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Dendritic Cell-Targeted Vaccinations: A Promising Immunotherapeutic Approach to
Cancer Treatment

by

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Abstract

Immunotherapy is an emergent form of cancer therapy that offers new and innovative techniques that work to enhance the body's natural ability to defend itself against harm. This thesis seeks to explore the efficacy of vaccines that target dendritic cells as one particular form of immunotherapy. Vaccination has the potential to provide antigen (Ag) that is accessible to be processed by dendritic cells, deliver the antigen to the dendritic cells, encourage dendritic cell maturation and further promote the ability of dendritic cells to present antigen to effector cells to encourage a tumor antigen-specific immune response. There are several different elements in the process of achieving these ends including delivery of antigen to dendritic cells in-vivo or ex-vivo, antigen selection, antigen-carrier use, addition of adjuvants and the targeting of antigen to dendritic cells through soluble mediators. These different components of dendritic cell-directed vaccinations will be detailed and reviewed in order to understand the efficacy and immune-stimulating potential of possible vaccines. Additionally, through a series of experiments, we were able to determine that primed dendritic cells dramatically increased production of pro-inflammatory cytokines after micro-particle endocytosis. These preliminary tests demonstrate that the tumor-Ag conjugated micro-particle matures dendritic cells to promote and activate an immune response. The results of the experiments that were performed and analysis of the various methods available for DC vaccination indicates that this method of cancer therapy has extensive potential and requires further testing to enhance efficacy and manufacturability of the vaccines.

Introduction

In the United States, over a woman's lifetime, she has a 1 in 3 chance of developing cancer. For men, this is a 1 in 2 chance [1]. Globally, 14 million people are diagnosed with cancer and eight million people die from the disease each year[2]. Despite the great strides that have been made in cancer research, treatment, and long-term therapies, many patients still continue to develop progressive, metastatic disease. At this point in time there are several different methods of trying to combat and eliminate cancer cells in the body. Traditional therapies include chemotherapy, radiotherapy, and surgical removal of tumors, but a new and promising line of cancer therapies has emerged from the discipline of immunotherapy. Immunotherapy seeks to harness the power of the immune system and direct it to fight tumor cells. These therapies are often used concurrently with traditional methods of treatment in order to maximize a patient's response to the therapy. These therapies are designed to elicit an immune response through targeting specific antigens in the body (such as a tumor antigen), priming immune cells to attack tumor cells selectively, trafficking appropriate immune cells to tumor sites and neutralizing the immunosuppressive environment around the tumor cell [3].

One type of immunotherapeutic approach involves vaccination. Cancer vaccines are intended to encourage the body's immune system to respond to antigens that indicate the presence of an abnormal or cancerous cell. The immune system recognizes a wide variety of antigens, which can be derived from exogenous or endogenously expressed proteins. [4]. For example, antigens generated from

exogenous proteins are typically derived from pathogens including bacteria or viruses. In contrast, endogenous proteins originate from within the body and include normal self-proteins and those produced by abnormal or cancerous cells [4]. The goal of tumor-specific vaccination is to use antigens to activate cytotoxic T cells capable of recognizing and responding to cancer-specific proteins. A promising method of initiating this activation is known as dendritic cell vaccination. Dendritic cells are a type of immune cell generated from the bone marrow that are critical in initiating an immune response [3]. Dendritic cells do this by collecting antigens, processing them and presenting them on their surface. This ability places them in a category of cells known as antigen-presenting cells (APCs) [3]. In the vaccination approach, dendritic cells are loaded with adjuvants that will cause maturation of the cell as well as tumor antigens that will be presented by the dendritic cell to other immune cells that will then locate and fight tumor-specific cells [5]. There have been various studies using this approach to combat a variety of cancer types. These studies will be reviewed and evaluated for their effectiveness in eliciting an immune response through dendritic cell vaccination. In addition, we will discuss our studies that used a tumor-specific antigen (SPAS-1) along with adjuvants in a dendritic cell vaccination (DC vaccination) model in order to explore one possible way in which DC vaccination could enhance the tumor-specific immune response.

Innate Immune System

The immune system is an integral part of the body's survival mechanism that evolved as the result of perpetual exposure to pathogens, organisms and foreign molecules. The immune system functions as an interconnected network of organs, cells and molecules that work to protect and repair the body. This system of defense is divided into two sections, innate immunity and adaptive immunity. Both of these systems operate in unique ways, but it is in their combined efforts that the body is able to defend its self from viruses, diseases, parasites and even internal threats such as cancer.

The innate immune system has developed from millions of years of evolution and can be viewed as the elemental defense system. Innate immunity is the first system to react once a pathogen has been detected in the body [6]. A pathogen can be defined as any organism or agent that can cause disease. The innate immune system is not pathogen specific; instead, it recognizes molecules that are found in many pathogens, but are not found in the body [7]. These molecules are also known as pathogen associated molecular patterns (PAMPs). Some of these stimulants include molecules on the cell surface of the pathogen such as lipopolysaccharide (LPS), teichoic acid, or bacterial flagella. PAMPs can also include short DNA sequences such as CpG, which is an un-methylated repetition of the bases guanine and cytosine [7]. Together PAMPs provoke the initial innate immune response.

There are several different cell types that function within the innate immune system. One of these cells is the macrophage. Macrophages are derived from blood-born monocytes. These cells are able to distinguish between "foreign" and "self"

molecules. This ability is crucial to protecting the body from attacking itself and thus deterring autoimmune responses. Macrophages also have receptors for antibodies and complement, which will be discussed in a further section. These receptors allow for the cell to enhance its phagocytic abilities [6]. Through phagocytosis, macrophages (and other phagocytic cells) can uptake, break down and destroy pathogens. Eosinophils are another kind of immune cell. These cells function primarily to protect the body from parasitic infections. These cells are not phagocytic and rely on the release of cytokines and cationic proteins to kill foreign invaders [6, 8] .

Natural Killer cells (NKs) are another group of cells within the innate immune system that protect the body from pathogens and normal (self)-cells that have been infected. NKs have many of the same morphological features of lymphocytes, however they do not possess specific antigen receptors. Instead NKs recognize abnormal or pathogenic cells through two different pathways. The first pathway is through the use of immunoglobulin receptors (FcR). NKs can use these receptors to bind to targets coated with antibodies. After binding to a target pathogen, the NK is able to attack using antibody-dependent cellular cytotoxicity [8]. This method induces the cell to terminate itself via apoptosis. The second pathway through which NK cells function in the innate immune system is through the monitoring of MHC class I levels. These MHC proteins are expressed on almost vertebrate cells. When an NK detects high levels of MHC proteins, it will not attack the cell. However, when the NK observes low levels of MHC class I proteins, it is able to selectively kill cells such as virally infected cells as well as cancer cells [7]. NKs

are extremely important to preventing viral replication as well as the expansion of cancer cells.

Another cell type within the immune system that plays an important role as a bridge between the innate and acquired immune system is the dendritic cell.

Dendritic cells are potent antigen-presenting cells that will be discussed in greater detail in following sections. Dendritic cells are covered with a variety of receptors including lipopolysaccharide receptors, mannose receptors as well as toll-like receptors and several others. These receptors allow the cells to recognize antigens, endogenous danger signals (such as those secreted by virally infected cells, or heat-shock proteins resulting from necrotic cells) [6].

One of the first steps to occur within the innate immune system is the release of cytokines from macrophages in what is known as an inflammatory response. Macrophages are long-living immune cells that “patrol” tissues in the body and engulf foreign pathogens through phagocytosis [7]. Cytokines are a group of soluble proteins that help regulate cellular activity within the body, particularly the immune system [6]. Once a macrophage has taken up a pathogen it becomes activated and releases a series of cytokines including granulocyte (G-CSF) and granulocyte-macrophage colony stimulating factors (GM-CSF). These cytokines stimulate the bone marrow to differentiate myeloid precursors into neutrophils, which are subsequently released by the millions into the blood stream [8]. These neutrophils are then recruited to the site of the invader and are able to uptake and kill the pathogen or microorganism.

The complement system is another key part of the innate immune system. The complement system is made up of a series of 20 proteins that are produced by the liver and that circulate through the blood and the extracellular fluid. These proteins are activated by both antibodies produced by B cells as well as by PAMPs to attack the pathogens through lysis or by making them more susceptible to phagocytosis [7]. The proteins within the complement system are activated in a “cascade sequence” that has a self-amplifying effect, which reinforces its rapid and destructive abilities. The complement proteins have limited pattern recognition abilities allowing them to primarily target invading pathogens, however some cells, such as resident microbes within the body can also be targeted by these proteins. This sequence must promptly be deactivated in order to prevent the complement proteins from attacking self-cells [7].

Aside from complement proteins, there are several other non-cellular elements that function within the innate immune system. Many of these factors serve as mediators, messengers and triggers that promote inflammatory responses, complement cascade activation as well as immune cell activation and recruitment. One such group of molecules is known as acute-phase proteins. The levels of these proteins increase in response to infection and inflammation and they serve to encourage tissue regeneration and promote resistance to infectious agents [6].

Cytokines are another group of soluble factors; they serve, for the most part, as messengers both within the immune system and between the immune system and other systems within the body [9]. Besides functioning as messengers, some cytokines also have the ability to function directly within defense responses. Some

of these functional cytokines play important roles in mediating tumor resistance and are increasingly being used as “therapeutic agents” in combination with glycoproteins such as interferons.

Another group of molecules closely involved in communication is the group known as adhesion molecules. These molecules are bound to the surface of immune cells and facilitate information transmission between cells. These molecules are critical in guiding movement of cells, promoting phagocytosis and regulating cellular cytotoxicity [8]. These adhesion molecules, once attached to the cell surface can encourage cell activation, cytokine production and promote the up-regulation of surface receptors.

The functions of the adhesion molecules, like those of cytokines, complement and acute-phase proteins are critical to enabling the immune system to recruit cells to sites of inflammation, defend against malignant or infected cells, “warn” other cells of the threat as well as promote a regenerative environment so that the body can begin to heal itself.

Together, innate immune cells and non-cellular immune factors work collectively to provide the first rapid response for defense against foreign pathogens and malignant cells that are detrimental to the host. Innate immunity non-discriminately safeguards the body. Its non-pathogen-specific design allows it to act quickly and broadly and defend against a wide array of threats. While the processes under innate immunity can be advantageous and useful in immune defense, in order to create long-lasting and pathogen-specific responses, there must be collaboration with the adaptive immune system. This collusion transpires largely through the

intermediary position of dendritic cells, which facilitate the cooperation of both parts of the immune system.

Adaptive Immune System

While the innate immune system plays an important role as the first line of defense against pathogens, the adaptive immune system, also known as the acquired immune system, works to produce highly developed and targeted responses. The innate immune system responds to the general presence of pathogens, on the other hand, the adaptive immune system mounts responses to specific pathogens encountered in the body. The adaptive immune system is engaged only when the innate immune system detects the presence of PAMPs and foreign Ag. This allows the immune system to develop specific responses only when there is a genuine threat and not when there is merely a benign presence [9].

The adaptive immune system functions primarily through white blood cells known as lymphocytes. Lymphocytes, which can be divided into two separate cell types - B cells and T cells, develop within the bone marrow through processes guided by stromal cells and cytokines [6]. B cells and T cells have antigen-specific receptors, which empower them to produce their own unique immune responses. B cells produce as antibody responses and T cells deliver cell-mediated immune responses [9]. Following the presentation of antigen (Ag) to its respective antigen-specific lymphocyte, the T and B cells are primed, activated and differentiated into a variety of effector cells with unique functions [8]. Together, B and T cells generate

pathogen-specific responses that allow for targeted, specific and long-lasting defenses.

Antibody responses constitute one crucial component of the adaptive and innate immune system. These responses are carried out by activated B-cells. In this mechanism, B cells are signaled by other immune cells to secrete antibodies, which belong to a group of glycoproteins known as immunoglobulins [9]. Antibodies are composed of two main parts: two identical heavy chains, two identical light chains [6]. At the N-terminal end of Ab, there are sites to which antigens can bind.

Antibodies have highly variable antigen-binding sites, which allows highly specific recognition of unique antigens. This enables the antibody to “recognize” the antigen. Antibodies serve several functions. They are able to activate complement, encourage phagocytosis of bacteria, and bind to tumors and infected cells, which allows effector and killer immune cells to target and attack the malignant cell [8]. Aside from the functions of antibodies that have been released into the body by the mature B cells, antibodies also play a key role in immature B cells. In the early stages of B-cell development, antibodies are imbedded in the cell surface. Here, early antibodies function primarily as receptor proteins that allow B-cells to capture antigen and subsequently process it to present it via MHC class II surface molecules to T-cells [8]. This process is similar to that which takes place in several other immune cells known as Antigen Presenting Cells (APCs). Through these unique functions of antibodies, B cells are able to contribute to both innate and adaptive immune responses.

Once an antigen is expressed on the cell surface of a B-cell, it can be presented to a particular subset of antigen-specific T cells. Just like B cells recognize specific antigen via embedded surface antibody receptors, T cells carry unique receptors designed to recognize distinct Ag presented by the B cell receptor. Once a B-cell and a T-cell have combined recognition of the antigen, the T cell begins to produce cytokines that promotes B-cell maturation, subsequently leading to the antibody release previously described [6, 9].

While T-cells play a role in B-cell maturation/activation, they also serve a variety of other functions within the adaptive immune system. Naïve T cells are, for the most part, contained within the lymphoid tissue such as the spleen and lymph nodes. Within the lymphatic system, the T cell will come in contact with an APC that is presenting its antigen on the cell surface. This process is largely executed by dendritic cells (DCs) that actively present antigen in the lymphoid tissues via MHC class I or class II molecules. Other APCs include B cells and macrophages, each of which promotes the T cell to develop and mature in unique ways. Once the T cell interacts with the antigen, the T cell undergoes an activation period [8].

There are several different subclasses of T lymphocytes, which are comprised largely of CD4+ and CD8+ cells. In their origins, T cells express both CD4 and CD8 co-receptors. During maturation in the thymus however, T lymphocytes lose expression of one of the two co-receptors and consequently become selective to the pathway of antigen recognition. These two emergent subclasses of T cells will recognize antigen through different pathways and develop into effector cells with unique abilities and functions [9, 10]. CD4 T cells only recognize antigen presented

via the MHC class II pathway, whereas CD8 T cells are uniquely able to respond to antigen presented through MHC class I molecules [8]. MHC class I molecules primarily function alert the immune system to the presence of virally infected cells by presenting peptide fragments derived from self-proteins [11]. MHC class II molecules, on the other hand, present antigen derived from exogenous sources [12].

In addition to stimulation via the MHC complex, T cells require co-stimulatory molecules to enable them to fully mature. TCR stimulation in the absence of the appropriate co-stimulatory signals usually results in anergy or apoptosis of the T lymphocyte. Dendritic cells (DCs) are one of the most effective cells at promoting T cell activation as they are very efficient at Ag processing and presentation and they express high levels of co-stimulatory molecules B7 and CD40. [8]. Dendritic cells play a unique role within both the innate and adaptive immune system and will be discussed in greater detail in the following section.

The innate immune system and the adaptive immune system, while utilizing decidedly distinct methods of defending the body from harm, are only effective through the inherent cooperation of both systems. These systems rely on intercellular communication, cellular and non-cellular components and on complex, interdependent pathways that work to defend the body against pathogens of all sorts and, critical to this discussion, against host-borne malignancies such as cancer. The immune system has the ability to defend itself against perpetual exposure to foreign elements. The potential for cancer-defense is self evident in the potent and effective design of the immune system. By understanding how and why the immune system works, it is possible to harness its extant abilities and direct them towards

cancer cells. In the following section the importance and use of dendritic cells in mediating the immune system will be illustrated.

Dendritic Cells

For decades the extent to which the immune system was able to uptake and respond to external stimuli was not fully understood by immunologists and biologists. Macrophages were viewed as the primary actors in the innate immune system, however there was little evidence showing that macrophages linked the innate to the adaptive immune system [13]. In 1973 Ralph Steinman's discovery of dendritic cells and subsequent research into their role in adaptive immunity forever changed the understanding of the immune system and how it functions as a unit rather than two separate entities. This discovery, which earned Steinman the 2011 Nobel Prize in Medicine or Physiology, is critical not only to understanding the immune system, but also how to manipulate it for medical advancements [14].

Dendritic cells (DCs) serve a unique role within the immune system as they function as a critical link between the innate and the adaptive immune system. DCs are the most potent of the professional antigen presenting cells (APCs), which include DCs, macrophages and certain B cells. In order to understand the extent to which DCs function as a critical bridge within the immune system, it is important to first understand DC biology, diversity and role in activating adaptive immune responses.

Dendritic cells are a part of the body's innate immune system. DCs are derived in the bone from bone marrow-derived precursors and subsequently leave

through the blood stream as immature DCs and seed themselves into peripheral tissues or directly in the lymph nodes [3, 15]. Immature DCs present in peripheral blood and tissues are able to capture and uptake antigen (Ag) through several integral processes (e.g. endocytosis and phagocytosis) [3, 14, 16]. Once a DC has taken up and processed the antigen, it then undergoes maturation through the direction of several cytokines [16]. In the maturation stage, the DC decreases its ability to uptake antigens, and up-regulates its surface expression of MHC Class I and MHC class II molecules, increasing the DC's capacity to express the processed antigen. Additionally, the DC up-regulates its expression of the chemokine receptor CCR7, which allows it DC to migrate to a location where it can present antigen to T-cells [14].

After the uptake of Ag, the tissue-resident DC migrates to the draining lymph nodes through afferent lymph vessel, which allow entry of cells into the lymph node through all parts of its periphery [3]. Concurrently, the DCs process the Ag into peptides, which are fragments of proteins, that then bind to and are presented on the DC surface by MHC class I and MHC class II molecules. Once a DC has processed the Ag and presented the ensuing peptide on the MHC complex, this complex is then presented to naïve T cells in the lymph nodes and lymphoid tissues [14]. Naïve T cells bind to the MHC-peptide complex and co-stimulatory molecules present on the DC surface (e.g. CD80, CD86 and CD40), resulting in the activation and differentiation of the T-cells. Typically, extracellular antigens such as bacteria, parasites and toxins are presented via the MHC class II molecules and are presented to CD4+ T cells. DCs present intracellular antigens including viral proteins and

tumor antigens expressed on MHC class I molecules to CD8+ T cells [14]. The process through which DCs are able to take up exogenous proteins and present them to CD8 T cells is known as cross-presentation. This aspect of DC antigen presentation is critical to the understanding of the adaptive immune system and its manipulation due to the fact that DCs are the only APCs that are able to present extracellular antigens to CD8+ T-cells.

Figure 1. When immature DC come into contact with the correct combination of antigen and maturation stimuli, they proceed to their mature, differentiated form and are then able to process and present antigen via cross-presentation to T cells. This figure was adapted from source # [17].

The location of uptake of Ag can lead to distinct responses by T-cells [3]. Lymph-node resident DCs acquire their antigen directly from the lymph and present their peptides to naïve CD4+ T cells. In the presence of the appropriate stimuli (co-stimulation, etc) presentation of peptides results in T cell priming, and the production of cytokines such as interleukin 2 (IL-2), which subsequently promotes T cell proliferation and expansion. This process works conjunctively with the tissue-resident DCs. The DCs that capture antigen in the peripheral tissues and

subsequently migrate to the lymph nodes to present their peptide already activated CD4+ T cells, which, in turn, promotes the formation of effector T cells, particularly CD4+ T helper cells [16, 18].

T-Cell Differentiation

The presentation of antigen to naïve T cells and their subsequent activation is dependent upon several key chemical pathways and co-stimulation by the DCs. Additionally, depending on the pathway, and the DC subset involved in the activation process, T cells can differentiate into a variety of effector cells with unique functions and abilities. As previously stated, DC up-regulate their co-stimulatory surface molecules, which interact with corresponding molecules on the T cell surface to trigger its activation [16]. DCs also secrete cytokines, which function to direct T cell differentiation. Naïve CD4+ T cells can differentiate into T helper 1 cells (Th1), T helper 2 cells (Th2), T helper 17 cells (Th17) or T follicular helper cells (Tfh) (and regulatory T cells). Th1 cells function as effector cells within cell-mediate immune system that combats intracellular viruses and bacteria. These cells also secrete cytokines which promote macrophage activation and cytotoxic T cell production [19]. The Th1 subset of T cells secrete pro-inflammatory cytokines that help to recruit cells such as neutrophils and macrophages to a site of inflammation [20]. Th2 cells are another variety of CD4+ effector T cell. Like Th1, Th2 cells encourage production of B cells and macrophage activation. Additionally, these cells have been associated with eosinophil and mast cell activation [21]. Finally, Tfh cells are critical in the formation of germinal centers, which are sites

located within the lymphatic system that promote B cell maturation and their subsequent differentiation into plasma cells and memory B cells [22]. Naïve CD8⁺ cells initially become effector cytotoxic T lymphocytes (CTLs) and then can further differentiate into various subsets of memory cells, including central and effector memory cells [3]. Each of these cells play a crucial role in immune responses and the ability to shape how T cells differentiate is key to creating a successful immune response against cancer cells.

Dendritic Cell Subsets

There are several different subsets of DCs and each subset has its own unique function within the immune system. Human DC populations can be broken down into two major subsets, plasmacytoid dendritic cells (pDCs) and myeloid dendritic cells (mDCs), also known as conventional dendritic cells (cDCs) [23].

Plasmacytoid DCs are distinguished by their expression of the surface molecule CD303⁺ and function primarily through the innate immune system. This subset of DC is primarily found in the blood, thymus, bone marrow and lymph nodes as well as other lymphoid organs. pDCs primary functions are to promote anti-viral and anti-microbial innate immunity [14]. They do this primarily through the secretion of type I interferons (IFNs) [23], which are a class of cytokine that can initiate a rapid CD8⁺ T cell response and promote the “immunogenic maturation” of other DC subsets [3]. pDCs are also capable of directing the transformation of activated B cells into plasma cells [3]. There is some evidence that pDCs can

infiltrate tumors, however, there is still debate as to whether they play an anti-tumor role or whether they can promote tumor growth [14].

While the pDCs demonstrate limited utility in combatting tumors and tumor growth, the mDCs show promise for use in cancer therapy due to the fact that they have strong capabilities at processing and presenting antigen and thus activating strong antigen-specific T cell responses. mDCs differentiate from myeloid progenitors into several further subgroups. Some of these subgroups include lymphoid tissue resident DCs, which include CD8⁺ DCs and CD8⁻ DCs as well as migratory DCs, which are the CD103⁺ DCs, CD11b⁺ DCs, epidermal Langerhans cells and dermal interstitial cells [3, 23]. The lymphoid-tissue resident DCs arise from precursors within the lymphatic system and are immature until stimulated by antigen within the lymphoid organs. The migratory DCs differ in that they are mature by the time that they reach the lymph [23].

One of the most important types of DCs in promoting an adaptive immune response are the lymphoid tissue resident CD8⁺ DCs. This DC subset produces particularly high levels of IL-12p70. IL-12p70 is an interleukin that stimulates the production of interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α), which facilitates the differentiation of naïve CD4⁺ T cells into Th1 cells [24, 25]. Additionally, CD8⁺ DCs are highly competent at capturing and presenting exogenous Ag via the MHC class I pathway to CD8⁺ T cells through cross-presentation [23]. These DCs are also proficient at capturing and processing dead or dying cells, which makes them valuable in anti-tumor immunity efforts. By being able to take up and

process tumor cells combined with their strong ability to cross present, CD8⁺ DCs are critical to initiating antigen specific T-cell responses against tumors.

While CD8⁺ DCs are highly capable of cross presentation through the MHC class I pathway, CD8⁻ DCs and the migratory DCs: CD11b⁺ DCs and Langerhans' cells, play key roles in driving B cell responses and are more effective at presenting antigen through MHC class II surface molecules. CD8⁻ DCs and Langerhans' cells are superior mediators at antigen presentation via the MHC class II pathway [23]. This pathway, as discussed earlier, typically functions to activate CD4⁺ T cells. This is evident in the ability of the migratory DCs to promote differentiation of naïve CD4⁺ T cells into Tfh-like cells [3]. These differentiated T cells are highly valuable in promoting lasting immunity through the production of memory B cells. Another key function of migratory DCs is their ability to shuttle Ag from where it was taken up to a lymph node where it can "give" the Ag to a CD8a⁺ DCs, which are able to cross-present with CD8⁺ T Cells [23]. The ability of the migratory DCs to donate their antigen for presentation by other DCs combined with their own ability to cross present Ag, allows for activation of both CD4⁺ and CD8⁺ T cells.

Maturation is a critical factor in DC directed immunity. Immature DCs are effective at taking up Ag, however, antigen presentation by an immature DC is more likely to induce immune tolerance to the Ag. Mature DCs, on the other hand, are capable of inducing immunity through their particular ability to stimulate an antigen specific T cell response coupled with their expression of potent co-stimulatory signals. DCs mature in response to exposure to "pathogens and their bi-products" [23]. Pathogens have certain unique signifiers that, combined with the

presence of an antigen, alert the DC to a threat and encourage the DC to mature in order to be able to stage an immune response. Some of these signifiers come in the form of pathogen-associated molecular patterns (PAMPs) and endogenous ligands. These pathogen bi-products are recognized by DCs through the Toll-like receptors (TLRs) on the cell surface. This leads to B7.1, B7.2 expression, IL-12 production, and increased MHC expression on their surface, all of which help boost their ability to stimulate naïve T cells in the lymph nodes. The ability to activate and promote maturation and differentiation of DCs through adjuvants will be a critical concept in the coming sections as this permits experimental manipulation of DCs and the adaptive immune responses that they initiate.

Dendritic cells are powerful players within the immune system. They are able to bridge the innate and adaptive immune systems to drive robust B and T cell responses; it is through this dynamic that the immune system has the capability to defend itself not only against foreign pathogens, but also from cancers originating from within the body. The next sections of this article seek to evaluate two methods of DC vaccination capable of enabling immune responses that fight against cancers: ex-vivo (in-vitro) and in-vivo vaccinations.

Ex-vivo DC Vaccination

The immune system is capable of fighting a range of pathogens including parasites, bacteria, and viruses. Through the systems in place designed to target and destroy invading elements, there is the potential for the immune system to be able to fight self-derived maladies, such as cancer. While the immune system can

eliminate early neoplastic cells that arise under normal conditions, once the cells begin to multiply and mutate too quickly the immune system no longer has the innate capacity to control the massing cells. In the beginning of a tumor growth, there is a point at which the immune system wins out over the growing cells and is able to eliminate them. In a second phase of tumor progression, there is a equilibrium between the multiplication of the neoplastic cells and the rate at which the immune system can destroy them. For these cells to progress to the point of becoming a tumor, the immune system loses its control in a process known as tumor “escape” [26]. One possible way of activating the immune system to combat cancer cells once they reach the point of escape is by targeting dendritic cells through vaccination.

Through vaccination, dendritic cells could be activated to help induce an immune response that directly targets cancer cells. There are two broad categories of DC-based vaccines: ex vivo and in vivo. Currently, Provenge (sipuleucel-t), is the only DC-based vaccine that has been approved by the FDA. Provenge is an ex-vivo generated vaccine that is used for patients with metastatic prostate cancer. The use of this dc-based vaccine has demonstrated a 4-month median increase in survival compared to patients’ treated with a placebo vaccine [14]. This success demonstrates that the future of DC-vaccinations is conceivable and that through proper research and development, full-scale vaccinations covering an array of cancers could someday be viable treatment options. In the following sections, ex-vivo and in-vivo DC vaccinations will be discussed and evaluated for their efficacy, their pragmatism, and their potential for inducing long-term cancer immunity.

Ex-vivo dendritic cell vaccinations are constructed by culturing haematopoietic progenitor cells or monocytes with antigen and cytokines that are able to induce DC maturation and increase their capacity to elicit tumor specific immune responses [3]. These progenitor cells and monocytes are typically obtained directly from the patient and as such, the preparation of ex-vivo vaccinations can be labor intensive and expensive. On the other hand, the vaccinations can be precisely tailored to the individual's needs and unique situation. As previously mentioned, there is only one FDA approved DC-based vaccine. There are several possible explanations for why DC vaccinations have largely been thus far futile. Foremost, the tumor microenvironment (TME) is highly successful at evading immune detection, misdirecting immune cells, and inducing anergy among cells that are potentially threatening to the tumor. Furthermore, to properly optimize dendritic cells to enhance effective response, there are many different factors that must be taken into consideration including: which kind of stimuli are used to activate the DC, which kind of antigen to present and in what form, where and how many primed DCs should be administered, how often and various other deliberations [14]. It is crucial to understand these various elements of creating the vaccine in order to generate a successful vaccine that is able to induce the immune system to directly target the tumor and bypass the TME.

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Figure 2. General Ex-Vivo Dendritic Cell Vaccination Strategy [27]: Ex-vivo derived DC vaccinations depend on the collection of tumor antigens through either physical collection or synthetic production. Subsequently the immature DC, typically a monocyte or a haematopoietic progenitor cell, is presented with the tumor antigen, which is then taken up by the DC through endocytosis. Finally, the DC is then introduced to a series of cytokines intended to induce maturation and promote expression of cell surface receptors integral to generating a targeted immune response. This figure was adopted from source #[27].

Antigen Selection

One key element of priming a DC ex-vivo is selecting the type and form of antigen to be captured and subsequently presented by the dendritic cell. Antigen can be given to the dendritic cell in various forms including whole-purified tumor-derived proteins, short peptides, cell lysate, tumor-derived RNA, or whole tumor cells [28, 29]. The type of antigen delivered to the DCs could have dramatic consequences in terms of how quickly the antigen is processed and presented, how

effective the antigen is at promoting an immune response or even whether or not the antigen will induce an immunosuppressive responses that would hinder T-cell effectiveness in attacking cancer cells.

Whole Tumor Cell Lysate

Whole tumor cell lysate is one of the many types of antigen sources and has demonstrated relative success in a number of clinical trials. One example of the use of whole tumor cell lysate supernatant as the source of antigen was in a study looking at DC vaccinations targeting recurrent ovarian cancer [30]. In this study, six patients underwent a series of treatments that began with intravenously administered bevacizumab (anti-angiogenic drug) and orally administered metronomic cyclophosphamide both of which function to inhibit angiogenesis. The metronomic delivery method is one in which the patient receives continuous low-doses of the drug, (i.e. once a day). This method is favorable in that it has demonstrated lower levels of toxicity and it allows the drug to work progressively, each administration compounding on the next [31]. Additionally, the lower doses of cyclophosphamide have demonstrated preferential depletion of regulatory T cells, which can inhibit anti-angiogenic progress. Next, the patients were vaccinated with DCs that had been pulsed with autologous tumor cell lysate. Finally, the patients were further injected with T-cells that had been previously exposed to the vaccine. Out of the six patients treated, four demonstrated anti-tumor immune responses. Overall, there was a 50 percent clinical benefit including a remission that lasted 14 months as well as one complete remission. While this treatment design

demonstrates a combinatorial approach, it also suggests to a certain extent that DC vaccination with tumor lysate as an antigen has the potential to promote anti-tumor T-cell immune responses [30]. The researchers in this study discuss several manners in which the treatment would be improved upon. First, the supernatants used in this study were derived from “freeze-thawed” tumor lysates; the authors suggest that using alternative techniques for obtaining the supernatants may have yielded superior results. Secondly, the DCs were not matured with any additional stimuli. The researchers also proposed that “stimulation with exogenous cytokines and TLR agonists could significantly enhance vaccine potency” [30].

Peptides

Another promising category of antigen able to prime DCs is tumor specific peptide fragments. These antigens are typically derived from tumor specific proteins rather than self-proteins as to avoid an autoimmune response. They can also, however promote targeting of over-expressed self-proteins like HER2 for breast, PSA for prostate cancer that are expressed on the tumor. These proteins do not activate T-cell responses in-vivo because the tumor cells lack the required co-stimulatory molecules[3, 29, 30]. One key benefit of using these peptide fragments is that they are present in many patients sharing the same kind of cancer, making it easier and cheaper to obtain and produce a vaccine that could benefit a large group of people. Additionally, the peptide fragments that are associated with tumor antigens can be created synthetically and uniformly allowing for a relatively cheap means of production compared to using autologous cell lysate, which has to be

appropriated from each patient individually [32]. In a study performed by Nestle et al., DCs were pulsed with several different tumor-derived peptides as well as keyhole limpet hemocyanin (KLH), a type of large carrier protein, and delivered to patients with melanoma. In this study, the peptide combinations used were tailored to the particular patients' haplotype, thereby maximizing the immune potential. The vaccine was injected directly into the lymph nodes. Out of the 16 patients participating in the trial, 11 demonstrated delayed-type hypersensitivity (DTH) to the peptide pulsed DCs as well as peptide-specific CTL recruitment. Overall, three patients manifested partial responses with regression of metastases and two patients achieved complete remission [32, 33]. This study highlights several key features about antigen selection for DC vaccination. First, an antigen, in this case specifically a synthetically produced cancer-related peptide, that produces a strong anti-tumor response in one individual, may not be ideal for another patient even one with the same diagnosis. Tailoring antigen selection to an individual's particular haplotype could provide a very valuable and effective treatment tool. This does, however, require individualization, which as discussed earlier, can be labor intensive and more extensive than a blanket treatment. Second, a combination of tumor-associated peptides may be more effective than the use of one sole peptide. This method provides the dendritic cell with the opportunity to deliver varied antigen and signals to the effector cells thereby creating unique responses and optimizing the potential for a successful immune reaction. Additionally, this method of ex-vivo DC vaccination demonstrated no indication of autoimmunity or severe reactions to the vaccine. This suggests that DC vaccination has the potential to be a

safe and less toxic form of treatment than chemotherapies and other kinds of cancer treatments.

Another factor that must be taken into consideration when developing a DC vaccination is the type of stimuli that must be added to encourage DC maturation and specialization. Thus far, many studies have used a standardized set of cytokines including TNF- α , IL-1 β , IL-6, which are all pro-inflammatory and PGE₂, which is a cytokine shown to promote up-regulation of MHC molecules on the DC surface molecules. While these cytokines have been successful to a degree at promoting DC maturation, it has been suggested that this composition of cytokines fails to induce IL-12p70 production and could potentially lead to the production of T-regulatory cells. IL-12p70 is a key cytokine produced by DCs involved in encouraging the differentiation of naïve T cells into Th1 cells [14]. A variation of this cytokine cocktail involves the use of TNF- α , IL-1 β , Poly(I:C), IFN- α and IFN γ . This blend of cytokines has shown to induce the IL-12 production that was lacking from the effects of the preceding combination.

In a study performed by Stift et al., ten patients diagnosed with medullary thyroid carcinoma (MTC) were administered an ex-vivo produced DC vaccination. The dendritic cells were matured from peripheral blood monocytes that were cultured with GM-CSF, IL-4, TNF- α and IFN- γ and tumor lysate antigen. The matured DCs expressed high levels of CD1a, CD11c, CD40, CD80, CD86, CD83 and MHC II. This indicates that the DCs were effectively activated to promote T-cell activation, differentiation and subsequent anti-tumor potency. In terms of clinical effects, seven on the ten patients demonstrated prolonged tumor marker decline.

Three patients demonstrated a partial objective response, one patient exhibited a minor response and two other patients demonstrated stable disease, thus no further tumor progression. The researchers propose that TNF- α and IFN- γ were critical to encouraging the expression of co-stimulatory molecules on the dendritic cells, the lack of which can lead to immune-tolerance [34]. The evidence presented in this article suggests that the cytokines used in this study could be effective maturation inducing agents. It is critical that the DCs not only be matured but also that they have the necessary surface molecules to present antigens and to successfully activate naive T and B cells through co-stimulation. For an ex-vivo derived vaccination to be effective, these conditions along with careful antigen selection and presentation must be met.

A final key consideration in the production and usage of ex-vivo DC vaccination is the site of administration of the matured and primed DCs. So far, part of the reason that DC vaccinations have had such limited clinical success is that once the DC vaccine has been injected, the vaccine fails to migrate to the secondary lymphoid organs[14]. Studies have demonstrated that less than 5% of injected DCs typically reach the draining lymph nodes, which is a critical site for cross-presentation to T-cells [35]. In order to improve the migration of DCs, there are several different possible measures that can be taken. First, the DC vaccine could be administered at several different sites, thereby maximizing the odds that of exposure to T-cells. Second, the DCs could be delivered directly into the lymph nodes [14].

In-Vivo DC Vaccination

Ex-vivo stimulated DCs have demonstrated immense potential for use in antigen-specific immune targeting through vaccination, however, the methods employed to produce positive results can be time consuming, labor intensive and extremely expensive. As an alternative, many researchers have turned to exploring the possibilities of targeting DCs in-vivo. This process eliminates the necessity of extracting and culturing autologous DCs, which can only be used for the individual from which they were extricated. Additionally, it changes some of the challenges that were presented with ex-vivo generation and delivery of the vaccine. For example, a main concern with ex-vivo vaccination is the delivery site. The dendritic cells must be injected into a location from which they can migrate. In-vivo vaccinations, however, depend on the natural migration patterns of DCs from origin to presentation of antigen to T and B cells. Under these circumstances, what becomes most important is the delivery of the injected antigen to the DCs. Similarly it is critical that the maturation cytokines contact the DCs to enable successful mediation of antigen specific effector cell responses.

In order for an in-vivo DC vaccination to be successful, endogenous DCs must be primed to present tumor antigens via both MHC Class I and MHC Class II pathways so that both CD4+ and CD8+ T cells are engaged in anti-tumor immune responses [14]. As with ex-vivo culturing of dendritic cells, there are several different options for antigen sources. Tumor associated antigens can be produced from oncogenic viruses such as HPV proteins, overexpressed variants such as Her2/neu, which is a oncogene, or self-antigens that are overexpressed specifically

on tumors [14]. The up-regulation and high expression level of tumor proteins alone is an essential defining factor in selecting a self-antigen in order to avoid an auto-immune response.

To enhance the probability of a directed and effective immune response in an in-vivo DC vaccination, the chosen antigen must be successfully administered and targeted to DCs in vivo. There are several different approaches currently being tested to achieve this goal. The first involves the use of soluble mediating proteins that interact with DC surface molecules [29]. In this model, the soluble mediators are combined with the antigen and subsequently administered to the patient. These mediators can play valuable roles in increasing not only the quantity of DCs, but also the quantity of antigen that are presented and the migration of the matured DCs. Several mediators that can be used to induce DC expansion and maturation in vivo include fms-like tyrosine 3 kinase Flt3 ligand (Flt3L) and GM-CSF [29, 36]. These mediators can also serve to cause differentiation of the DCs. GM-CSF generates CD11b⁺ and CD8a⁻. With the addition of Flt3L, however, naïve DCs differentiate in to CD11b⁺CD8a⁻ and CD11b⁻CD8a⁺ DCs, each with unique priming and co-stimulatory abilities [29]. Heat shock proteins (HSPs), another kind of soluble mediator, can bind to DC surface receptors and induce DC maturation and promote presentation of antigens via the MHC class I pathway. Cytosine phosphate guanosine oligodeoxynucleotide (CpG ODN) is a particularly useful mediator that interacts with TLR-9 on DCs and are particularly useful in priming pDCs to stimulate T-cells [29, 36].

Particulate Antigen Delivery

Figure 3. Representations of the different varieties of nano-particulate antigen delivery systems; this figure was adapted from source #[37].

Another method of antigen delivery involves the use of particulate vaccines. Particulate vaccines have the benefits of long-term release of antigen, selective antigen and adjuvant presentation, as well as the ability to target DCs specifically and even particular subgroups [38]. There are several different kinds of particulates that can be used in vaccines. The first delivery system uses virus-like particles (VLP). VLP are typically derived from viruses including Hepatitis B (HB) and parvovirus (PPV). These viruses, among others, have the ability to rearrange

themselves into particles that can encapsulate antigens [29]. Exposure to VLPs will cause DCs to mature and secrete inflammatory cytokines [29, 39]. Additionally, VLP can stimulate both CD4⁺ and CD8⁺ T cells in vivo. In several ways, VLPs are particularly useful in that they act as both a carrier and an adjuvant. A key point to acknowledge is that VLP are not infections and are thereby will not adversely affect immune or other cells. The Hepatitis B VLP is particularly useful in protecting antigen from deterioration. The HB VLP can be taken up by both macrophages and DCs, which then present the delivered antigen via the MHC class I pathway. The particles that sized in the range of 0.04-0.1 microns were, however, more effectively taken up by CD205⁺DCs, while the larger VLPs were taken up by macrophages [40]. PPV-VLPs are taken up exclusively by CD8a⁻ and CD8a⁺ DCs making them very useful in solely targeting these DC subsets in vivo [29].

Synthetic Particles

Synthetic nano- and micro-particles are an example of a different type of particulate that can be used to deliver antigen to DCs in vivo. In this design, antigens can be conjugated to or encapsulated in synthetically created particles that can be taken up by CD8a⁺ DCs and processed both in vivo and in vitro. One key advantage to this approach is that there is greater flexibility for antigen selection, more uniformity across particles, higher yield and increased stability than with the use of VLPs or soluble mediating factors. There are two major categories of synthetic particles that can be used; biodegradable and non-degradable.

Biodegradable Particles

Biodegradable particles are typically created from hydrolytically degradable polyesters such as poly (D,L-lactic-coglycolic acid) copolymers (PLGA), poly (D,L-lactide) (PLA), poly (ortho esters) (POE) or from liposomes or ISCOMs. Most of these particles are not conjugated with danger signals and therefore do not produce strong inflammatory side effects [29]. This makes the particle more tolerable to the patients and potentially limiting the detrimental effects of the cancer therapy. PLGA is a particular effective particle choice due to the fact that these particles have demonstrated the ability to incorporate a wide variety of antigens, which makes PLGA particles useful in a wide spectrum of cancers. PLGA particles are able to protect the antigen from deterioration and they deliver the antigen to APCs, in particular DCs, in a targeted manner. Typically, antigens by themselves have a presentation time of approximately 12 hours by dendritic cells. In a study performed by Shen et al., it was demonstrated the antigen delivered via PLGA particles was effectively presented through the MHC Class I pathway for over 96 hours. This demonstrates the increased viability and stability of the antigens introduced through this particulate system as well as the ability of these particles to deliver antigen that targets CD8⁺ T-cell responses [41]. There is also evidence that particulate antigens are more effectively utilized by DCs in cross-presentation and activation of T cells [42]. PLGA particles also have the capacity to integrate and deliver immune-modulators to DCs, such as TLR ligands, which can both encourage DC immune capacity as well as reverse the effects of tumor-induced immunosuppression of DCs [42]. Another benefit of using PLGA, is that it has been

approved by the FDA for the use of several different clinical application including drug delivery [43]. This makes it a valuable material in terms of accessibility and patient trials.

PLA and POE largely incorporate plasmids or tumor DNA as their primary antigen. These delivery systems have been shown to be more successful at producing antibody, CD4 and CD8 T cell immune responses than when DNA was administered without a delivery system. PLA and POE protect the fragile DNA from deterioration within the body. These systems, however, have limited antigen selection capabilities making them useful only in certain types of cancer [29].

Liposomes, another type of antigen delivery system, are small phospholipid vesicles that have been regularly utilized in vaccine designs. They, like the other particles discussed, are able to encapsulate the selected antigen into their system and deliver them to APCs. Liposomes are able to protect the antigens against degeneration by the APC and thereby help to prolong the presentation time of the antigen. This kind of delivery system helps to facilitate presentation of antigen via the MHC class I pathway [29].

A fourth and final variety of biodegradable particles are immune-stimulatory complexes, also known as ISCOMs. ISCOMs are spherical “cage-like” structures that are composed of antigen, saponin, cholesterol and phospholipids [29, 44]. These particles are highly immunogenic and are promptly and effectively endocytosed by DCs. Delivery of antigen through the ISCOM particles, promote the presentation of antigen through the MHC Class II pathway[29]. Studies have shown that ISCOM-based vaccines are highly effective at stimulating antibody responses in addition to

facilitating antigen specific CD4+ and CD8+ T cells, including CTL responses. Additionally, these vaccines have demonstrated long-lasting immune responses, which are critical for enduring tumor eradication [44]. ISCOMS have demonstrated greater efficacy when combined with adjuvants such as LPS that contribute to DC maturation and thereby the immune response[29]. Functionally, ISCOMS are best suited to incorporate recombinant proteins [44].

Non-biodegradable particles

Non-biodegradable particles constitute the second kind of antigen delivery system that can be created synthetically. Typically, these particles are made from latex, gold, silica, iron or polystyrene. These particles are particularly useful in that not only can antigen be incorporated on the surface of the structures, but they also provide a surface to which adjuvants can be attached. Additionally, due to the non-biodegradable nature of the particles, they endure inside the body allowing for repeated exposure of antigens to DCs and therefore prolonged and recurrent immune stimulation [37]. Another advantage of the use of non-biodegradable particles is that their synthesis can be highly controlled and their size and shape can be manipulated to meet the needs of the vaccine [37, 45]. Non-biodegradable particles have demonstrated the ability to be engineered to present antigen highly effectively through both MHC class I and class II pathways. These particles represent one of the most customizable groups of antigen delivery systems. One of the most favorable materials that have been used so far for the construction of non-degradable particles has been silica. Silica-based nanoparticles (SiNPs) are

biocompatible, effective at selective tumor targeting and are able to be monitored in-vivo real time with multi-modal imaging [45]. Another key aspect of SiNPs is the presence the silanol groups on the particle surface. These silanol groups can be used to deliver a variety of molecules and adjuvants such as ligands, peptides and antibodies. By using the silanol groups, the particles can be modified in ways that allow for increased cellular recognition, increased efficacy at being taken up by APCs, absorption of biomolecules, and higher ability to be directly targeted to cancer cells [45, 46]. These factors that contribute to the effectiveness of the SiNPs as well the other benefits discussed relating to non-biodegradable particles as a whole demonstrates their potential for being a compelling antigen delivery system for DC activation in-vivo. There is, however, some concern that because these particles will not degrade naturally in the body, there is the potential for toxicity and accumulation of particles within certain tissues [29]. In order to be certain that non-biodegradable particles are safe for human treatment, these concerns must be addressed and tested for optimal dosing and minimal toxic effects.

Figure 4. Antigenic Delivery by Nanoparticle: There are several different methods of mediating antigen delivery through nanoparticle delivery. Antigens can be delivered through conjugation, where they are chemically attached to the nanoparticle. Encapsulation involves delivery of the antigen in which the antigen is enveloped within the nanoparticle. Adsorption relies on a charge or hydrophobic interaction between the particle and the antigen to connect the two. The final method of introducing antigen and nanoparticles together in a vaccine relies simply on mixing the two, without any direct interaction of the particle and the antigen [45]. This figure was adopted from source [45].

Targeting DC Surface Molecules

Up to this point, we have primarily discussed methods of encapsulating or securing selected antigens through various particles as a means of delivering it to APCs, particularly DCs, *in vivo*. These methods are utilized to protect the antigen from degradation, increase uptake by APCs, prolong antigen presentation time, and increase the quantity of the selected antigen directed to DCs. Another aspect of

delivering antigen to DCs in vivo effectively involves targeting DC receptors specifically. Several different adjuvants and antibodies specific to DC surface receptors can be introduced either alongside or integrated into antigen delivery particles in vaccines to increase their ability to selectively deliver antigen to DCs. This is possible through the exploitation of the receptors present on the DC surface; antibodies can be modified so that their variable region “fits” the DC receptors similarly to how antibodies can selectively bind to antigens. Additionally, the ability to target antigen to specific DC receptors consequently allows antigen and antigen carriers to be targeted to different DC subsets, thereby tuning immune responses. DC targeting can also be used to target antibodies, which instead of providing co-stimulation with antigen delivery directly, induce signals that aid in DC activation and maturation [3].

Antigens are largely targeted to DCs through the use of DC surface lectins. Many of these receptors belong to the C-type lectin receptor (CLR) family. CLRs constitute a group of lectins that share a carbohydrate-recognition domain (CRD), which allows them to bind to a select group of sugar residues. This aspect of CLRs is valuable in that they can be present on the cell membrane and selectively bind to passing sugar groups, connecting them to the DC. Several different CLRs that are particularly useful for DC targeting include the mannose receptor, DEC-205 (also known as CD-205), and DC-SIGN [36].

The mannose receptor is present on immature DCs. Targeting antigen to this receptor increases antigen presentation via both the MHC class I and class II pathways [36]. The mannose receptor can be targeted in vivo by fusing antigen to an

anti-mannose receptor monoclonal antibody (anti-MR mAb) [47]. These fused complexes have been demonstrated to elicit both CD4⁺ and CD8⁺ antigen specific T cells. Furthermore, MR targeted antigen vaccination strategies have shown to promote anti-tumor responses [47].

DEC-205 is another type I CLR that is highly expressed on mature DCs and largely functions to facilitate antigen presentation. DEC-205 is expressed in only very low levels on other cells making it an ideal candidate for selectively targeting DCs. In terms of research and trials, it is important to note that DEC-205 is restricted to CD8⁺ DCs in mice, but it represented more widely in the human DC population. This means that low level responses in the testing of mice, might not necessarily reflect the response that would be exhibited in humans. Delivery of antigen to DCs through the targeting of the DEC-205 receptor promotes CTL responses as well as CD4⁺ T cells that provide prolonged induction humoral immune responses [36]. A key point, however, in the targeting antigen to the DEC-205 receptor, is that without proper stimulation with additional adjuvants, the DCs could induce antigen specific tolerance in lieu of tumor specific immune responses [48]. In order to avoid anergic responses, the antibody targeting DEC 205 (α -DEC-205 Ab) should be administered in conjunction with adjuvants that induce DC activation such as TLR3, TLR7 or CD40 agonists [48].

DC-SIGN is a surface receptor that is primarily expressed on immature dendritic cells, and at lower levels on mature DCs. The antibody targeting the DC-SIGN receptor effectively locates DCs that are present in the draining lymph nodes, which is an ideal site for DC activation, stimulation and subsequent presentation of

antigen to the naïve T cells that settle in the lymph nodes [36]. This method of targeting antigen to DCs has demonstrated that the presentation of antigen through both MHC class I and II molecules and promotes memory T-cell, CD4+ T-cell, CD8+ T cell as well as humoral responses leading to a multi-faceted and antigen specific immune response. Currently, there is a clinical trial in progress examining the effects of a DC-SIGN-targeted vaccine based upon the use as of a modified lentiviral vector. Thus far, the use of the lentiviral vector in conjunction with the DC-SIGN-targeting approach has been shown to initiate aggressive anti-tumor T cell responses [49].

SPAS-1 Conjugated Micro-particle Project

Several different methods have been described throughout this article as possible ways to initiate and enhance immune responses against cancer cells through the exploitation of dendritic cells' natural abilities as antigen presenting cells. Overall, the methods discussed have been designed to introduce dendritic cells to a selected cancer-specific antigen and subsequently induce the presentation of these antigens to immune effector cells. In order to further explore the potential and efficacy of some of the approaches designed, we decided to test a possible vaccination strategy utilizing iron micro-beads as a non-biodegradable antigen carrier, SPAS-1 as the antigen, and several different adjuvants to promote DC maturation and activation. The processes used to create these particles along with tests performed to evaluate the ability of the micro-particles to affect DCs will be detailed and assessed in the following sections.

SPAS-1 is a protein produced by the murine prostate cancer cell line TRAMP-C1. SPAS-1 is a useful target for cancer therapy because it can be recognized by a subset of tumor-specific CD8 T cells, it is upregulated by prostate cancer cells during tumorigenesis and it is not expressed on normal cells. This means that SPAS-1 can be targeted effectively by the immune system and that it will not promote autoimmune immune responses. In order to elicit an immune response against the SPAS-1 antigen, we examined the effects of using a micro-particle vaccine containing the SPAS-1 antigen combined with lipopolysaccharide (LPS), CL264, and α DEC-205. Lipopolysaccharide (LPS) is a molecule found on the outer membrane of some bacteria. It is one of the pathogen associated molecular patterns (PAMPs) that can be recognized by immune cells with pattern recognition receptors (PRRs). When encountered by macrophages, dendritic cells or some B-cells it elicits a strong immune response encouraging these cells to secrete pro-inflammatory cytokines. CL264 (in this case biotin labeled) is a ligand that promotes dendritic cells to secrete the pro-inflammatory molecule interferon-alpha (IFN- α) and to activate the NF- κ B pathway. NF- κ B is a protein that is critical in regulating genes and up-regulating genes that are involved in T-cell development, maturation and proliferation. These proteins are able to activate immune cells to respond to the presence of the tumor. α DEC-205, the antibody that binds to DEC-205, was used as the DC targeting antibody.

The micro-particles were created and tested for efficacy through administration to DCs in-vitro, however, the end goal of this particle was in vivo

antigen delivery to and subsequent activation of DCs in order to prime tumor Ag-specific CD8 T cells.

SPAS-1 Purification

The first step in the process of creating the micro-particle was to obtain a purified version of the SPAS-1 protein that could be used as an effective antigen. In this study, the SPAS-1 protein was derived from a 6xhistag-GFP-(green fluorescent protein) tagged SPAS-1 recombinant *E. coli*. To separate the GFP-tagged SPAS-1 from the *E. coli*, the recombinant specimen was subjected to a series of buffer combinations aimed at solubilizing the SPAS-1 protein, thereby separating it from the *E. coli*. The most effective buffer cocktail consisted 100 μ L base solution (10mM Imidazole, 50mM Sodium Phosphate, 5mM arginine and 0.01% tween 20 in 200mL distilled water), 125 μ L 2M Urea, 125 μ L 750mM NaCl, and 25 μ L 0.1% Triton. The solubilizing solutions were evaluated for their ability to separate the soluble from the insoluble portions of the recombinant *E. coli*. Due to the fact that the recombinant *E. coli* was GFP-tagged, the relative fluorescent intensities of both the soluble as well as the insoluble portions were able to be determined through the examination of the portions using a using a 510-590 wavelength filter. The soluble to insoluble fraction ratio was determined using the fluorescent intensities for each buffer solution. The buffer solution that produced the highest yield of the soluble portion (i.e. the SPAS-1) relative to the insoluble portion was selected as the most effective buffer solution.

In order to continue purifying the remaining SPAS-1 recombinant *E. coli*, the recombinant *E. coli* was subjected to the optimal solubilizing solution. The mixture was then run through a centrifuge, separating the soluble and insoluble fractions. Next off, the soluble fraction was filtered through a three filter series of 1.2 μ , 0.45 μ and 0.2 μ filters. This process resulted in approximately a 50% SPAS-1 protein extraction efficiency when compared to the amount of SPAS-1 present in the original recombinant solution.

The filtered solubilized SPAS-1 solution was then further purified from background materials that were not removed through the preliminary extraction of the SPAS-1 protein from the SPAS-1 recombinant *E. coli*. This was achieved by using the “Akta Pure” which uses the Nickel affinity purification process. First the SPAS-1 protein solution was run through the purification column. This was followed by a load binding/ wash buffer consisting of 10mM Imidazole, 50mM Sodium Phosphate, 5mM Arginine, 2M Urea, 0.01% Tween-20, 2M Urea and 0.1% Triton x-100 in ddH₂O. This wash buffer was used to wash away anything that was not sticking to the nickel column. After this, an elution buffer (made of 250mM Imidazole, 50mM Sodium Phosphate, 1.5M Sodium Chloride and 5mM Arginine in ddH₂O and 0.5M NaOH) was run through the column and the flow-through was collected. The elution buffer functions to strip the column of the target SPAS-1 protein. Next, the elution buffer was run through once again. A slightly altered version of the elution buffer was then run through the column, in which the concentration of Imidazole was increased to 500mM and concentration of Sodium Chloride was increased to 2M. These runs were followed by another wash flow-through, an elution flow through, a

NaCL flow through and finally a Nickel solution flow through. The collected flow through samples were then examined for their levels of fluorescence.

Figure 5. Average MFI of Products of SPAS-1 Purification Process

The collected samples and their respective mean fluorescent intensities (MFIs) demonstrated that the flow through from the primary elution yielded the highest level of purified SPAS-1. The results demonstrated that the Elution #1 yielded the highest concentration of the SPAS-1 protein. This elution, along with the third elution run through were subsequently concentrated for use in the following experiment.

In order to examine the progression of the purification throughout the varying purification steps and to estimate the final yield of the SPAS-1 protein, the solutions present through the various stages of protein purification were run

through the Thermal Cycler and subsequently added into nine different wells of the gel for a SDS-PAGE (sodium-dodecyl sulfate poly-acrylamide gel electrophoresis) test. In order to be able to examine the results, dye was added to stain the proteins present in the gel and the gel was imaged. The following stages of protein purification were added to the wells in their respective orders: 1) insoluble SPAS-1 Recombinant E. Coli 2) Soluble SPAS-1 Protein 3) Soluble and Filtered SPAS-1 Protein 4) Purification Binding Flow-through 5) Purification wash flow-through #2 6) Elution #1 7) Elution #3 8) Concentrated Elution #1 9) Concentration Elution #3.

Figure 6. 3-D Representation of SDS-PAGE results on varying stages of SPAS-1 purification process

In well number two, we can see that the original insoluble SPAS-1 recombinant E. coli has a large amount of many different proteins in it. As the purification process progresses, the amount and variation of the proteins decreases

until we are left primarily with our target protein, which is shown in the number 9 sample which was a concentrated version of elution #1. It was surprising to see that the concentrated elution 1 was very similar to the well number four which represents the filtered soluble SPAS-1 solution. This shows that the nickel affinity purification process was not as successful as we might have hoped. This gel does however show us the yield of the SPAS-1 protein from the purification process. Well 1 represents the standards column from which other levels of proteins can be determined. We compared the amount of SPAS-1 protein in well 9 to the control in well 1 and we can see that we have approximately 500ng of the protein in 20 μ L (or ~25ng/ μ L) of the concentrated elution solution #1.

Figure 7. Overall SPAS-1 Purification Process

Micro-Particle Production

Next, we created a variety of micro-particles and determined the relative amount of antigen and antibody bound to the micro-bead. Iron-oxide micro-beads with streptavidin were used as a form of non-biodegradable antigen carrying micro-particle. We used a tumor-specific antigen (SPAS-1) plus LPS, CL264 and α DEC-205. The combination all the adjuvants on a micro-bead creates what are called dendritic cell activating receptor-targeted particles (or DARTs). The iron-oxide micro-beads were covered in streptavidin molecules. Streptavidin has an extremely high affinity for biotin. In order to determine the most effective micro-particle we created five different variations. The variations were as follows: 1) iron-oxide beads + LPS, 2) beads + CL264, 3) beads + α DEC-204, 4) beads + SPAS-1, 5) DARTS + SPAS-1.

In order to connect the α DEC-205 to the micro-bead, we had to add an anti-rat secondary antibody with a biotin molecule that could bind to the bead. Because the α DEC-205 used in this experiment is a rat antibody it connects to the anti-rat secondary antibody. The SPAS-1 requires an additional step. First, an anti-rabbit secondary antibody, which has a biotin molecule, connects to the streptavidin molecule. Second, an anti-6x-histag of rabbit origin connects to the secondary antibody. Third, because the SPAS-1 antigen was created with a 6x-poly-histidine tag, it can be added and connect to the anti-6x-histag.

Figure 8. Example of Micro-Particle Assembly: This image depicts a DARTs + SPAS-1 micro-particle arrangement in which all of the adjuvants as well as the SPAS-1 antigen have been conjugated to the iron-oxide micro-bead through the streptavidin molecule and a series of binding antibodies.

Figure 9. Image of DC2.4 Dendritic Cells with DARTs + SPAS-1 Particles

Because the α DEC-205 and SPAS-1 antigen contain a fluorescent tag, the amount retained on the micro-particle can be tracked. Known amounts of α DEC-205 antibody and SPAS-1 antigen were added to the micro-particle. After washing, the particles the amount of SPAS-1 and α DEC-205 that were retained on the micro-particles was determined. For the beads + α DEC-205, approximately 11% was retained after the wash. For the DARTS and the added α DEC-205 only 4% was retained. There were approximately 22,000 α DEC-205 molecules on the beads + α DEC-205 microparticle and only 8,000 on the DARTS + SPAS-1 molecule. This is likely due to the competition for space on the DARTS particle. On the DARTS there is not only α DEC-205 but LPS and CL264 as well. On just the beads, the SPAS-1 antigen was retained at 17.1% after the wash and on the DARTS 16.8% remained attached. We determined that there were approximately 25,000 SPAS-1 molecules per iron-oxide micro-bead for both the beads + SPAS-1 micro particle as well as the DARTS + SPAS-1 molecule. These higher numbers indicate strong bonds allowing the SPAS-1 to securely attach to the micro-particle.

Figure 10. Mean Fluorescent Intensity of α DEC-205 before and after wash

Figure 11. Mean Fluorescent Intensity of SPAS-1 on Beads before and after wash.

Figures 11 and 12. Figure 10 depicts the mean fluorescent intensity of 12 wells of the α DEC-205 before and after wash per condition. Figure 11 depicts the mean fluorescent intensity of 12 wells of SPAS-1 per eight conditions shown above.

Micro-particle Testing

In order to determine the relative effectiveness of the engineered micro-particles, several different tests were performed to examine the ability of the micro-particles to be taken up and processed by DC2.4 dendritic cells and thus induce an immune response. The first test performed was an immunocytochemical test, which was used to assess the expression of CD40, CD86, MHC Class I and MHC Class II molecules on dendritic cells after exposure to the different micro-particles. The dendritic cells exposed to the micro-particles were incubated with fluorescently tagged anti-CD40, anti-CD86 and anti-MHC Class I/II antibodies. (Approximately ## hours or min later), the dendritic cells were scanned to determine the expression of the indicated surface molecules.

Figure 12. Fluorescence of Treated Dendritic Cell: Columns 1-3 contain DCs treated with nothing. Columns #4-6 depict CD40, #7-9 CD86 (B7.2), and #10-12 MHC I/II expression. DC2.4 cells were treated with: A) nothing (media), B) beads only, C) beads + LPS, D) Beads + CL264, E) Beads + DEC-205, F) Beads + SPAS-1, G) DARTs + SPAS-1.

For this experiment we focused on four surface receptors: CD40, CD86, major histocompatibility complex (MHC) class I and MHC class II molecules. These surface receptors are essential components for provoking an immune response and activating T cells. Thus, their up-regulation as a result of exposure to the micro-particles would demonstrate that the particles are successfully priming the dendritic cells to activate T cells.

The beads + LPS treatment demonstrated the most up-regulation of the target surface receptors in the dendritic cells. CD40 expression was increased 60%

over baseline expression, CD86 was increased over 40%, MHC class I expression increased over 30% and MHC class II expression increased by over 200% over the baseline expression in untreated dendritic cells. All of the treatments besides the beads only treatment demonstrated increased levels of surface receptors. However, we observed significant experimental variation, thus these data need to be repeated to ensure the results are consistent. We believe that much of this error is a result of the timeframe in which the experiment was done. The expression levels were read approximately 24 hours after the dendritic cells were exposed to the micro-particles. It is possible that had the cells been able to incubate longer with the particles, there may have been less error in the readings and a more accurate representation of the levels surface receptor expression.

The next test performed was an enzyme-linked immunosorbent assay (ELISA) that was used to test for production of the cytokines TNF-alpha and IL-6. The ELISA was performed as dictated by the eBioscience ELISA kit.

Concentration of IL-6 Secreted By Dendritic Cells After Treatment

Figure 13. Concentration of IL-6 Secreted by Dendritic Cells after Treatment: Mean concentration of IL-6 secretion of eight wells per treatment.

Concentration of TNF- α Secreted by Dendritic Cells After Treatment

Figure 14. Concentration of IL-6 Secreted By Dendritic Cells After Treatment: Mean concentration of TNF- α secretion of eight wells per treatment.

In the previous experiment, we were looking at the surface receptors being expressed by dendritic cells after exposure to the micro-particles described in experiment 5. In this experiment, we are looking at what cytokines the dendritic cells secrete to determine how these treatments affect DC polarization. To do this we looked at two cytokines, IL-6 and TNF- α . IL-6 is a cytokine that is involved in promoting inflammation and in the maturation and differentiation of B cells. Tumor Necrosis Factor Alpha (TNF- α) is an inflammatory cytokine involved in tumor necrosis, inhibition of tumorigenesis, neutrophil proliferation and apoptosis. The

increased production of these cytokines by a dendritic cell indicates that the dendritic cell has recognized a potential threat and is promoting an immune response against that threat.

We established baseline levels of IL-6 and TNF- α being produced by dendritic cells. These dendritic cells produce an average of 0.33ng/mL of IL-6 and 0.35ng/mL TNF- α . The IL-6 ELISA shows that dendritic cells exposed to beads + LPS, beads + DEC-205 demonstrate a slight increase, while the cells exposed to beads + SPAS-1 and DARTS + SPAS-1 secrete over 9 times the amount of IL-6 than the untreated cells. This suggests that while LPS and DEC-205 can influence a dendritic to secrete IL-6, exposure to a specific antigen drives a more extreme response. Furthermore, the combination of the antigen with the DARTS complex drives an even slightly higher concentration of IL-6. This suggests that the combination of antigens and adjuvants is the most successful at encouraging dendritic cells to secrete IL-6.

In the TNF- α ELISA, the beads + LPS, beads + SPAS-1 and DARTS + SPAS-1 all show highly increase concentrations of TNF- α . The LPS treatment results in a TNF- α concentration of 1.46ng/mL, the beads + SPAS-1 treatment a concentration of 2.27ng/mL and the DARTs + SPAS-1 treatment a concentration of 2.38ng/mL. The beads + CL264 treatments shows a small increase while the beads only and beads + DEC-205 treatments have almost no effect on TNF- α secretion. These results suggest that the LPS, which has a pathogen-associated molecular pattern, and the SPAS-1 antigen have the most effect on the dendritic cells, the combination of which drives the highest response and thus the highest concentration of secreted TNF- α .

Conclusions

Overall this project has illustrated several key promising features of dendritic cell vaccinations and it has also identified several areas that could be improved upon and re-worked for stronger anti-tumor immune responses. When exposed to the micro-particles, the beads + LPS micro-particles demonstrated the highest level of up-regulation of cell surface receptors including CD86, CD40, MHC Class I and MHC Class II molecules. The DARTs + SPAS-1 micro-particle only demonstrated up-regulation of CD86 and MHC Class II molecules over the baseline. As discussed previously, it is possible that a prolonged incubation period could lead to different results, however the results do indicate interesting aspects of dendritic cell activation. First, LPS is a PAMP, which was used in this experiment to instigate a strong immune response in the dendritic cells. It is evident that this was true in the case of the up-regulation of cell-surface receptors. It is interesting that there was significantly less prevalence of the surface receptors when the dendritic cells were exposed to the DARTs + SPAS-1 micro-particle. This indicates that due to the competition for space on the micro-bead, less LPS was attached to the micro-particle and therefore we did not see as strong of response. Additionally, it indicates that the SPAS-1 antigen had little to no effect on inducing an up-regulation in surface receptors on the DCs.

While the cell surface receptors were not highly impacted by the DARTs+ SPAS-1 micro-particles, the cytokine production demonstrated significantly higher levels of secretion by dendritic cells that were exposed to the beads + SPAS-1, and even more so by the DARTs+SPAS-1 micro-particles. These results indicate that the

SPAS-1 antigen plays a crucial role in promoting cytokine secretion by the DCs. Additionally, the results show that the addition of the adjuvants further promote this function.

While this was just one study used to examine the effects of antigen and adjuvants through a micro-bead delivery system on dendritic cell function, there are several different things that could be improved upon in subsequent testing. First, while the purification process was relatively effective in singling out the SPAS-1 antigen, as can be seen in Figure 6, there are still other proteins present in the solubilized solution that was created. A purer form of SPAS-1 may result in better results and stronger DC responses. Furthermore, as described in previous sections, the use of non-biodegradable antigen delivery systems has several benefits, but these particles also have the potential to have toxic side effects, build up in the tissues as well as limited re-vaccination potential. The use of a biodegradable antigen carrier such as a PLGA or ISCOM micro-particle could have the same immunogenic effects without some of the concerns relating to non-biodegradable systems. Finally, it may also be beneficial to tinker with different combinations of adjuvants in order to determine the optimal combination for maturing DCs, increasing DC surface receptor expression and promoting cytokine secretion, all of which are crucial to antigen presentation, T-cell activation and differentiation and an overall stronger anti-tumor immune response.

Summary

Cancer therapy has taken considerable strides in the past years. The introduction of immunotherapy as a viable form of cancer treatment has provided a new platform for growth, innovation and a powerful tool for fighting cancers by harnessing the innate prowess of the immune system. One area of advancement in the immunotherapy originates from the ability to enhance the immune system's natural abilities with the addition of adjuvants and stimuli through vaccination. In this study, we sought to explore the immunogenic potential of vaccination of both ex-vivo primed dendritic cells as well as vaccinations aimed at targeting and activating dendritic cells in-vivo.

Several different factors have been discussed in the examination of dendritic cell vaccinations. These factors include ex-vivo versus in-vivo targeting of dendritic cells, antigen selection, adjuvant selecting, antigen-carrier options and DC targeting strategies. In order to create an effective vaccine that can stimulate a lasting and targeted anti-tumor response, all of the components of a vaccination must be considered not only for their ability to function collaboratively, but also to work for the individual cancer scenario. Effectively, this means that there is not one perfect cure all for cancer, however, there is the potential for different vaccines to work productively for particular cancers and at different stages of cancer progression.

The individual project presented in this article addresses some of the factors that can contribute to the success of a dendritic cell-based vaccination strategy. The adjuvants selected for vaccination can play unique and critical roles in priming dendritic cells for antigen presentation and subsequent T-cell activation and

differentiation. In the current project, the adjuvants I selected played a crucial role in promoting dendritic cells to produce surface cell receptors critical to antigen presentation and co-stimulation of T-cells. In contrast, the specific antigen selected had much less of an impact on cell surface receptors, but still had a tremendous effect on inducing cytokine secretion by the DCs. These unique functions of the adjuvants and antigen demonstrate their interdependence and the importance of creating a balanced vaccine that contributes sufficiently to both aspects of DC activation and functionality.

Overall, vaccinations aimed at inducing dendritic cell responses that have the ability to direct targeted, tumor specific immune attacks, have the potential to become not just one type of cancer therapy, but one of the most effective and non-toxic cancer eradication methods. Dendritic cell vaccinations are customizable, workable and largely innocuous. These vaccinations are geared at wielding the pre-existent defense mechanisms and functions of the immune system and enabling them to recognize cancer cells as malignancies. In addition to a prompt immune response, vaccinations have the potential to induce lasting immunity, which serves to prevent recurrent tumor growth. With further refinement and testing, immunotherapy through vaccination has the capacity to become a powerful tool in cancer therapy.

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