immunotherapies with other treatments to increase CD8 T-cell populations. However, the combination therapy has yet to be investigated in patients with thyroid cancer. So, she agreed that for her papillary thyroid cancer patients with high PD-1 expression and low TIL CD8+ cells (and very poor prognosis), those patients might benefit from a combination therapy of a high number of TILs (containing CD8+ cells) and anti-PD-1 checkpoint therapy, although to her knowledge that experiment has not yet been done.

With respect to the topic of **TIL numbers** versus patient prognosis, some scientists have observed a difference between a high number of infiltrating *neutrophils* versus a high number of *CD8+ cytotoxic killer cells*. An example is the paper: Liu et al., 2018, The prognostic values of tumor-infiltrating neutrophils, lymphocytes and neutrophil/lymphocyte rates in bladder urothelial cancer. *Pathology Research and Practice*, 2018 May 20. pii: S0344-0338(18)30352-2. In this paper, the authors investigated the roles of tumor-infiltrating neutrophils (TINs), tumor-infiltrating lymphocytes (TILs), the neutrophils/lymphocytes ratio (NLR), and clinical outcomes in patients with bladder cancer (BC). They analyzed 102 bladder cancer patients. Immunohistochemistry was used with CD66b antibodies to score neutrophils, and with CD8 antibodies to score lymphocytes. Their results indicated that high TINs and high NLR ratios associated with poor overall patient survival, while higher TILs correlated with longer survivals (P < 0.01). We interviewed the corresponding author on the article: **Dr. Erlin Sun**, Department of Urology, Tianjin Institute of Urology, The 2nd Hospital of Tianjin Medical University, Tianjin, China. When asked his opinion of why the high tumor-infiltrating neutrophil count correlated with poor patient outcome, while high TILs correlate with better patient prognosis, he replied: "We know that neutrophils can be polarized into either an anti-tumoral (N1) or a pro-tumoral (N2) phenotype, so they show different functions. N1 neutrophils are anti-tumoral effector cells by inducing cytotoxicity, mediating tumor rejection and anti-tumoral immune memory. In contrast, N2 phenotype neutrophils support tumor progression by promoting angiogenesis, invasion, metastasis and immunosuppression. According to the results of our study, we suspect that tumor-infiltrating neutrophils have pro-carcinogenic [N2 phenotype] effects on tumor progression. However, tumor-infiltrating CD8+ lymphocytes are found to be favorable prognostic factors in our study, which may play a role in tumor suppression by immune process. Our results are just a clinical retrospective analysis, the mechanism still need to be researched". So, Dr. Sun thinks that the reason a high tumor-infiltrating neutrophil count correlates with poor patient prognosis is that the infiltrating neutrophils are of a N2 phenotype that is pro-tumorigenic. N2 neutrophils promote angiogenesis, invasion, metastasis, and immunosuppression. So, it will be important in future studies to assay for exactly which types of immune cells have migrated to the tumor site.

Results for CAR Vaccines

A problem noted throughout our Lit Review is the induction of **side-effects** caused by the vaccine. In the case of CAR vaccines, the side-effects can be caused by the presence of target antigen in normal tissues (so the T-cells target and kill those normal cells). An example of this is the paper: Richman et al., 2018, High-Affinity GD2-Specific CAR T Cells Induce Fatal Encephalitis in a Preclinical Neuroblastoma Model, *Cancer Immunology Research*, 2018 Jan; 6(1): 36-46. In this article, the authors studied neuroblastoma xenograft mouse models with a vaccine containing GD2-CAR T-cells. The CARs showed enhanced anti-tumor activity, but in some cases the treatment caused death of the mice. The authors observed brain T-cell infiltration and proliferation (which seems necessary for killing the tumor cells), but the T-cells may have also targeted nearby GD2 in normal brain cells. We interviewed the

corresponding author for the paper: **Dr. Michael C. Milone**, Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania. When asked his opinion whether targeting GD2 in normal cells can be avoided in human patients, he replied: "…The T cell activity in the brain is due to targeting low level GD2 expression on neurons and glial cells. While some scientists believe it is possible to design a CAR T-cell therapy that can distinguish the generally higher expression in tumors compared to the normal expression, I think this will be challenging with current technology". So, Dr. Milone agrees that the sometimes fatal encephalitis he observes in his mouse models treated with GD2-CAR cells likely results from the targeting of GD2 in normal neurons and glial cells expressing low levels of GD2. He believes it will be challenging to design a CAR to distinguish the high GD2 expressors from the low expressors. So, more research should be done identifying antigens almost exclusively expressed in the tumors, if possible.

Another example paper dealing with **CAR-induced side-effects** is Locke et al., 2017, Phase 1 Results of ZUMA-1: A Multicenter Study of KTE-C19 Anti-CD19 CAR T Cell Therapy in Refractory Aggressive Lymphoma. *Molecular Therapy*, 2017 Jan 4; 25(1): 285-295. In this study, the authors designed CAR KTE-C19, an autologous CAR T-cell therapy targeting CD19 that also uses CD3-zeta and CD28 as co-stimulators to treat patients with refractory B-cell leukemia. The CAR was was used to treat 7 patients. 1/7 patients (14%) experienced dose-limiting toxicity of grade-4 cytokine release syndrome (CRS), 1/7 patients (14%) showed grade >3 CRS, and 4/7 patients (57%) showed neurotoxicity. All >grade-3 events resolved within 1 month. 3/7 patients showed clinical responses (stabilizations) at 12 months. To get an idea of the main cause of the side-effects, we interviewed the corresponding author for the paper: **Dr. Frederick L. Locke, MD**, Department of Blood and Marrow Transplantation, Moffitt Cancer Center, Tampa, FL. When asked his opinion on the cause of the neurotoxicity observed in 4 of your 7 (57%) phase-I patients, he stated: "More and more evidence suggests that there is breakdown of the blood brain barrier, so the CAR T-cells get into the CSF where they continue to secrete cytokines. This likely leads to endothelial damage and dysfunction, which appears to be the main driver of CNS toxicity". So, Dr. Locke indicates that the evidence from his lab suggests the neurotoxicity observed with his KTE-C19 CAR therapy is caused by a breakdown of the blood brain barrier (BBB), allowing CAR cells to enter the cerebrospinal fluid (CSF), and in a deteriorating cycle of events the CAR T-cells continue to secrete cytokines in the CSF that causes endothelial cell damage. Thus, the side-effects are not only caused by a targeting of a specific antigen in normal cells, it is also caused by the continued elevated production of cytokines by the T-cells.

The most frequently observed **side-effect** is cytokine release syndrome (CRS). Thankfully, in most cases, the CRS was manageable by treating with corticosteroids and IL-6-receptor blocking antibodies. In order to shed light on how cancer vaccine treatments can cause CRS, we interviewed the corresponding author on a paper where CRS was observed: Young et al., 2018, Activity of Anti-CD19 Chimeric Antigen Receptor T Cells Against B Cell Lymphoma Is Enhanced by Antibody-Targeted Interferon-Alpha. *Journal of Interferon and Cytokine Research*, 2018 Jun; 38(6): 239-254. The corresponding author is: **Dr. John M. Timmerman**, Division of Hematology & Oncology, Department of Medicine, Center for Health Sciences, University of California at Los Angeles, Los Angeles, CA. In this paper, the authors investigated methods for lengthening the survival of CD19-CARs in patients with B-cell lymphomas. Because interferons (IFNs) have the ability to promote T-cell activation and survival, the authors tested whether antibody-targeted IFN therapy could enhance their CAR therapy. They produced a new type of CAR anti-CD20-IFN, containing an antibody-like portion against the CD20 lymphoma target fused with a potent type-1 IFN isoform alpha14 (α14). The combination approach was found to enhance lymphoma cell killing *in vitro*. Pre-treatment of the lymphoma cell lines with the fusion peptide (anti-CD20-hIFNα14) markedly increased cytokine production by the subsequently added CARs, enhancing several CAR activities, but it was unclear to us whether this novel approach might increase the incidence of cytokine release syndrome. When asked whether his new anti-CD20-hIFNα14 treatment might cause increased cytokine release syndrome, Dr. Timmerman replied: "Maybe in some patients. But there are many patients that don't have a clinical response [to the CD19-CAR] or much CRS, so if we

knew how to predict those patients, adding the [anti-CD20-hIFNα14] fusion protein might help them". So, Dr. Timmerman agreed with us that his new drug could indeed increase the incidence of cytokine release syndrome, but that the drug is strongly needed to treat the lymphoma patients that do not respond to CD19-CARs, as it targets a different antigen (CD20) on the lymphoma cells, so this warrants its use.

Sometimes **tumor location** is important. For example, solid tumors are difficult to treat with cancer vaccines due to their location and general inaccessibility. Over the past decade, the use of CAR cells has led to strong improvements in patients with hematopoietic malignancies which are readily treated by systemic infusions on of the CAR cells. But CAR treatment of solid tumors is hindered by challenges inherent to an organized tumor mass, such as abnormal vasculature, migration through a dense stroma, and an elevated tumor interstitial pressure (IFP). Some scientists are trying to overcome these problems by performing a localized delivery of the CARs directly to the tumor. An example of this is the paper: Hardaway et al., 2018, Regional Infusion of Chimeric Antigen Receptor T Cells to Overcome Barriers for Solid Tumor Immunotherapy. *Journal of Vascular and Interventional Radiology*, 2018 Jul; 29(7): 1017-1021. The authors take advantage of the fact that hepatic tumors derive their blood supply from the hepatic arterial circulation (where the scientists deliver CARs), while normal hepatocytes mainly subsist off the portal circulation (not encountered by the CARs). This regional delivery approach has already been used with chemotherapeutic, radiotherapeutic, and chemoembolic agents for liver tumors. The regional approach has also been used for the treatment of of hepatic and pancreatic malignancies with dendritic cells, macrophages, or lymphokine-activated killer cells (LAKs). We interviewed the corresponding author for the paper: **Dr. Steven C. Katz, MD, FACS**, Associate Professor of Surgery, Director, Complex General Surgical Oncology Fellowship, Director, Office of Therapeutic Development, Roger Williams Medical Center, Providence, Rhode Island. When asked his opinion about whether his regional CAR delivery method would not only treat the cancer but help diminish the adverse side-effects, he replied: "We believe the overall therapeutic index would be enhanced, in general, for regionally infused products". So, Dr. Katz agreed that his regional method for delivering CAR T-cells to solid tumors not only reduces the tumors better, it also decreases the adverse side-effects.

With respect to CAR vaccines, some researchers are working on new methods for delivering the CAR receptor gene to the T-cells without using lentiviruses (**non-lentiviral delivery methods**). The lentiviruses tend to integrate at random sites and can be harmful. A new technique delivers the RNA encoding the CAR receptor directly to the T-cells, as seen in the paper: Svoboda et al., 2018, Non-viral RNA chimeric antigen receptor modified T cells in patients with Hodgkin lymphoma. *Blood*, 2018 Jun 20. pii: blood-2018-03-837609. The authors treated 4-5 patients with classical Hodgkin lymphoma (cHL) using CAR T-cells. To limit potential toxicities, they used non-viral RNA CART19 cells, which the authors expected to express the CAR receptor protein only for a few days off the delivered RNA, as opposed to CARs generated by viral vector transduction which expand *in vivo* and retain CAR expression via the integrated DNA. Their results showed no severe toxicities. To our knowledge, this is the first CART19 clinical trial to use non-viral RNA gene delivery. But it was unclear to us how efficient the RNA technique was relative to the highly efficient lentivirus technique, so we interviewed the corresponding author on the paper: **Dr. Jakub Svoboda**, Lymphoma Program, Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA. When asked what percent of the patient's T-cells can be treated with the RNA technique versus treatment with a lentivirus encoding the CAR gene, he replied: "That is a good question, but difficult to answer since every patient is different. [In our study] the number of infused modified RNA CART19 cells varied (the dose was based on weight, the protocol allowed for a range). These cells [RNA-treated CAR cells] are transient (last few days, do not expand) and they likely represent only a tiny portion of the lymphocytes in relation to the total number in the human body (the total number of lymphocytes in the human body is estimated to be about $2x10^{12}$). For the lentivirus transduced CART19 cells, these tend to expand *in vivo*, but the expansion varies from patient to patient, but again - likely a tiny portion". So, Dr. Svoboda indicated that it is difficult to determine the percent of T-cells transduced with his RNA technique to deliver the CAR gene versus using a lentivirus, but in any

case, the end numbers vary for each patient depending on the extent of CAR expansion, not just based on the percent transfected.

Results for Checkpoint Inhibitor Treatments

One of the recent trends in the checkpoint vaccine field is uncovering various ways that some tumors are **resistant to checkpoint therapy**. For example, an exciting find in 2018 was the discovery that a high level of TGFβ hormone in the tumor micro-environment acts as a primary mechanism of immune evasion for tumor cells (Tauriello et al., TGFβ drives immune evasion in genetically reconstituted colon cancer metastasis, *Nature*, 2018 Feb 22; 554: 538-568). In order to gain information on the feasibility of using a combination of a checkpoint therapy with anti-TGFβ antibody, and determining how TGFβ levels might be assayed in patients, we interviewed the first author on the article (whose name was provided by the corresponding author): **Dr. Daniele Tauriello**, Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, Barcelona, Spain. When asked her opinion of whether a clinical trial might be feasible combining a checkpoint inhibitor vaccine with anti-TGFβ antibody, she replied: "We think that, at least in CRCs [colon cancer metastases], high levels of TGFβ prevent or strongly diminish a full activation of T cells. Furthermore, T cell infiltration is low, which could be a direct result of T cell dysfunction, but might also be a separate mechanism by which TGFβ affects immune evasion. If these are the two main ways in which high TGFβ levels suppress anti-tumour immunity, then inhibition of the TGFβ pathway (for example with TGFBRI inhibitor Galunisertib), should lead to full activation of T cells….. and a combined inhibition of stromal TGFβ signalling with blockade of the PD-1/PD-L1 checkpoint could be an effective way to overcome immune evasion in patients with advanced/metastatic CRC". When further asked whether there is an assay for clinicians to quantitate the levels of TGFβ in the tumor microenvironment (to predict which patients might benefit from the combined therapy), she responded: "….in our lab's previous papers we showed that high TGFβ mRNA levels strongly predict poor prognosis in stage I+II+III patients, so if primary tumours could be removed by surgeons and analysed for TGFβ mRNA levels (or analyzed for TGFβ-controlled gene expression levels), …we expect that the combined insight will lead to more power to clinically intervene". So, Dr. Tauriello indicated that the way to measure TGFβ levels in patients would be for the surgeons to remove the primary tumors, and then for lab personnel to assay for for TGF-beta gene transcription levels (presumably using something like RT-PCR). The high TGFβ patients would have a poorer prognosis but should respond to their dual therapy.

All cancer immuno-therapies have **side-effects**, and checkpoint inhibitor treatments are no exception. But the presence of side-effects does not necessarily mean the drugs should be discontinued. In most cases, the side-effects observed were mild and transient, and are likely far outweighed by the poor prognosis of a relapsing cancer patient. To gain more insight on the topic of checkpoint inhibitor side-effects, we interviewed an expert on liver injuries induced by checkpoint inhibitor treatments, **Dr. Eleonora DeMartin**, Centre Hépatobiliaire, Hôpital Paul Brousse, Groupe Hospitalier Paris Sud, DHU Hepatinov, Villejuif, France. Dr. DeMartin's contact information was provided by the corresponding author of the paper: De Martin et al., 2018, Characterization of liver injury induced by cancer immunotherapy using immune checkpoint inhibitors, *Journal of Hepatology*, 2018 Jun; 68(6): 1181-1190. When asked her opinion of whether the benefits of checkpoint inhibitor therapy outweigh the side-effects caused by the treatments, she replied: "I agree that most of the immune-mediated hepatitis side-effects provide a far better prognosis than the prognosis from cancer relapse/progression. Liver injury induced by cancer immunotherapy either improves spontaneously, or it responds to corticosteroid therapy in most of the patients. Interestingly, it seems that patients who develop immune-related adverse-effects (iRAEs) better respond to therapy. However this finding needs to be confirmed". When asked whether there is any way for a clinician to predict which patients are more likely to develop liver injuries following checkpoint therapy, she replied: "No, unfortunately we haven't been able to identify predictive factors for hepatic

IRAEs. We are running a multidisciplinary study now in order to answer this question". When asked whether she thinks that autoimmune diseases resulting from checkpoint inhibitors are severe enough and common enough to prohibit inhibitor usage, she replied: "The answer is no. They remain a rare complication, and in most cases they are not severe. Considering the revolutionary results of cancer immunotherapy I encourage its use". So, Dr. DeMartin, indicated that in the case of her patients treated with checkpoint therapies, the side-effects are minor or rare, and are less important than attempting to save the patient's life from a relapsing cancer.

Continuing on the topic of **side-effects** caused by checkpoint therapies, we interviewed **Dr. Michael A. Postow**, Memorial Sloan Kettering Cancer Center and Weill Cornell Medical College, New York. Dr. Postow was corresponding author on: Postow MA, Sidlow R, Hellmann MD (2018) Immune-Related Adverse Events Associated with Immune Checkpoint Blockade. *New England Journal of Medicine*, 2018 Jan 11; 378(2): 158-168. When asked his opinion about whether the side-effects are outweighed by the potential benefits of the checkpoint therapy, he replied: "I think the benefits dramatically outweigh the possibility of side-effects. Untreated metastatic cancer invariably leads to death at some point. Immunotherapies offer patients the hope that they can keep their cancer under control for a long time if they respond well. This is an amazing potential benefit and well worth the possibility of side-effects. And even if side-effects occur, they can be well managed and do not usually lead to permanent disability. The side-effects usually resolve within weeks to months". When asked whether there is any way for physician's to determine which patients will develop side-effects, he responded: "Unfortunately we do not really know. There are many research programs going on to identify predictive factors that are associated with immunotherapy side-effects. It may have to do with germline genetics, or with other "host" immunologic factors. Some people think that patients with less heavy of a burden of cancer are more likely to have side effects, perhaps because these patients with lower tumor volume are less immunosuppressed". And when asked his opinion about whether there is a subset of patients that should absolutely not receive checkpoint therapy, such as immunocompromised patients or patients on immunosuppressant drugs, he replied" Although efficacy may be a little lower in patients on immunosuppressants or otherwise immunocompromised, since these patients have been shown to benefit from immunotherapy in some situations, I think it is reasonable to try immunotherapy when no other good cancer treatments are available". Thus, Dr. Postow is a strong advocate of attempting to use immunotherapies on patients with otherwise very poor prognoses, even if some side-effects develop.

One serious side effect of checkpoint inhibitor therapy is the occasional development of **T-cell tumors**. The point of checkpoint therapy is to activate the patient's inhibited T-cells, and sometimes tumors develop. A paper covering this topic is: Ludin and Zon, 2017, Cancer immunotherapy: The dark side of PD-1 receptor inhibition. *Nature*, 2017 Dec 7; 552(7683): 41-42. doi: 10.1038/nature24759. We interviewed **Dr. Aya Ludin Tal**, Postdoctoral Fellow, Zon Lab, Harvard Department of Stem Cell and Regenerative Biology, Harvard Stem Cell Institute, Harvard University, Cambridge, MA. Dr. Tal's contact information was provided by Dr. Zon, the corresponding author on the paper. When asked whether there is any way that physician's can determine which patients will develop T-cell tumors, she replied: "…. In some cases, the patient already has a T-cell tumor, [and in these cases] various options of treatment should be considered. ….If their T cells are cancerous, it might not be a good idea to expand this population of cancer cells by driving them into proliferation using PD-1. PD-1 inhibition is not yet used in hospitals with patients with T cell-based cancers, but since it is gaining interest in multiple types of cancers, the authors warn against possible side effect that is not seen in other types of cancer". Their paper also showed that anti-PD-1 activates different subsets of T-cells, so we asked her which types are more effective for their patients. She replied: "Different subsets of T cells have different roles in fighting or protecting cancer. Cytotoxic [CD8+] and helper cells [CD4+] help fight tumors, whereas regulatory T cells [T-reg] can protect the tumors". And when asked whether the overall risks of immunotherapy are different or on par with other cancer treatments like radiation or surgery, she replied: Each treatment has its own risks, so it's hard to say. It also depends on the type of immunotherapy used, each has its own

risk, such as the induction of autoimmune effects through CAR T-cell administration (uncontrolled T cells). The risks of the different immunotherapies are still being investigated. However, anti PD-1 and anti-CTLA-4 therapies have already been approved clinically for many types of cancers, suggesting that the benefit for the patient is higher than the risk. The efficiency of treatment, whether it be immunotherapy, surgery or irradiation is an important factor considered, and many times these treatments are combined together". Thus Dr. Tal indicated that checkpoint therapy is not yet used clinically for patients with T-cell tumors (as it could further activate the tumors), but warns the current emphasis on expanding checkpoint therapy to other types of cancers should consider the effects on T-cells. And with respect to the overall risk, she pointed out that checkpoint therapies have already received FDA approval, so a panel of experts have already determined that the benefits outweigh the risks.

An interesting recent finding in the checkpoint inhibitor field is the discovery that the **gut microbiome** (the type and quantity of bacteria present in the gut) strongly affects the response to checkpoint therapy. An example is the article: Gopalakrishnan et al., 2018, Gut microbiome modulates response to anti-PD-1 immunotherapy in melanoma patients, *Science*, 2018 Jan 5: 359(6371): 97-103. We interviewed the first author on this paper **Dr. Vancheswaran Gopalakrishnan**, Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA. Dr. Gopalakrishnan's contact information was provided by the corresponding author on the paper. One of the findings of the paper was that patients with a gut microbiome enriched for *Ruminococcaceae* family bacteria respond better to anti-PD-1 therapy, so we asked Dr. Goplakrishnan whether they plan to move forward with clinical trials by supplementing patient microbiomes with *Ruminococcaceae* microbes? He replied: "We are planning a clinical trial to test the hypothesis that modulation of the gut microbiome will enhance therapeutic responses. Several strategies will be used, including fecal microbiota transplant and designer probiotics that enrich for favorable bacteria……. FMT [fecal microbiota transplants] have already proven to be successful for treatment of *Clostridium difficile* infections, but there is limited data for oncology [and FMTs]. In addition to the above, one may also consider lifestyle changes such as dietary modifications to modulate/maintain the microbiome". When asked how difficult it is to assay a patient's microbiome, he replied: "This can be done in several ways. The most commonly used approach is called 16S rRNA sequencing. The 16S gene is a ubiquitous bacterial marker that has both conserved and variable regions. The conserved regions act as targets for PCR-primers. The variable regions can be amplified, sequenced and compared with reference databases to identify bacterial diversity and composition. Another approach is whole genome shotgun sequencing. This gives greater resolution of bacteria at the species level and also gives a sense of their functional capabilities. But sequencing takes time, with a turnaround of approximately 1 month, so this is not yet feasible at a per-clinic basis. But this is surely something that people are invested in". Thus, Dr. Gopalakrishnan provided some very useful information on: 1) how indeed they are moving forward to test their hypothesis that modifying the gut microbiome affects the success of PD-1 therapy, 2) there are several different ways they plan to modify the microbiome in patients (including fecal transplants and probiotics), and 3) the methods for assaying species type and number in the biome include sequencing ribosomal RNAs, and performing bioinformatics to determine the species type and abundance.

CONCLUSIONS and RECOMMENDATIONS

Based on the research performed for this IQP project, our team has developed several conclusions and recommendations. We focused on two main problems prevalent in the area of cancer vaccines: 1) the fact that only a subset of patients respond to a particular therapy, and 2) the fact that most cancer vaccines cause adverse side-effects. We emphasized new advances in the field for overcoming these problems.

Cancer Vaccine Non-Responding Patients

With respect to the topic of **non-responding patients**, our research indicates that tumors resistant to cancer vaccine treatments occur for all types of cancer vaccines and for all types of cancers. These tumors are non-responsive for a variety of reasons. Some of the most important reasons are discussed below along with potential solutions:

1) Down-Regulation of the Target Antigen: The major mode of resistance of some tumors to immune therapy is a loss of target antigen on the surface of the tumor cells. For reasons that are not yet understood, in some cases a patient's tumor stops making the target antigen, or lowers its expression. When this occurs, a potential remedy is to follow the initial failed therapy with a different one that targets another antigen, if available. This combination approach is most feasible for the lymphomas and leukemia where multiple target antigens are already available. One of the best practices we observed in our review of the cancer vaccine clinical trials was the constant monitoring of target antigen expression on the tumor. This is easily done by using immunohistochemistry (IHC) to measure the levels of target protein, or by using reverse transcriptase polymerase chain reaction (RT-PCR) to measure the levels of target mRNAs. The best patient prognoses were observed for patients with tumors strongly expressing the target antigen.

2) Tumor Heterogeneity: A recent discovery in the cancer field is that some tumors are not homogeneous; they consist of regions that differ from each other. In some cases, portions of the tumor may lack expression of the target antigen, so those cells will not be killed by the vaccine and will continue growing. In other cases, different areas of a tumor express different neo-antigens (new proteins made by a tumor caused by different DNA mutations over time). In these cases, different areas of the tumor are genetically distinct from each other. Each has their own growth rates, metabolic pathways, and overall aggression. In these cases, if it is possible to resect genetically different parts of the tumor without harming the patient, perhaps different tumor regions can be identified with their corresponding antigens, then the patient could be treated with a combination vaccine against the antigens of each section.

3) Immunosuppression Induced by the Tumor: In some cases, the patient's tumor inactivates the patient's immune system, lowering removal of a cancer (or blocking a cancer vaccine). In some cases, this inactivation occurs via checkpoint inhibition on nearby T-cells to keep them in check. This immuno-suppression keeps the T-cell from killing the tumor. The discovery of key components of checkpoint inhibition (including PD-1, PD-L1, CTLA-4) has in recent years allowed the use of antibodies against the components to re-activate the patient's immune system. This type of cancer "vaccine" has increased exponentially in the past few years. A very recent 2018 discovery is the strong role of TGFβ in the tumor microenvironment at inhibiting T-cells or DCs that have migrated to the tumor, and this finding opens up the possibility of treating these resistant patients with anti-TGFβ antibodies, or with antibodies that block TGFβ signaling.

4) Immunocompromised Patients: In some cases the patient is immunocompromised due to their pre-treatment with chemotherapy (which destroys actively dividing cells in the body). In these cases, the patient may lack the necessary immune components for killing the tumor. In the case of monovalent

antibody treatments, the binding of the vaccine antibody to the tumor cell by itself does not kill the cell, and other components of the immune system must recognize the bound antibody and help kill the cell. In the case of bispecific antibodies designed to bind the target antigen on the tumor cell while also binding to a T-cell to bring them together, an immunocompromised patient may lack a sufficient number of T-cells to be recruited to the site. So, perhaps these patients could be treated with an ADC vaccine or a CAR T-cell vaccine, which by themselves can kill a tumor cell.

5) Short Vaccine Half-Life: In some cases, the cancer vaccine was ineffective in a patient due to a short half-life. This was especially a problem with passively administered antibody vaccines that are rapidly cleared from the body. A recent trend with antibody vaccines is to encode the antibody genes in a deliverable vector (such as a harmless virus), and deliver the vector to the patient's bloodstream. The DNA continues to express the antibody gene far longer than with a delivered protein antibody. The short half-life of some CAR T-cell vaccines appears to have resulted from a lack of co-activation of the T-cells, but this problem has now been remedied through the inclusion of co-stimulation domains on the engineered CAR receptor (such as CD28 or CD3-zeta).

6) Altered Tumor Growth Pathways: In some cases, a tumor became resistant to therapy when the tumor mutated to switch growth pathways. Thus, the original vaccine treatment attempting to block the pathway failed. In these cases, perhaps the tumor resistance could be overcome by switching drugs to block the new growth signaling pathway the tumor now depends on. This is especially a problem when using antibody vaccines to block specific receptors. If the tumor is no longer dependent on that particular signaling pathway, blocking the pathway's receptor will not prevent tumor growth.

Cancer Vaccine Side-Effects

With respect to the topic of **side-effects**, our research shows that all types of cancer vaccines cause adverse side-effects. The side-effects varied considerably, depending on the type of treatment and the type of cancer, ranging from very mild and transient, to patient death in a few cases.

1) Patient Deaths: In clinical trial, three patients died from *E. coli* or *Candida* sepsis caused by treatment with a CD19 x CD3 bispecific antibody that eliminated B-cells from the leukemia patients, hindering their ability to make antibodies against the endogenous pathogens. And seven patients died in one year (2016) in CAR clinical trials when the CAR cells caused fatal brain swelling. So, patient deaths indeed occasionally occur with cancer vaccines, and these deaths are not unexpected for patients with relapsed cancers. However, the patient deaths are rare from the vaccines, and most of our interviewees argued are worth the cost of trying to save lives from certainly fatal relapsing cancers.

2) Cytokine-Release Syndrome: The second most serious side-effect seen with cancer vaccines is cytokine release syndrome (CRS). CRS is a hyper-elevation of cytokine hormones that typically cause fevers, hypotension, and hypoxia, but can be deadly when it causes organ failure. Although the cytokine elevation is actually a desired outcome of cancer vaccines as an outcome of immune system activation, the activation can become too prolonged and harm the patient. CRS was sometimes seen in the antibody therapy trials, and in the adoptive T-cell therapies. In a large trial of patients with aggressive non-Hodgkin's lymphoma, about 18% of the patients developed CRS. But in most cases, the CRS was treatable with corticosteroids (to lower inflammation) and with cytokine blocking antibodies (such as anti-IL6-receptor antibody), so it was not usually life threatening.

3) Autoimmune Disorders: In the case of checkpoint vaccines, which are designed to ramp up the patient's immune system, one of the more serious side-effects observed was autoimmune disease (where the patient's immune system attacks the body). This is especially a problem with autoimmune myocarditis, an immune attack on the heart, which can be fatal. While we identified a few papers that

warned of such events, our interviews with physicians performing these therapies indicated they saw these disorders only rarely in their patients.

Scientists are still trying to determine what causes the side-effects, but they appear to be caused by a variety of mechanisms. In some cases, they appear to be caused by expression of the target antigen in normal cells, thus those cells are destroyed by the therapy. Our interviews with scientists who designed vaccines showed that designing a cancer vaccine that can only target a tumor cell expressing high antigen levels while ignoring normal cells with medium expression levels of the antigen is not currently possible. So, we recommend continuing the search for new target antigens that are only found in tumor cells and not in normal cells.

In other cases, the killing of normal cells in the body appears to have resulted from a "bystander effect". In this case, the normal cells are killed due to their location near a targeted cell. This is especially the case for antibody-drug conjugate vaccines (ADCs) where the highly cytotoxic drug can be released nearby normal cells. While this killing could be beneficial if the bystander cell is a tumor cell no longer expressing target antigen, in most cases the bystander effect is against normal tissue.

Overall, the side-effects in the vast majority of cases were relatively mild grade-1 and -2 events, transient (lasting only a few days), and treatable. The side-effects were especially treatable if they were detected early, so these events should be constantly monitored throughout the entire treatment. With respect to whether the chance of side-effects should deter physicians from using these treatments, the individuals interviewed for our project were strongly in favor of continuing the treatments, arguing that the prognosis from the side-effects was far better than very poor prognosis they patients face from the relapsing cancer. The side-effects they observed were relatively minor or rare, and are less important than attempting to save the patient's life from their relapsing cancer. As stated previously, most of the patients participating in these cancer vaccine trials have run out of other treatment options.

Future Trends

We have observed several trends in the cancer vaccine field that hopefully will help solve some of the problems mentioned above.

1) Combination Therapies. The simultaneous or consecutive use of two types of therapies has increased drastically in the past few years. In some cases, the physicians are attempting to treat patients that have down-regulated one target antigen by using an agent that targets a different antigen. In other cases, the combination approach is designed to kill the tumor by two different mechanisms which hopefully will synergize. A good example of this is the combined use of a checkpoint vaccine (to remove the immuno-suppression caused by the tumor) with a second vaccine to target the tumor (such as a DC, TIL, or CAR).

2) Altered Method of Cell Killing: In some cases, tumor remission was achieved by switching from recruiting T-cells to the tumor, to recruiting natural killer (NK) cells to do the killing. This has especially been effective with bispecific antibodies, where one domain is against CD16A on NKs (to recruit them) while the other domain is against CD30 (targeting the NKs to the lymphoma cells). The approach was successful and is now being tried in other tumors.

3) Careful Patient Selection: Due to some of the mechanisms discussed above for tumors blocking the immune system, a recent strategy is to closely monitor the patient's tumor for potential upregulation of inhibitors such as TGFβ, PD-1, PD-L1, or CTLA-4. When any of these are found to be elevated in the tumor microenvironment, the appropriate modifier should be delivered, such as delivering antibodies against TGFβ signaling if TGFβ is found to be elevated.

4) Personalized Medicine: Neo-antigens are newly expressed proteins (or carbohydrates) on the surface of tumor cells that form following DNA mutations. Because neo-antigens are not expressed during human development, they are viewed as foreign in the body once they are expressed, so they make good target antigens. The use of new rapid DNA sequencing methods allows a patient's tumor cells to be sequenced to analyze for neo-antigen formation, and if found, these could be used to prime DC or TIL vaccines. But the process needs to be fast, as some patients have a very poor prognosis, and can die within weeks.

5) CRISPR/Cas9 Editing of CAR T-Cells: One of the most promising and recent innovative uses of CAR cells combines CAR receptor engineering with the use of CRISPR/cas9 gene editing technology to eliminate a host gene. For example, the CRISPR system has been used to eliminate the gene TRAC which is associated with allo-recognition in the body. Thus, its elimination could result in "universal" CAR cells that could be used in any patient without rejection, eliminating the need to isolate the T-cells from each patient.

6) Gut Microbiome: An interesting recent finding in the checkpoint inhibitor field is the discovery that the gut microbiome, the type and quantity of bacteria present in the gut, strongly affects the response to immune checkpoint therapy. The microbiota from mice non-responsive to checkpoint therapy when transplanted into naïve mice induced them to become non-responders, and microbiota from responder mice when transplanted into naïve mice made them become responders. One research team found that mice with a gut microbiome enriched for *Ruminococcaceae* type bacteria respond better to checkpoint therapy. Our interviews with the lead scientist of this study indicated he plans on moving into clinical trials, either by supplementing the patient's diet with this type of bacterium, or by using fecal microbiota transplants (FMTs) enriched for this bacterium.

7) TIL Numbers and Composition: Several studies have shown that the presence of a high number of tumor-infiltrating lymphocytes (TILs) in a patient's tumor correlates with a better patient prognosis. This finding has now been extended to show that treating patients with a high number of TILs is more effective than using low numbers. In addition, we learned from our interviews that it is important to determine the composition of the patient's TILs, because a high number of infiltrating CD8+ (cytotoxic T-lymphocytes) correlates with a good prognosis, while a high number of infiltrating neutrophils correlates with a poor prognosis. It was recently shown that infiltrating neutrophils are of an N2 phenotype which is pro-tumorigenic (promoting tumor angiogenesis, invasion, metastasis, and immunosuppression). So, it will be important in future studies to assay for exactly which types of cells have migrated to the tumor site.

8) Tumor Location: Diffuse tumors such as lymphomas and leukemia have been treated by the intravenous perfusion of cancer vaccines for several years now. But solid tumors are difficult to treat due to challenges inherent to an organized tumor mass, such as abnormal vasculature, migration through a dense stroma, and an elevated tumor interstitial pressure (IFP). It was recently shown that some of these problems can be overcome by using a localized delivery of the vaccine directly into (or nearby) the solid tumor. Our interviews indicated this localized delivery approach might also lower the incidence of side-effects, as fewer tissues in the body would encounter the vaccine.

Overall, we conclude that cancer vaccines represent a fascinating method for fighting cancer, and in some cases these approaches have provided complete remissions for relapsing cancers that are impossible to treat using any other approach. The cancer vaccine field is complex (with treatments ranging from simple antibody treatments, to genetically modifying T-cells), but the field provides a variety of approaches for potentially treating a patient's tumor. While each type of therapy can induce side-effects in a portion of the patients, we agree with our interviewees that the side-effects are usually minor, transient, and treatable, and are far less important than attempting to save the patient's life from their relapsing certainly fatal cancer.

APPENDIX

SAMPLE QUESTIONS

1. Cancer Vaccine Side-Effects:
 A. Do you agree that most cancer vaccines cause side-effects?
 B. In your clinical trials have you observed any serious (grade-3 and grade-4) side-effects?
 C. If the side-effects were serious, were they at least *treatable* (manageable)?
 D. If the side-effects were manageable, does the potential for a cure for a patient with a fatal disease *outweigh* the risk associated with a potentially fatal side-effect?
 E. In your opinion, how can cancer vaccines be *improved* to cause fewer side-effects? Do we need more research to identify target antigens on different types of cancers that are not present in normal cells? Would a *personalized medicine* approach to identify patient-specific neo-antigens on a patient's tumor help decrease off target side-effects?

2. Lack of Vaccine Efficacy:
 A. In some cases, a cancer vaccine appears to not work in a particular patient. In your opinion, what kinds of events can cause this: loss of expression of the target antigen? Inactivation of the perfused T-cells by the patient's tumor cells? Lack of vaccine accessibility to a solid tumor site?
 B. For patients who do NOT respond to a cancer vaccine treatment, do you think it would be beneficial to test a *combination* treatment with two or more vaccines that work by different mechanisms?

3. Best Correlates of Protection:
 A. It is not clear to us what factors best correlate with a vaccine's success. For TIL and CAR T-cell vaccines, some studies indicate success correlates best with a *high load* of TIL or CAR cells. This suggests that developing methods for amplifying the number of T-cells perfused into the patient should be a high priority. Other studies show the worst side-effects occur at the highest T-cell doses. What is your opinion?
 B. TIL vaccines isolated from a patient sometimes contain a mix of T-cells targeting a variety of surface antigens, while engineered CAR vaccines typically target one key antigen. What is your opinion about which strategy is best? Would the best approach vary from patient to patient?
 C. Some studies with TIL vaccines suggest that enriching and amplifying the T-cells from the patient that are directed against a specific target antigen provide the best prognosis, while other students suggest that using a *mixture* of TILs against a variety of antigens is most successful. What is your opinion? How would we know which procedure to use in advance?

4. Clinical Trial Patients:
 A. Early cancer vaccine clinical trials have been criticized as having a relatively low number of patients. Lately, the number of patients in cancer vaccine clinical trials has grown exponentially to the point that some people worry there might not be a sufficient number of refractory cancer patients to enroll in the trials. Do you think this is a problem?

5. Patient Chemoablation Treatments:
 A. For TIL and CAR T-cell vaccines, some studies indicate that pre-treating the patient with chemotherapy to obliterate (chemoablate) the patient's own immune system prior to

infusing the T-cell vaccine is critical for eliminating cells that can inhibit the vaccine. Other studies indicate this pre-treatment is not needed. In your opinion, what dictates when chemoablation should be used?

6. Immune Checkpoint Vaccines:

    A. It appears that a recent trend in the cancer vaccine field has been to use *combination vaccines* of 1) a checkpoint inhibitor plus 2) a vaccine against a specific target antigen. Do you think this combination approach has been mostly successful?

    B. What do you think of the recent failed Incyte Phase-III clinical trial using a PD-1 inhibitor (Opdivo) plus an inhibitor of the enzyme IDO? Their phase-I and II data looked very promising, but the phase-III data with the combination was no better than Opdivo alone. What is your opinion?

    C. Immune checkpoint vaccines have been shown in some cases to cause an over-activation of the immune system (such as inflammation). Do you now routinely monitor for these effects?

7. Personalized Medicine:

    A. Some scientists argue that cancer surface antigens vary from patient to patient, thus using a personalized medicine approach to identify a patient's own surface antigens is better than using a vaccine that targets a generic surface antigen. But how expensive is this personalized approach? It must be expensive to sequence the DNA from a patient's tumor to identify "neo-antigens" present on that particular tumor.

    B. How long does a typical DNA genome (exome) sequencing project take? Our readings indicate it can take weeks to sequence the DNA, analyze it for neo-antigen formation, and synthesize the neo-antigens chemically. Some patients don't have weeks to live, so should the speed of this approach be accelerated?

INTERVIEW PREAMBLE

We are a group of students from the Worcester Polytechnic Institute in Massachusetts, and for our research project we are conducting a series of interviews to investigate problems associated with the field of cancer vaccines which have recently shown both strong successes and failures.

Your participation in this interview is completely voluntary, and you may withdraw at any time. During this interview, we would like to record our conversation for later analysis. We will also be taking notes during the interview on key points. Is this okay with you?

Can we also have your permission to quote any comments or perspectives expressed during the interview? This information will be used for research purposes only, and we will give you an opportunity to review any materials we use prior to the completion of our final report, which will be published on-line in WPI's archive of projects.

If the subject does not agree to be quoted, we will respond as follows: "Since you would not like to be quoted during this interview, we will make sure your responses are anonymous. No names or identifying information will appear in any of the project reports or publications."

Your participation and assistance is greatly appreciated, and we thank you for taking the time to meet with us. If you are interested, we would be happy to provide you with a copy of our results at the conclusion of our study.