A REVIEW OF TUMOR-ASSOCIATED MACROPHAGE-TARGETED CANCER
THERAPIES

By

Emma Katherine Wandscher

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Approved by:

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Maryam Ahmed, Ph.D., Thesis Director

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Cole Edwards, Ph.D., Second Reader

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Jefford Vahlbusch, Ph.D., Dean, The Honors College

I. Abstract

Tumor-associated macrophages, or TAMs, make up a significant component of the tumor microenvironment and contribute to the human immune response to cancer. TAMs can either take a pro-tumorigenic form, promoted by pro-inflammatory cytokines, that facilitates tumor growth and metastasis through the formation of new blood vessels, or an anti-tumorigenic form, promoted by interferons and lipopolysaccharides, that recognizes cancerous cells as malignant entities for destruction. This investigation seeks to explain how macrophages affect the body's response to cancer and how understanding these mechanisms can be used to develop anti-cancer therapeutics. Several different therapeutic strategies that focus on TAMs are detailed, including biophosphonate therapy to prevent macrophage production, altering levels of cytokines (i.e., CCL2, CXCL12, CSF-1) and adipocytokines to prevent macrophage recruitment, repolarizing macrophages to an anti-tumorigenic form, limiting vascular endothelial growth factor (VEGF) production by macrophages, and inducing apoptosis in M2 macrophages with minigene vaccines or engineered apoptotic agents. There are many exciting possibilities for research in this rapidly evolving field, and the hope is that future research will get us one step closer to making cancer treatment as effective and risk-free as treating a common sinus infection.

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II. Introduction

"Cancer" is an umbrella term used to refer to several diseases that cause unregulated cell replication and division within the body. These diseases typically start in a single body zone, for example, the heart or lungs. This uncontrolled cell division often leads to the formation of tumors. Tumors are masses of cells that form solid "lumps" that can sometimes be detected from the surface. Though many tumors are benign and do not contain cancerous cells, this project will focus on cancerous tumors, specifically. Cancer cells are unique in their ability to metastasize. NIH's National Cancer Institute defines metastatic cancer as having "spread from the place where it first started to another place in the body." This means that if an individual develops cancer in a primary location such as the breast, it may potentially spread to new locations within the body and continue growing cancerous cells. Metastasis not only makes cancer more difficult for healthcare providers to locate on a patient, but also makes it significantly more difficult to treat. If a cancer has metastasized, it requires treatment both to shrink or remove the existing tumors as well as prevent cancerous cells from spreading to even more locations within the body. Metastasis is responsible for approximately 90% of modern cancer deaths (Seyfried and Huysentruyt 2013). For example, the Mayo Clinic states that only 5% of patients with metastatic lung cancer will survive for longer than 5 more years (Mayo Clinic Staff 2020). Not only is cancer an issue for human health and rights, but it also ties into the economy, with approximately $80.2 billion spent in the US alone on cancer-related medical costs (American Cancer Society Medical Content Team 2018). Only by studying the details of the cellular functions of cancer and the human immune response to these functions can we learn how to effectively treat cancers.

The goal of this thesis was to develop a better understanding of the different therapeutic approaches to cancer that are available. To accomplish this, I began working in the laboratories

of Drs. Maryam Ahmed and Darren Seals, studying tumor-associated macrophages (TAMs), podosome development, and oncolytic virotherapy. The overarching goal of the work continuing in this laboratory is to determine the efficacy of vesicular stomatitis virus (VSV) as a potential oncolytic virotherapy. For my project, I investigated the ability of polarized tumor-associated macrophages to produce podosomes and how the number of podosomes produced changes with VSV infection. From this initial research, I became familiar with macrophages and their role in the tumor microenvironment, and subsequently developed this thesis to further investigate and better understand other types of TAM-targeted therapeutic approaches.

A. The Tumor Microenvironment

Studying cancer in the human body requires a focus on more than just the cancerous cells themselves. Studying the cells surrounding tumors and the way these cells interact with the tumor is necessary to gain insight as to how the tumor can obtain the resources it needs to develop while effectively evading the immune response. Understanding these complex interactions requires investigating the cancer environment at the cellular and molecular level. The tumor microenvironment (TME) refers to the cancerous cells, immune cells, and extracellular matrix in the area surrounding and including the tumor. It "is comprised of proliferating tumor cells, the tumor stroma, blood vessels, infiltrating inflammatory cells, and a variety of associated tissue cells" (Whiteside 2008). The cells within the tumor microenvironment play a large role in the development and behavior of tumor cells, including their ability to metastasize. One of the body's primary responses to the presence of a tumor is the activation of an inflammatory response. This response aims to attack the tumor by creating a hypoxic environment that prevents cell survival and encourages release of inflammatory cells

such as macrophages (Whiteside 2008). However, over time the presence of inflammatory cells leads to the production of reactive oxygen species and pro-tumor cytokines. Therefore, the initial inflammatory response to a tumor, if sustained over a long period of time, becomes a mechanism to prolong the survival of the tumor. The components of the immune response within the tumor microenvironment and the roles that they fulfill subvert our expectations of what the immune system is and what it does. While immune cells protect our bodies from pathogens and other invaders, the human immune system is just as capable of furthering diseases that can lead to sustained damage.

B. The Immune Response to a Non-Foreign Invader

When the body senses a foreign or unusual substance in the body such as bacteria, viruses, or cancer, the immune response is triggered. This involves the development, proliferation, migration, and activation of several different cell types. The human immune system can be divided into two subsystems, the innate immune system and the acquired immune system. The innate immune system is composed of cells and proteins that the body has produced as a response to foreign invaders since birth. These cells form the "general" immune response that is dependent on white blood cells found throughout the body to target and kill pathogens. The acquired (or adaptive) immune system is generated after the initiation of the innate immune responses and consists of cells that respond to specific pathogens. The cells in this system can "remember" antigens that the body has been previously exposed to and produce the appropriate antibodies to fight the invader upon re-exposure to the pathogen. This project focuses on understanding immune cells that are part of the innate immune response and their role during carcinogenesis.

The immune response can be a double-edged sword when it comes to detecting and fighting cancer. Cancer detection can be particularly difficult for the immune system because these cells, though they are malignant, are still technically recognized as "self" cells, thus making them difficult to identify as foreign and dangerous to the body. Earlier studies have identified that the body does eventually produce antibodies that are specific to the proteins associated with their individual tumors (Stockert et al. 1998). However, this study showed that this phenomenon only occurred in a small portion of the skin, ovarian, lung, breast, and colon cancer patients, with only 25 of the 234 patients in the study, or just over 10%, developing anti-tumor immunity (Stockert et al. 1998). These antibodies were detected via an enzyme-linked immunosorbent assay, or ELISA. This type of assay is used in many different areas of healthcare and immunology and is not limited just to cancer. ELISA allows us to separate, measure, and identify the cellular components of blood serum. Furthermore, we cannot rely on antibody therapy for cancer due to the difficulty in targeting different forms of cancer with distinct protein signatures. Not only would this require the development of better detection systems, including highly specific ELISAs like the ones utilized in this study, but it would require a large investment of time, research space, and money, all of which are in short supply during the COVID-19 pandemic.

One of the largest regulators of tumor growth and proliferation is the inflammatory response within the tumor microenvironment. It has been shown that in an environment with chronic inflammation, whether caused by consistent infections, prolonged inflammatory responses, or autoimmune issues, tumors are more likely to form. Once tumors have formed, they are able to efficiently grow and may have the capability of invading surrounding tissues.

Invasion is facilitated by angiogenesis, the formation of blood vessels that contribute greatly to metastasis via the bloodstream (Goradel et al. 2021). For example, individuals that abuse alcohol consistently show chronic inflammation in their livers and pancreases and have higher rates of liver and pancreatic cancer (Lin and Karin 2007). The "double-edged sword" in this case is that the inflammatory response is the body's attempt to fight off the infection, while unknowingly providing support for cancer cells and their ability to migrate and invade other areas in the body.

The primary regulators of the inflammatory response are signaling molecules known as cytokines. There are several different cytokines associated with the tumor microenvironment, most of which are proinflammatory and therefore pro-tumorigenic. The most well-characterized cytokine associated with tumor proliferation is the pro-inflammatory cytokine tumor necrosis factor-alpha, shortened to TNF-α, which activates the nuclear factor kappa-chain-enhancer for B cells, or NF-kB, kinase signaling pathway (Karin 2006). NF-kB is a transcription factor which regulates the expression of genes that play a role in reducing tumor cell apoptosis, tumor cell cycle progression, and plays a key role in metastasis (Karin 2006) . This molecule is released from both tumor cells and host immune cells, including macrophages. This cytokine is also proposed to assist with the initial formation of a tumor by promoting the production of nitric oxide (NO) and reactive oxygen species (ROS), which can cause the DNA damage associated with the uncontrolled proliferation of cancer cells (Hussain et al 2003). Studies have found that genetic polymorphisms that enhance the production of TNF-α are associated with an increased risk of several different cancers, including multiple myeloma and cancers of the bladder, liver, stomach, and breasts (Mocellin et al 2005). The inverse has also been found in that tumor cells treated with an antibody that neutralizes TNF-α showed widespread cancer cell death (Pikarsky

et al 2004). IL-6 is another proinflammatory cytokine associated with tumor proliferation and resistance to apoptotic molecules. IL-6 acts as a signaling molecule, and triggers the phosphorylation and activation of STAT1, which is a tumor growth inhibitor, and STAT3, which is a tumor growth promoter (Hodge et al 2005). This means that the function of IL-6 can vary widely in the tumor microenvironment and it is likely too unpredictable to use as a target for broad-spectrum cancer therapy. Another cytokine that may contribute to tumor progression is IL-23, which is mainly produced by activated antigen-presenting cells and other phagocytic cells, and its receptors are mainly found on T-cells, natural killer cells, and natural killer T cells (Trinchieri 2003). IL-23 induces the production of IL-17 and promotes inflammation in the end stages of cancer. Ongoing studies are also aimed at determining whether IL-23 can induce TNF-α production by dendritic cells. Dendritic cells, or DCs, are immune cells that act as a bridge between innate and adaptive immunity by phagocytosing antigenic material, breaking it down into smaller pieces, and presenting those pieces on their surfaces to alert T cells and the rest of the adaptive immune system (Trinchieri 2003). In general, numerous studies have indicated that many inflammatory cytokines are associated with tumor proliferation and progression.

In response to the detection of a tumor, the body can also produce anti-inflammatory cytokines to reduce the likelihood of the tumor to metastasize. One of the most effective of these is TRAIL, which is short for TNF-related apoptosis-inducing ligand and is produced by T cells and NK cells and is capable of inducing apoptosis in various cancer cell types with minimal effects on healthy cells (Lin and Karin 2007). TRAIL can bind to five different receptors, two of which are "death receptors" that flag cells for caspase-dependent apoptosis (LeBlanc and Ashkenazi 2003) Mice with TRAIL knockouts or that were treated with antibodies to neutralize

the TRAIL produced by their systems showed a much higher chance of developing tumors, whether through experimental induction or naturally (Takeda et al 2002). TRAIL likely plays a large role in mediating tumors as they occur, but it is limited in its application. Not all tumor cells are sensitive to the effects of TRAIL, and if TNF-α has already activated NF-kB in the tumor cells, they are already resistant to TRAIL-mediated apoptotic signaling (Luo et al 2004). This means that naturally occurring TRAIL cannot be used in its original form for therapy, but a recombinant form may be developed that is more effective against more varieties of cancer. Another anti-tumorigenic cytokine is IL-10. This cytokine suppresses the immune system, reduces inflammation, and inhibits the NF-kB pathway through mechanisms that have yet to be characterized by the scientific community (Schottelius et al 1999). The suppressive effect of this cytokine means that proinflammatory cytokines such as TNF-α, IL-6, and IL-12 are not produced. IL-10 is known for modulating apoptosis and preventing further angiogenesis as it promotes the regression of a tumor (Kundu and Fulton 1997). It accomplishes this through a downregulation of MHC class I molecule expression, leading to higher rates of natural killer cell attacks on cancerous cells (Kundu and Fulton 1997). Like IL-23, IL-12 is another anti-inflammatory cytokine produced by APCs and other phagocytic cells, and its receptors are found on T cells and NK cells. Like IL-23, it also induces a proinflammatory response. Despite this, IL-12 is associated with tumor reduction. IL-12 has given promising results in laboratory testing, with mouse models showing that IL-12 can not only prevent further spread of existing tumors but can also cause them to recede back to a smaller size (Trinchieri 2003). This is aided by interferon gamma (IFN-γ), which has a cytotoxic effect on cancer cells and the blood vessels that they produce. Despite its efficacy, IL-12 is not safe to use as a human therapy because it can induce high levels of IFN-γ leading to severe side effects for patients and toxicity (Trinchieri

2003). Though the body naturally produces these anti-tumor substances, they typically are not expressed at levels high enough to overcome a cancerous tumor on their own.

C. Macrophages and Cancer

A large component of the innate immune response is made up of monocytes and their descendant cells, macrophages. Macrophages are immune cells that "sample" their surrounding environment by consuming substances throughout the body (Mills 2012). In the tumor microenvironment, they act as an activator, sampling the material in the area, analyzing it, and recognizing whether to deploy either a "fight" or "fix" response. This allows the immune system to determine the difference between a wound and an infection. Macrophages exhibit plasticity based on environmental stimuli and can be coerced into numerous populations with distinct phenotypic markers and functions. Unactivated or 'naïve' cells are called M0 macrophages. This resting state occurs before they are alerted to a foreign presence and prior to phagocytosis. These M0 macrophages polarize into either M1 or M2 macrophages after they encounter and absorb environmental material. These two categories of macrophages have opposing functions. M1 macrophages, or "classically activated macrophages," support and encourage the immune response by producing NO. This chemical inhibits metastasis by preventing tumor cells from being able to grow and spread efficiently. This is what Mills (2012) refers to as the "fight" response. M2 macrophages, or "alternatively activated macrophages," inhibit the immune response by preventing pro-inflammatory signaling and producing ornithine, which supports repair. However, this response backfires in the TME by encouraging metastasis and strengthening bonds between the cancerous tumor cells and the cells and extracellular matrix surrounding it instead of healing healthy, normal cells. Therefore, M2 macrophages are considered pro-tumorigenic due to their ability to suppress the body's natural immune response

to tumor formation, while M1 macrophages are considered anti-tumorigenic as they support the natural immune response.

While M1 macrophages are considered beneficial for the well-being of the body, the tumor microenvironment is rich in IL-10, a cytokine which promotes polarization of monocytes into M2 macrophages (Sica et al 2006). In contrast, tumors do not secrete LPS and IFN-γ, which promote polarization of monocytes into M1 macrophages (Sica et al 2006). Cases have been observed where tumor-associated macrophages make up to 50% of the mass of the tumor, and macrophage levels are used as a biomarker to estimate the severity of cancer (Poh and Ernst 2018). There are several ways in which tumor-associated macrophages (TAM) promote the proliferation of cancer. Not only do TAMs express lower levels of inflammatory cytokines, but studies have shown that accumulations of these TAMs can promote endothelial cell migration (Sica et al 2006). TAMs can also contribute to tumor growth via stroma formation and the formation of new blood vessels through release of platelet-derived growth factor (Sica et al 2006). Furthermore, TAMs have been shown to form clusters near blood vessels and promote more invasive behavior through the secretion of epidermal growth factor nd other chemoattractants that recruit more cancerous cells to the initial tumor location (Yamaguchi et al 2006). This allows the tumor to strengthen its hold in the initial location and digest the extracellular matrix surrounding it. As the tumor entrenches itself in its initial location, it prepares for pieces of itself to break off and begin to spread to new locations in the body.

D. Podosomes and Extracellular Matrix Degradation

One of the key means by which M2 macrophages efficiently invade the tumor is through the development and use of actin-rich protrusions known as podosomes. Podosomes are made up of a dynamic and constantly replaced core of polymerized actin surrounded by proteins responsible for adhesion and scaffolding, such as Tks4, Tks5, cortactin, Src, and MT1-MMP, a metalloprotease (Murphy and Courtneidge 2012). These podosomes act as contact points between macrophages and the ECM surrounding them. There is somewhat of a language debate on this topic, specifically on the differences between "podosomes" and "invadopodia." Murphy and Courtneidge (year) describe the two as being essentially the same structure, with the only difference being that invadopodia are associated with cancerous cells and podosomes are associated with all other cell types. For this reason, I will use the term podosomes, as I am focused on this structure in macrophages, not the tumors with which they are associated.

According to Murphy and Courtneidge (2012) the presence of podosomes and invadopodia is directly related to the ability of a tumor to metastasize. Though podosomes are utilized for other adhesive and degradative purposes, I focus specifically on how podosomes are associated with the ability of tumor-associated macrophages to alter the extracellular matrix in the tumor microenvironment. They accomplish this using integrin proteins, which facilitate communication between the macrophage and the ECM surrounding it (Linder and Aepfelbacher 2003). After binding to the ECM, these podosomes can pull the matrix apart into smaller pieces, which can then be moved to new locations or degraded. Cancerous cells within a tumor help to facilitate the formation of podosomes through the secretion of colony-stimulating factor 1, or CSF1 (Yamaguchi et al 2006). This cytokine acts as a regulator for the building and distribution

of podosomes. Once the formation of new podosomes is stimulated, N-WASP facilitates the rapid formation of the dynamic actin core, which results in the cell membrane projections associated with macrophages (Yamaguchi et al 2006). These projections, when combined with recruited proteins, become podosomes. Tumor cells also produce proteinases, enzymes that degrade the extracellular matrix that work in conjunction with podosomes to facilitate metastasis of cancer and movement towards the bloodstream (Yamaguchi et al 2006). Therefore, podosome formation by macrophages contributes to the metastatic process, but much work is necessary to understand the mechanisms by which podosomes promote carcinogenesis.

E. Traditional Cancer Therapies

There are many different treatments available to patients who have been diagnosed with cancer. One of those primary treatments is chemotherapy. Literally translated, chemotherapy is "chemical-based therapy." This type of treatment uses drugs given topically, orally, or intravenously that are intended to destroy existing cancer cells and prevent new ones from forming (National Cancer Institute 2015). Many of these drugs were developed to combat a specific biochemical pathway discovered in the mid-1950s when Charles Heidelburger at the University of Wisconsin identified that cancer cells in rats had a greater uracil uptake than average (DeVita and Chu 2008). These drugs act as a broad-spectrum treatment for cancer, though more specific treatments are being developed that represent modern treatment options for patients.

Another common cancer treatment is radiation therapy, or radiotherapy. Approximately 50% of all cancer patients receive radiotherapy at some point during their treatment (Baskar et al 2012). Radiotherapy-concentrated X-ray radiation that is used to kill cancer cells and reduce the

size of tumors. It accomplishes this by damaging the DNA in the cells, and triggers programmed cell death, or apoptosis, which continues for several weeks. There are several different ways that this type of therapy can be administered. Radiation can be directed at the body via external beams, implanted devices, or liquid drugs that are administered orally or intravenously (National Cancer Institute 2015). Not only is radiation effective against a variety of different cancers, but it is also one of the most cost-effective cancer treatments, making up only 5% of the total cost of cancer care (Baskar et al 2012). The most reported side effect of radiation is fatigue, but many of the other side effects are similar to those observed during chemotherapy, including diarrhea, nausea, changes in skin, and tenderness (National Cancer Institute 2015). However, radiotherapy has the benefit of causing less damage to healthy cells than chemotherapy because healthy cells are more capable of repairing DNA damage than cancerous cells (National Cancer Institute 2015).

One of the more invasive techniques used to treat cancer is surgery. There are several surgical options associated with cancer diagnosis and care. First, surgery may be used for diagnosis. When a tumor is discovered, it is typically biopsied and analyzed under a microscope to observe unusual patterns in cell growth. Surgery is also used to determine what "stage" cancer is in, to remove tumors from specific locations in the body, to remove the bulk of a cancer by "shaving down" a tumor, to treat pain or systemic effects, to place medical devices, reconstructive surgery to restore the appearance and function of the body after treatment, and preventative surgery to remove tissue that is likely to become cancer (National Institute of Health 2015). These different types of surgery options can be combined with other cancer therapies for a well-rounded treatment plan.

III. Therapeutic Strategies to Target Tumor-Associated Macrophages

One of the treatments for cancer that is increasingly being explored is macrophage-specific therapy. TAMs are key targets because they are highly abundant at basement membrane degradation sites and at sites surrounding invading tumors (Poh and Ernst 2018). In breast cancer, up to 50% of the tumor mass can be attributed to macrophages (Tariq et al 2017). Not only can macrophages act to facilitate cancer metastasis, but they also can be used as a biomarker for cancer severity and can guide medical providers in the patient treatment plans. Measuring TAM levels for cancer patients can also help identify those who are more likely to have positive outcomes when given chemotherapy after surgery because TAMs can be re-educated to prevent tumor proliferation (Poh and Ernst 2018). Figure 1 shows the different strategies employed to target TAMs. These include the prevention of macrophage development and recruitment to the tumor site, induction of macrophage re-polarization, targeted cell killing, inhibition of angiogenesis, and oncolytic virotherapy. Each of these strategies will be discussed in detail in the following section.
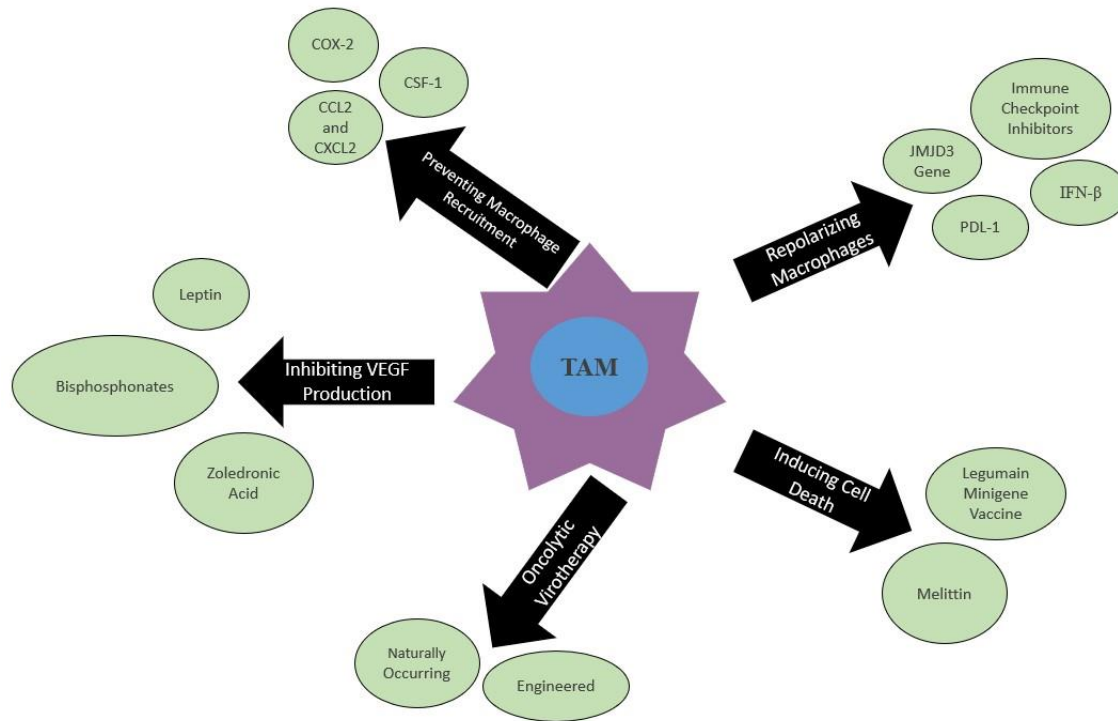
Figure 1. An overview of macrophage-targeted cancer therapies, including prevention of macrophage recruitment, macrophage repolarization, vascular endothelial growth factor (VEGF) production inhibition, apoptosis induction, and oncolytic virotherapy.

A. Preventing Macrophage Production

A macrophage-specific cancer therapy strategy is preventing the development of macrophage populations. This technique has been shown in studies to limit tumor growth and spread, as well as promote cancer cell response to chemotherapy (Poh and Ernst 2018). Drugs such as trabectedin can be used to inhibit the production and release of pro-tumoral cytokines, including CCL2 and IL6. Biophosphonates have been shown to have a cytotoxic effect on myeloid cells, progenitors for monocytes, which become macrophages when polarized. In essence, this class of drugs can be used to prevent the development of macrophages by starting further "upstream" in the polarization process.

B. Preventing Macrophage Recruitment

Preventing macrophages in the body from associating with malignant tumors represents a viable macrophage-targeted treatment strategy. Some of the first potential targets for therapy are CCL2 and CXCL12, which facilitate the recruitment of TAMs into the cancer tumor and promote polarization to the M2 state (Tariq et al 2017). Obese mice with a CCL2 gene knockout showed a significant reduction in macrophage invasion into the tumor tissue and an increased production rate of inflammatory cytokines (Tariq et al 2017). The opposite effect was also observed in mice that overexpressed CCL2, where more TAM infiltration and reduced cytokine production was observed (Tariq et al 2017). Drugs that target these signals have shown significant reduction of both tumor growth and metastasis in breast cancer and prostate cancer (Poh and Ernst 2018). This makes targeting CCL2 and CXCL12 a viable option for cancer therapy. There are already drugs under investigation that take advantage of this pathway. Siltuximab (CNTO 328) is an anti-IL-6 antibody that shows reduced tumor vascularization and reduced production of macrophage chemoattractants, including CCL2 and CXCL12 (Tariq et al 2017). Bindarit, which acts as an anti-inflammatory agent, has been shown to inhibit CCL2 secretion, and therefore the infiltration of macrophages into a tumor, in breast cancer and prostate cancer during preclinical trials (Tariq et al 2017). Treatments targeting CCL2 and CXCL12 may reduce the effect TAMs have on tumor growth, proliferation, and angiogenesis.

Colony-stimulating factor (CSF) also plays a large role in macrophage recruitment to a tumor (Tariq et al 2017). For this reason, CSF, and specifically CSF-1, which is responsible for TAM recruitment, must be investigated as a potential therapeutic target. In mouse models with CSF-1 blocking, tumor growth rates were reduced, and overall survival likelihood increased. In

breast cancer, elevated CSF-1 has been directly correlated with increased tumor vascularization and a decreased overall chance of survival. This means that, especially in areas with an already large concentration of existing blood vessels, CSF-1 can play a direct role in determining how much a tumor can grow and spread via metastasis. The mechanism by which CSF-1 functions is better understood than that of CCL2 and CXCL12, hence there are several different types of drugs in development and in clinical trials that focus on this specific molecule. Small molecules have been discovered that inhibit expression of CSF1R, the receptor that works in conjunction with CSF, and have been developed into drugs including Plexxicon and Pexidartinib. Results from preclinical trials of Plexxicon (PLX-3397) show that M2 macrophages are significantly reduced in the TME and that there is a higher proportion of cytotoxic T cells able to infiltrate the tumor and facilitate cell death. Another way to target CSF-1 is through antibodies. Anti M-CSF antibody treatment has been shown to inhibit tumor growth by up to 40% in human breast cancer cells used in mouse model. It also can reduce the number of TAMs recruited to a tumor entirely, of both the M1 and M2 varieties. These treatments have great potential, especially if used in conjunction with other, more well-established cancer treatments. Anti-CSF1 combined with paclitaxel (PTX), a commonly utilized chemotherapy agent, has been shown to significantly decrease tumor progression and improve the efficacy of cytotoxic immune cells in breast cancer.

Adipocytokines are a class of chemical signaling molecules that are secreted by white adipose tissue, which is found subcutaneously throughout the body. These chemicals initiate the process of inflammatory cytokine secretion, including CCL2, IL-1, IL-6, and TNF-α, as well as contribute to the tissue activation of the NF-kB pathway to induce further inflammatory response (Tariq et al 2017). These molecules can be especially important for cancers that occur in fattier

areas of the body, especially breast cancer. One of the most well-characterized of these adipocytokines is COX-2, which acts as an inflammatory mediator that affects the expression of genes associated with cancer and stimulates the formation of prostaglandins, lipids associated with injuries. There are several drugs that utilize this pathway and show exciting preliminary results. Celecoxib, a COX-2 inhibitor, has been shown to inhibit inflammatory mediators including COX-2, angiogenesis, and progression of breast cancer in studies. Curcumin, a known anti-inflammatory agent, has also demonstrated efficacy as a potential anti-cancer treatment as it suppresses release of IL-6, IL-8, and IL-8 by inhibiting the NF-kB and COX-2 pathways. NSAIDS also produce a similar effect, reducing the production of pro-inflammatory pathways. Aspirin has been shown to reduce the incidence of epithelial cancers and inhibits the secretion of adipokines, CCL2, and PAI-1. Even if adipocytokines are not particularly relevant to every type of cancer, their healing potential may be harnessed as a treatment for cancers in high-fat areas.

C. Changing Macrophage Phenotype and Function

Yet another class of macrophage-specific cancer therapy strategy involves the reprogramming of existing pro-tumorigenic macrophages to change them from the M2 pro-tumorigenic state to the M1 anti-tumorigenic state. Studying the genetic code of M2 macrophages has allowed scientists to identify genes and signaling pathways that regulate polarization. The JMJD3 gene is one potential target identified by gene sequencing. This gene acts as a transcription activator for genes associated with the M2 macrophage polarization state and becomes a transcription inhibitor for M1 macrophage genes (Tariq et al 2017). This means that theoretically, if this gene were knocked out or silenced, there would be more M1

macrophages and fewer M2 macrophages produced. The inhibition of certain genes not only can promote the repolarization of macrophages but can also act as a booster for the remainder of the immune system (Poh and Ernst 2018). Another therapeutic mechanism under investigation is manipulating the expression of PDL1, which is associated with reduced tumor cytotoxicity of macrophages (Tariq et al 2017). When IL-10 and TNF-α are produced by activated monocytes, PDL1 expression is enhanced (Tariq et al 2017). Studies that blocked the expression of PDL1 showed enhanced patient immunity against cancer (Tariq et al 2017). Drugs and other treatments aimed at reducing the expression of this protein may prove effective with future research. A third mechanism for macrophage repolarization under investigation is IFN-β (Tariq et al 2017). DMXAA (dimethylxanthenone-4 acetic acid) has been shown in studies to increase the repolarization of M2 macrophages to M1 macrophages by upregulating the IFN-β signaling pathway (Tariq et al 2017). When T-cells and the rest of the immune system are inhibited or reduced, repolarization favors the M1 state. Daclizumab, a drug utilizing CD25-specific monoclonal antibodies, has been shown to eliminate T regulatory cell populations, removing many of the checkpoints that limit production of immune cells and the repolarization of macrophages from M2 to M1 (Tariq et al 2017). These drugs have been used for cancer for years and were approved by the FDA for use as a cancer treatment in 2011 (Tariq et al 2017). Therapeutic strategies that repolarize M2 macrophages into M1 macrophages instead of killing them altogether are favorable for cancer patients as they do not deplete macrophages but coerce them to fight the tumor.

D. Targeting the Production of Vascular Endothelial Growth Factor

Vascular endothelial growth factor, or VEGF, induces angiogenesis in tumors (Tariq et al 2017). VEGF is produced by pro-tumorigenic M2 macrophages. There are several different therapeutic approaches available to target VEGF production. VEGF is associated with leptin receptors in the body, and treatment of breast cancer cells with leptin receptor repressors leads to decreased macrophage production of VEGF (Tariq et al 2017). Further characterization of how leptin and VEGF interact with each other may elucidate further development of this technique. Another treatment already in use for many cancers is bisphosphonate drugs. These drugs act as inhibitors of bone resorption and are capable of inhibiting angiogenesis (Lipton 2008). They are already commonly used to strengthen the bones of patients with malignant bone diseases such as osteoporosis. These drugs may also have the potential to arrest the cell cycle, induce apoptosis in tumor cells, and inhibit metastasis (Lipton 2008). Bisphosphonates are also associated with decreasing VEGF production from macrophages. Patients with cancer that had metastasized from the initial tumor location to their bones were treated monthly with pamidronate, a bisphosphonate drug (Lipton 2008). Their basal VEGF levels were shown to have decreased rapidly and significantly at 1-, 2-, and 7-days post-infusion with the drug (Lipton 2008).

Another drug associated with bone disease and VEGF production is zoledronic acid. Patients with metastatic bone disease from breast cancer provided a zoledronic acid infusion showed significantly reduced serum VEGF levels 3 weeks after their infusion, fewer skeletal-related events (bone fractures, spinal cord compression, etc.), and a delayed disease progression (Lipton 2008). This discovery led to a study to evaluate whether low dose, weekly zoledronic acid inhibits angiogenesis in patients whose cancer had metastasized to their bones. Patients were found to have an overall decrease in circulating VEGF after the first week of treatment, and this

suppression continued for the entirety of the 84-day study (Lipton 2008). Zoledronic acid seems to be the most promising of the drugs that reduce VEGF production, and it may become a more popular treatment for bone cancer in the future. Overall, strategies to decrease the ability of M2 macrophages to express factors associated with angiogenesis are continuing to be explored as effective anti-cancer therapeutics.

E. Depletion of TAMs

There are several therapeutic approaches that attack and kill M2 TAMs preferentially while leaving M1 TAMs relatively unscathed. The first of these approaches is a minigene vaccine that targets Legumain, which is coded by the LGMN gene and is only expressed in M2 macrophages (Tariq et al 2017). This therapy is exciting because of the minimal side effects it has, including leaving existing M1 macrophages relatively unharmed. Researchers have developed an oral minigene vaccine against mouse expression of Legumain. The minigene vaccine strategy works similarly to normal vaccine strategies in that it facilitates a cytotoxic immune response to a specific antigen. In this case, the antigen is a small peptide chain that is only 8–10 amino acids long (Lewēn et al 2008). This allows for very specific targeting that ignores any irrelevant antigen epitopes to prevent unnecessary reactions that would present as side effects in patients. This minigene vaccine was tested in syngenic BALB/c mice and was determined to successfully inhibit vascularization of breast cancer cells by facilitating a specific cytotoxic T cell response (Lewēn et al 2008). These data represent exciting developments in the world of immunology and cancer biology as soon we may be able to use a similar vaccine as a prophylactic measure to prevent cancerous tumors from forming in the first place.

Another mechanism to induce apoptosis in M2 macrophages is through melittin (MEL), a component of bee venom, fused with KLA, a mitochondrial membrane-disrupting agent (Lee et al 2019). MEL has been shown to bind preferentially to macrophages that express CD206. CD206 has been shown to be more highly expressed on M2 macrophages in preclinical cancer models and is associated with angiogenesis. In fact, CD206 has historically been used as a prognostic indicator for metastasis and general outcomes for lung cancer . Lastly, KLA is a naturally occurring protein with antibacterial properties, showing a preference for prokaryotic cells while leaving eukaryotic cells unaffected. Once KLA has been absorbed into a cell, the mitochondrial membrane is punctured, releasing cytochrome C into the internal environment of the cell and inducing cell death. MEL-dKLA, the fused molecule, was shown to decrease M2 macrophage viability by about half and inhibit further tumor growth in studies using mice with subcutaneous tumors. Not only did the tumors not grow, but tumor size and weight were found to have decreased in mice who received the MEL-dKLA treatment. In addition, the ratio between M1 and M2 macrophages was found to be significantly higher in treated mice than those who received control treatments. This approach, like the minigene vaccine, appears to have great potential, but requires further research.

F. Oncolytic Virotherapy

A potential treatment for cancer that works in conjunction with TAMs is oncolytic virotherapy. This treatment involves exposing a patient to an oncolytic virus (OV), a specific class of viruses, either naturally occurring or human-engineered, that replicates within and eventually kills cancer cells while sparing healthy cells from damage (Fukuhara et al 2016). This therapeutic approach was previously investigated from 1949–1980 but yielded low success because of the lack of genetic tools to downregulate viral pathogenicity (Fukuhara et al 2016).

After major strides in the disciplines of virology, immunology, and genetics, as well as the development of tools for investigation of oncolytic agents in the 1980s and early 1990s, Matuza (1991) showed that a version of the type I herpes simplex virus with modifications to the gene coding for thymidine kinase could potentially be used as a therapy for brain cancer. This began a resurgence in research on OV and how they can be used to treat cancers.

i. Ongoing OV Research: Vesicular Stomatitis Virus

My work in the laboratories of Drs. Ahmed and Seals focused on the OV, vesicular stomatitis virus (VSV). VSV is associated with disease in cattle and other mammalian livestock species but exhibits low virulence in humans. However, its pathogenic mechanisms in cancer cells make it potentially useful as an anti-cancer treatment. Wild-type VSV induces programmed cell death through an intrinsic pathway, meaning that once a cell is infected, it detects errors within itself and begins apoptosis (Hastie and Grdzelishvili 2012). Recombinant forms of this virus were initially developed by researchers as vectors for vaccine delivery and were genetically engineered to contain mutations in the viral matrix (M) protein, which is responsible for inhibiting host transcription and nuclear-cytoplasmic transport of host mRNA through binding with nuclear pore components and transcription factors (Kopecky et al 2001). This leads to the suppression of host gene expression in infected cells, including expression of genes in the host antiviral immune response, thus promoting viral replication (Ahmed et al 2003). Unlike the wild-type form, M protein strains of VSV kill through the extrinsic pathway, meaning that cells are destroyed from external processes that result in ligands from outside of the cell binding to receptors on the cell surface to trigger apoptosis as compared to internal apoptotic signaling (Hastie and Grdzelishvili 2012). Research into this virus and its mechanisms of action are

ongoing in the lab of Dr. Ahmed as well as other laboratories interested in developing VSV-based oncolytic agents and vaccines. Studies have shown that VSV replication is tightly controlled by the type I interferon (IFN) response. If a normal cell is infected with VSV, virus replication is attenuated through activation of antiviral genes in the type I IFN pathway (Ahmed et al 2004). However, the type I IFN response pathway is commonly defective in cancer cells, thus making them susceptible to virus infection and killing. Therefore, to further increase the tumor selectivity of this virus while promoting safety, cancers can be pre-treated with IFNs or infected with M protein mutant strains with an ability to induce IFN production in infected cells (Ahmed et al 2004). Large strides are being made in this research field, and VSV carries great potential as an oncolytic agent.

Studies in the laboratories of Drs. Ahmed and Seals are currently investigating the use of VSV as an anti-cancer agent that has the capability of targeting M2 TAMs. Studies have shown that the M protein mutant strain of VSV (rM51R-M virus), can effectively coerce M2 macrophages to a M1-like proinflammatory state. Furthermore, rM51R-M virus can inhibit podosome formation in M2 macrophages, thus having the potential to decrease the metastatic potential of cancer cells. The Ahmed and Seals labs seek to investigate the effects of VSV infection on macrophages and determining whether VSV can be used to "switch" macrophages from their M2 state to their M1 state. This would make them transition from promoting the growth and blood vessel formation of the tumor to attacking it. My project in the laboratory was focused on the podosomes produced by these macrophages and how their numbers differed when the macrophages were infected with the rM51r-M strain of VSV. Preliminary results supported my hypothesis that infection with recombinant VSV would reduce the overall number of macrophage podosomes produced. I was able to conclude that infection with rM51r-M VSV

appears to reduce the pro-tumorigenic activity of macrophages in the tumor microenvironment. The next phase of research into this line of inquiry would be to obtain primary immune cells as opposed to the THP1 laboratory cell line utilized in our previous experiments and replicate our previous studies to determine if this viral strain is also effective in primary cells. Investigations using primary immune cells are already underway and will hopefully yield promising results.

ii. The Future of Oncolytic Virotherapy

As of 2016, several oncolytic viruses have been approved for therapeutic use. In 2015, the United States, the European Union, and Australia approved the use of T-vec, a genetically engineered oncolytic virus containing two genetic deletions affecting the ability of the virus to infect healthy cells and the rate at which the virus replicates once it has infected cancer cells (Fukuhara et al 2016). This therapy has been shown to be an effective treatment against several different types of cancer. In 2015, reoviruses were classified as "orphan drugs" for ovarian cancer, pancreatic cancer, and some brain and spinal cancers, meaning that they are potentially useful as pharmaceuticals, but require further study (Fukuhara et al 2016). These viruses are naturally occurring, and show enhanced replication in "transformed," or cancerous, cells than in normal cell lines (Fukuhara et al 2016). However, this type of research tends to be underfunded, as pharmaceutical companies are reluctant to provide resources to a long-term project that will not immediately generate a profit. Despite the financial risks involved, there is a currently ongoing clinical trial for using an intravenous injection of a strain of VSV engineered to express NIS and human interferon beta as a clinical treatment for certain types of lung, head, and neck cancers. I find this really exciting because if this proves to effectively promote tumor regression and even promote tumor apoptosis, we could see many other strains of VSV and other oncolytic viruses being brought into clinical trial settings. If this form of virotherapy proves to be effective

for the non-small cell lung cancer and head and neck squamous cell carcinoma patients in this study, it will pave the way for other oncolytic viral therapies that treat other forms of cancer, such as breast cancer, small-cell lung cancer, or pancreatic cancer in the future.

Obtaining funding for research and clinical trials is not the only challenge facing oncolytic virotherapy. There are also considerations that must be made surrounding safety and efficacy of these viruses before they can be widely used as drugs. Firstly, it must be ensured that the viruses are safe to use in patients and patients do not suffer for toxic side-effects. Many of these viral strains are known to be normally pathogenic, so for approval in humans, the viruses must be genetically modified to reduce pathogenicity. However, this approach can be taken too far, and a virus may be weakened so much that it is defeated by the patient's immune system before it reaches the bloodstream. There are several different mechanisms to achieve this "happy medium," including immune checkpoint inhibitors, anti-tumor gene insertion into the viral genome, and coupling of the virus with other anti-tumor cells, including macrophages (Fukuhara et al 2016). Another limitation to oncolytic virotherapy is engineering a strain that can permeate between tumor cells from the initially infected cells. To do this, strains that are capable of overcoming the tight epithelial junctions of tumor cells that make them resistant to take up larger molecules including oncolytic viruses (Goradel et al 2021). Researchers also must find viruses that are able to survive and replicate effectively in hypoxic environments, such as the tumor microenvironment (Goradel et al 2021). The largest challenge faced by researchers investigating oncolytic virotherapy is that viral dosage and side effects can vary wildly between patients. This means that for this approach to work, the treatment plan must be tailored to each case specifically, meaning that this treatment requires more time on the part of scientists and

physicians and more money to compensate for this on the part of the patient and their insurance company. This is especially important for patients who have already received other forms of cancer therapy and likely have a compromised immune system because of it. Overall, OVs have the potential to be a treatment for cancer but require more research before they can be used as safely and regularly as traditional cancer treatments like chemotherapy and radiation.

IV. Conclusions

Macrophages and other immune cells play an important and necessary role in cancer. However, extensive research into how the immune system interacts with cancer shows that the immune response is not always beneficial to the patient, sometimes it promotes carcinogenesis. Therefore, it is important to understand the complex interaction of immune cells with tumors. In the case of macrophages, they represent a major component of the tumor microenvironment and studies on how they contribute to tumor growth and metastasis may lead to important information on how to harness their power for good. Through the manipulation of various factors, pro-tumorigenic macrophage activity can be thwarted. This can occur with drugs that prevent the development of macrophages. However, this type of strategy, which depletes macrophages, appears to be a "double edged sword" that may leave patients vulnerable to other infections. With the existing knowledge in this area, preventing recruitment of macrophages or selectively killing M2 macrophages seem like the most likely therapeutic methods. In addition, these strategies may lead to minimal side effects on cancer patients compared to traditional chemotherapy. There are many possible therapeutic mechanisms to target TAMs, but most require further study and characterization before they can safely be used in humans. Future research in this area will likely focus on the differences between M1 and M2 macrophages and

establishing mechanisms to reduce the M2 macrophage population without inhibiting the tumoricidal activity of M1 macrophages. All in all, this is an exciting area of research that will likely surge in the coming years, especially following the COVID-19 pandemic. With the development of more effective anti-cancer therapies, including those detailed here that target macrophages, a cure for cancer is on the horizon, and with more time and research, it may be discovered.

V. References

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