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Immune Modulation by Tumor-Derived Extracellular Vesicles in Glioblastoma

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1. Introduction

Glioblastoma multiforme (GBM) is a highly aggressive and malignant brain tumor that is largely resistant to surgical, radiological, and chemotherapeutic intervention. It is character‐ ized as a WHO Grade IV astrocytoma with high infiltrative capacity into brain parenchyma, resulting in debilitating cognitive deficits. Features such as diffuse tumor margins, high vascularity, and necrosis are cardinal indications for GBM diagnosis [1]. Current treatment regimens include a combination of surgery and radiotherapy, along with adjuvant temozolo‐ mide. This line of therapy has been successful in extending patient survival to 15 months [2]. However, the significant emotional and financial cost incurred by patients and their families during this process can be devastating. All three approaches employ a shotgun approach with nonspecific effects leading to reduced quality of life and increased burden of disease. Despite advances in the management of many other tumor types, glioblastoma has not experienced the same success and median survival still remains 15 months. An alarming 8% average survival at 3 years stresses the urgent need for more effective treatments [3]. Emerging novel therapeutics aim to manipulate the immune system for selective destruction of cancerous tissue, while leaving healthy brain tissue undisturbed. These approaches offer promise for a selective approach of tumor elimination with reduced side effects and improved quality of life. However, immunotherapeutic success has also been limited, due to lack of understanding of the influence of cancer cells on the immune system. Better understanding of the glioma and immune interface will allow for more effective immune manipulations in order to generate anti-tumor responses. Recent studies have identified intercellular signaling modalities as critical components for tumor growth. Extracellular vesicles released from GBM cells have been revealed to be potent modulators of the local environment to facilitate malignancy and mitigate destruction of the tumor by immune cells. Specific mechanisms of vesicle-induced changes are becoming more evident and offer novel targets for future therapies. Elucidating

intracellular changes and signaling cascades that lead to phenotypic deviation of naïve immune cells will reveal points of regulation to manipulate for tumor destruction. This chapter aims to examine the multifaceted roles of GBM-derived extracellular vesicles on immune cells. We will discuss the current state of knowledge about the mechanisms by which extracellular vesicles alter the tumor microenvironment, along with new findings relating to transformation of T cells by extracellular vesicles. Phenotypic changes, functional pathways, and protein profiles of immune cells will be discussed to gain an understanding of the means of transformation. These changes will also be assessed over time to highlight potential early markers of tumor influence that may be used for diagnostic or prognostic application. Finally, we will discuss considerations for new immunotherapies relating to immune cell transformation induced by extracellular vesicles.

2. Tenacity of brain tumors

2.1. Failure of current therapies

2.1.1. Traditional therapies

Despite recent advancements in approaches to cancer treatment, GBM remains a complex and problematic cancer to target. Diffuse borders of GBM tumors invade sensitive brain tissue and make complete surgical resection particularly difficult. Incidentally, cancerous cells are left behind, which allows the tumor to repopulate the resection cavity and enables recurrent tumor growth. Radiation therapy is commonly utilized to eliminate portions of tumor remaining after surgery and in recurrent settings. Although radiation has shown to provide survival benefit, innocent bystander damage to nearby brain structures still remains a major obstacle in this field. Newer technologies, including gamma knife surgery, allow for a more targeted approach to access remaining tumor and reduce the risk of complications from a second surgery [4]. Despite this, damage to healthy tissue still occurs and the inability to target every cancerous cell constituting the tumor remains problematic. The addition of the alkylating agent temozolomide to GBM treatment has extended overall survival and progression-free survival for patients receiving surgical resection and radiation [5]. A multimodal approach in the treatment of GBM offers the most promising outcome for patients. However, prognosis remains dismal and quality of life is poor because these treatment options do not specifically target glioma cells. In recent years, alternative approaches have been explored that more specifically isolate cancerous cells and remove support from surrounding structures. Gliadel wafers implanted into the surgical resection bed provide survival benefit by releasing anti-tumor agent, carmustine, locally to target areas of infiltrating tumor. Since most GBM tumors will recur in a 2 cm margin surrounding the resection cavity, carmustine impregnated wafers are able to focus on this area, while sparing other sensitive structures. The ease of implantation and regional release of drug makes this method attractive, yet overall survival and quality of life is equal to temozolomide alone, and wafers offer no benefit in recurrent settings [6]. Electric field devices offer a novel approach to GBM destruction by emitting alternating electric fields to slow and prevent tumor progression. However, incomplete understanding of the mechanism by which electric fields can slow tumor growth has hindered success of this therapy. The

noninvasive nature of electric field technology offers promise for enhanced quality of life and ease of treatment delivery integrated into patient's daily lives. Unfortunately, these devices have shown to confer minimal benefit in survival over chemotherapy [7].

2.1.2. Monoclonal antibodies

Utilization of natural defense mechanisms offers the most potential to support the failing triad of traditional therapies in GBM. One element of immunotherapy that has gained momentum in recent years has been the use of monoclonal antibodies. Monoclonal antibodies offer antigenic specificity and have been successful in controlling a variety of other cancers in different stages including breast, lung, and melanoma [8]. The strength behind antibody treatment lies in their ability to circulate to identify distant metastasis and specifically target tumor-specific antigens. The first major obstacle for antibody therapy in GBM is delivery across a selective blood-brain barrier and maintenance of the antibody binding pocket once penetration is complete. Since GBM almost never metastasizes outside the cranial cavity, antibodies must be able to access the tumor *in situ* by crossing over the blood-brain barrier easily. The second major barrier for antibody therapy is the existence of few tumor-specific antigens on the surface of GBM cells. Although some antigens have been identified to date, tumors have been shown to adapt quickly to change expression of surface molecules in the face of antigen-specific attack. Thus, even if these antigens have roles in aiding tumor progression, they are not ideal targets because they are not necessary for tumor survival. In addition, the vast heterogeneity of GBM tumors precludes a single target approach. Therefore, soluble factors constituting the tumor microenvironment have been targeted. GBM tumors are highly vascularized and release abundant amounts of vascular endothelial growth factor (VEGF) into the external environment to induce local angiogenesis. Their high metabolic needs require sustained nutrient delivery directly to the tumor site, so much that the cores of GBM tumors contain areas of necrosis. Bevacizumab is a monoclonal antibody that binds and neutralizes VEGFA in the brain tumor environment to reduce vascularization and limit nutrient delivery. It is currently the only monoclonal antibody approved for use in recurrent GBM and is implemented during various disease states in clinical trials [9]. Bevacizumab remains a passive treatment option that has been successful in restricting tumor growth, but does not actively kill cancerous cells. Some studies have suggested bevacizumab promotes development of structurally competent blood vessels around the tumor, rather than the compromised leaky vessels that typically grow in tumor sites [10]. This modification may help ameliorate symptoms caused by cerebral edema, but does not slow nutrient delivery to GBM tumors. To date, bevacizumab has conferred minimal overall survival benefit, although time to recurrence and quality of life are reportedly improved [11]. Currently available treatment options offer a variety of approaches that demonstrate clinical benefit, but none substantially extend survival or decrease burden of disease for patients.

2.1.3. Tumor-specific immunotherapy

The heterogeneity and adaptability of GBM tumors needs to be met with a dynamic and adaptable treatment strategy in order to maintain remission or eliminate the tumor altogether. Manipulation of the adaptive immune system against GBM is the goal of future immuno-

therapies as a means to specifically identify cancerous cells for destruction. Vast molecular differences between patients and within individual patient tumors have prevented identification of common components to target. New approaches employ *ex vivo* stimulation of patient's adaptive immune cells with their own tumor followed by injection of the activated cells back into circulation. The goal of this approach is to initiate a level of immune stimulation that will specifically target tumor antigens typically hidden *in vivo*, while protecting the patient from autoimmune complications. The promise for initiation of an adaptive response stems from identification of lymphocytes within GBM tissue during autopsies and the later understanding of tumor specificity of these cells [12]. Although unable to eliminate the tumor, it became clear that lymphocytes play a role in antitumor immunity. Activated lymphocytes have a unique ability to migrate into brain parenchyma, thus *ex vivo* activation of the adaptive immune response has been explored as a means of eliciting antitumor responses [13]. Variations in type of tumor material, incubation conditions, adaptive cell types, and course of treatment have been under rigorous investigation to optimize tumor destruction [12,14,15]. Tumor-specific antigens continue to be ideal targets, but tumors rarely rely on a single antigen, and targeting overexpressed molecules could critically disrupt healthy cells that need those antigens for survival. Molecular targets including EphA2, IL-13R2a, EGFRvIII and survivin have been implicated in GBM progression and offer potential pathways and signaling modalities for cellular immunotherapies to target [16–19]. Other potential sources of tumor antigens include protein complexes, chaperones, RNA, and whole cell lysate [20]. These approaches offer the benefit of stimulating a varied approach of tumor destruction because of the immune cells' innate ability to distinguish harmful from harmless. The potential for response against multiple tumor antigens is greater with unbiased access to native tumor material (antigens), thus the immune response has a better chance to elicit a multidimensional attack against the heterogeneous GBM. The two primary approaches employing these strategies include autologous T cell transfer and dendritic cell vaccines.

Autologous T cell transfer has been referred to as passive immunotherapy because the T cells are stimulated *ex vivo* and reintroduced to the patient as antigen specific, activated cells. Dendritic cell vaccines are an active type of therapy, in which dendritic cells are isolated from the patient and pulsed with tumor antigen before reintroduction to the patient. The dendritic cell then has the opportunity to present antigen in secondary lymphoid tissue to lymphocytes in the ideal environment for activation. Both approaches continue to be studied in order to identify the optimal approach to adaptive immune activation. Sipuleucel-T was the first FDA-approved dendritic cell vaccine to exhibit survival benefit for any type of cancer and created a new realm of potential for personal immunotherapy [21]. Utilization of this treatment strategy for GBM is much more difficult, primarily because Sipuleucel-T relies on the presence of a tumor antigen found on the subset of cells that respond well to treatment. Dendritic cell vaccines for GBM currently under investigation in clinical trials employ differential antigenic approaches in hope of producing an ideal cocktail [22–24]. However, passive and active immunotherapies have not yet transformed the landscape of GBM due to complexity of the disease. In fact, very few patients have exhibited tumor regression for any substantial period of time [25]. A major obstacle that must be overcome when developing these immunogenic responses is the immunosuppressive environment induced by GBM. Once the burden of

immunosuppression is alleviated, immunotherapeutic strategies and their effectors will have a better chance to infiltrate, identify, and kill cancerous cells.

2.2. Immune manipulation by gliomas

Malignant brain tumors are known to correspond with multiple mechanisms of immune suppression. Although phenotypic changes have been well documented, specific mechanisms inducing the alterations are less well understood. Presence of modified immune cells transformed by the tumor environment presents obstacles investigators need to consider when developing new therapeutics utilizing the immune system. GBM cells can modulate their external environment through release of soluble factors and expression of discrete molecules on the cell surface. One soluble factor released from GBM that appears to have a major role in tumor progression is transforming growth factor beta (TGFβ) [26]. TGFβ has a wide range of effects capable of suppressing immune responses including inhibition of IL-2 receptor on T cells, down regulation of MHC molecules on antigen presenting cells, and even neutralization of activated lymphocytes entering the tumor environment ([26,27]. Inhibitors of TGFβ and neutralizing antibodies have shown promise in mitigation of glioma growth and immune suppression [28]. To further enhance immune suppression in the local environment, GBM recruits and converts naïve cells. Transformation of monocytes to immunosuppressive macrophages is one of the most significant changes GBM produces in the microenvironment. Tumor associated macrophages (TAMs) can comprise of classically activated M1 macrophages or alternatively actived M2. TAMs are predominantly of the M2 phenotype and are the most common immune infiltrate in glioma [29]. Evidence has shown TAMs can comprise up to 40% of the tumor mass in GBM, thus the suppressive roles are highly apparent [30]. Unlike M1 macrophages, M2 TAMs do not release proinflammatory and chemoattractive factors, but instead aid in tumor progression and attenuate activity of infiltrating lymphocytes. TAMs can arise from both circulating monocytes and resident microglia near the tumor site [31]. Gliomas ability to tip the balance towards the M2 phenotype is important for tumor survival and progression because of the protection conferred to evade immune destruction. TAMs isolated from glioma express very little MHCII or costimulatory molecules CD80/86 and this phenotype can be transferred to naïve monocytes introduced to the same cell culture media [29,32]. Impaired release of TNFα from glioma macrophages can also be transferred to naïve monocytes in cell culture, providing a regionally dependent inhibitory modulation of monocytes in the GBM environment. In addition to reductions in TNFα release, TAMs actively suppress the extracellular space by constitutively expressing immunosuppressive cytokines IL-10 and TGFβ [33]. This active inhibition is supported by impaired cytotoxic capabilities of TAMs, which generates a highly immunocompromised environment for GBM to grow [34]. Converted TAMs further propagate the glioma environment through release of matrix metalloproteases and growth factors for endothelial cells and tumor cells alike [35]. In order to remove the inhibition caused by glioma TAMs, it will be necessary to tip the balance back towards the classically activated M1 phenotype without causing autoimmunity or inflammatory consequences.

Glioma patients exhibit varying degrees of lymphopenia, but often a great proportion of remaining cells are regulatory T cells. This phenomenon has been observed in many types of cancer and is thought to act as a driving force to maintain immunosuppression and propagate tumor cells. It is unclear whether regulatory T cell production is driven primarily by MDSC, tumor-released proteins, alternative signaling pathways, or carefully selected from the natural T cell population. Anti-CD25 treatment options have shown benefit in the glioma setting, which could help tilt the balance back towards an immunostimulatory environment [36]. Although the presence of a high concentration of regulatory T cells persists, some T cells have the capability to become active and access the tumor bed. Tumor-infiltrating lymphocytes (TILs) are antigen-specific lymphocytes capable of penetrating the blood-brain barrier in an effort to confer immunity against a growing tumor. The presence of TILs in gliomas indicates patients are not entirely tolerant to the tumor and attempt to mount a response against it, thus yielding a more favorable prognosis for patients. However, the TILs do not respond to mitogenic stimulation and proliferate very poorly in the face of a challenge [37]. The exact origin and capabilities of TILs are not clear, but their ineffectiveness in tumor destruction generates an opportunity to learn about transformation. Similar activation abilities have been reported with B cells isolated from peripheral circulation of GBM patients, whereas immuno-globulin production is hindered when cells were stimulated [38].

3. Extracellular vesicles

3.1. Genesis

Extracellular vesicles (EVs) arise primarily from two distinct biogenesis pathways during healthy and pathological conditions. Release of vesicles can occur by direct shedding of plasma membrane (microvesicles) or though invagination of an endosome to form a multi-vesicular body, which later fuses with the plasma membrane (exosomes) [39]. Distinction of origin is controversial, since components of the plasma membrane and intracytosolic proteins are found in both. Overlapping similarities in size, density, membrane proteins and morphology suggests differential nomenclature is not necessary, but the specific difference between the types of extracellular vesicles is not fully understood [40]. Shuttling of contents into EVs occurs through random capture during vesicle formation and through selection based on mechanisms remaining to be elucidated [41]. EVs are released by many cell types in normal and pathological states, including lymphocytes, dendritic cells, neurons, reticulocytes, and tumors [42–46]). These vesicles contain distinct cargo that resemble the cell from which they were released and can be identified in circulating fluids such as urine, breast milk, plasma, saliva, cerebrospinal fluid, and amniotic fluid [47]. EVs vary in size from 30-2,000 nm in diameter, which ultimately dictates the amount of cargo they can carry. Enhanced release of extracellular vesicles from cancer cells is an indicator that they may have a role in aiding in tumor progression [48]. Release is further heightened in cancer cells exposed to radiation or hypoxic conditions [49,50]. Due to their presence in these fluids and the specific signature they contain, EVs are ideal vehicles for transfer of large amounts of information to modulate the external environment and signal immune complexes.
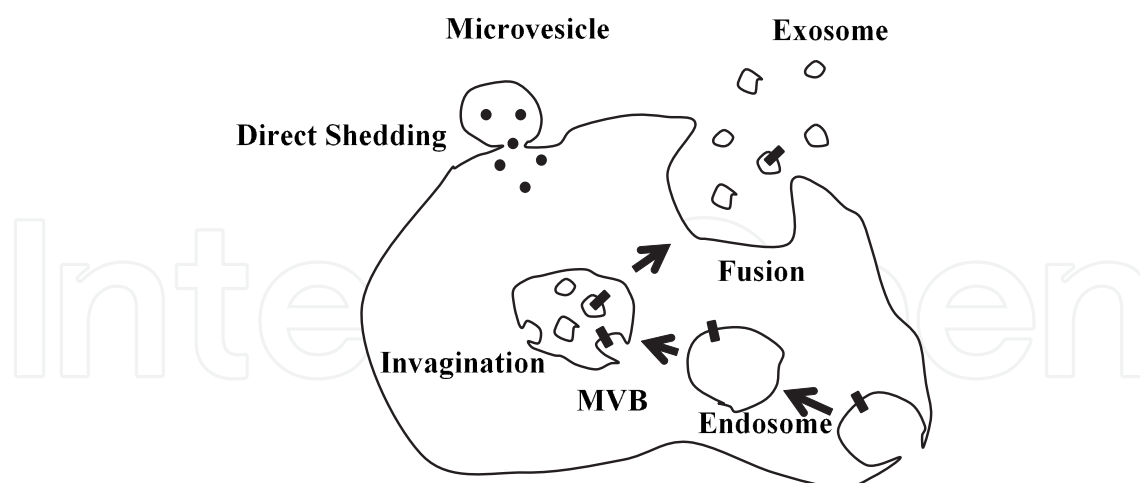
Abbreviations used in this figure. MVB: Multivesicular body

Figure 1. Release pathways for microvesicles and exosomes. Microvesicles are directly shed from the cellular membrane and capture contents by inclusion of nearby particles. Exosomes are formed through endosomal invaginations, which form multivesicular bodies and fuse with the plasma membrane. Shuttling of exosomal contents is more selective due to machinery available

3.2. Waste receptacles

EVs are responsible for a multitude of roles in intercellular communication. Enrichment of discrete molecules suggests well-organized machinery dictates packaging of internal contents before release. These small packets of information can be transferred long distances with specificity for a recipient cell. However, their pleiotropic roles in a healthy setting are still not fully understood. They were originally thought to be waste receptacles for cells lacking lysosome machinery, since release was first identified during reticulocyte maturation [45]. Other studies have confirmed packaging of waste products into extracellular vesicles, along with cytotoxic substances, including chemotherapeutic agents [51]. This evidence provides a novel mechanism of drug resistance in cancerous cells and a potential target to induce sensitivity to toxic drugs as well. Controlling drug transport can allow for delivery of chemotherapeutic drugs at a lower concentration and provide increased efficacy. This is particularly important in GBM in order to preserve healthy brain tissue typically damaged by toxic drugs during treatment.

3.3. Immune response

In addition to waste disposal, EVs are now known to be important factors in antigen presentation [42]. Immunostimulatory properties of EVs were first recognized by Raposo et al. when they found MHC class II molecule enrichment on the surface of extracellular vesicles released from EBV-transformed B cells. These EVs were sufficient to elicit an antigen-specific T cell response. These data present a mechanism for rapid amplification of specific immune response via presentation of peptides bound to MHC II molecules. Later studies confirmed presence of co-stimulatory molecules on the surface of vesicles and

amplification of CD4+ and CD8+ responses through horizontal transfer of MHC II loaded EVs from activated dendritic cells to maturing dendritic cells [52]. Further mechanisms of immune activation by EVs include activation of NK cells and monocytes [53,54]. Extracellular vesicles represent an ideal booster for immune responses, but also contain a kill switch to dampen response and tightly regulate clearance of pathogens. Activated lymphocytes release EVs containing Fas ligand and APO2 ligand, which are both capable of inducing cell death [55]. It is postulated that release of cytotoxic components from activated cells ensures moderation of excessive immune responses and reduces autoimmunity. The expression and composition of EVs in a normal immune response is highly controlled and deliberate to ensure modulation at appropriate times.

3.4. Horizontal transfer of information

Intercellular communication via EVs allows for transfer of large amounts of information to maintain homeostasis and normal physiological function. Some functions that have been identified to date include tissue repair, stem cell regeneration, synaptic plasticity, neuronal communication, and viral transmission [56–59]. EVs contain many components that resemble the host cell, but also contain discrete pieces of information that may be useful for identification of specific signaling capabilities. Valadi et al. first described packaging and transfer of messenger RNA (mRNA) and microRNA (miRNA) in extracellular vesicles in 2007 [60]. EVs from healthy mast cells were harvested and analyzed based on genetic content. Interestingly, no DNA was found within EVs, which suggests they signal exclusively through epigenetic changes and signaling mechanisms in the recipient cell. mRNA found in EVs released from mast cells exhibited functionality and was found to be unique from the donor cell. This discovery revealed a new complexity to intercellular communication by means of modifying protein expression and gene regulation through horizontal transfer of packets of information. Later studies have been able to identify distinct miRNA signatures in pathological conditions not found in normal cells. These signatures have been well documented in ovarian cancers, lung cancer, and GBM [61–63]. mRNA contained within GBM extracellular vesicles control a variety of cellular functions to promote tumorigensis, including enhancement of angiogenic, proliferative, and migratory pathways. Identification of cargo released from cancerous cells offers critical information about components that are deliberately shuttled out of the cell, possible influence on the local environment, and the potential to act as a peripheral biomarker for diagnostic or prognostic applications. Genetic signatures are particularly valuable in the brain tumor arena because of their ease of collection and sensitivity to identify of tumor progression.

4. Tumor-derived EVs

4.1. EVs and tumor microenvironment

The Taylor group first identified release of tumor-derived EVs in 1979 [46]. Their seminal study was met with antagonism because it was not believed that small particles like these

could have any substantial effect on tumor propagation. Later studies confirmed these small cellular surrogates could act locally and peripherally and their bioactive cargo had significance in the cancer setting [64,65]. EV cargo varies between cells and disease states and the significance of inclusion of certain cargo is just beginning to be understood. EVs are capable of carrying proteins, lipids, carbohydrates and nucleic acids in endless combinations. The restrictive size of the EV compartments dictates the amount of material that can be packaged, thus tumor cells must selectively package opportunistic cargo to facilitate tumor progression. Tumor specific antigens MART1 and HER2/Neu have been identified in EVs released from cells with respective mutations, implicating a role for EVs in horizontal transfer of cancer promoting agents [48]. Much like transfer of viral peptides, EVs offer a mechanism to exponentially facilitate cancer progression through delivery of disease-specific antigens. In the brain tumor setting, EVs have been shown to carry and transfer the truncated EGFRvIII from GBM cells containing the mutation, to GBM cells without the mutation [66]. EGFR mutant variant III may be associated with over 50% of GBM tumors and indicates a negative prognosis for patients [67]. Rapid onset and growth of this subset of GBM relies on the presence of EGFRvIII on the plasma membrane surface. EV delivery of the mutated receptor to neighboring cells presents a partial explanation for the relentless aggressiveness of these tumors. Besides tumor antigens, EVs also carry factors to modify the extracellular environment to ensure tumor survival. Active matrix metalloprotease (MMP) and other matrix remodeling enzymes have been identified as EV cargo and are capable of compromising the rigidity of the extracellular matrix [68]. GBM tumors are notoriously invasive around the borders of the tumor margin and it is likely that EVs play a role in promoting invasion. Other factors carried by GBM EVs include angiogenic proteins VEGF and IL-6, which aid in recruiting new blood vessels to the tumor site. Many other pieces of cargo have been identified to date, but the functional consequences of their presence is not clear. Identification of a tumor signature from tumor-derived EVs offers promise for noninvasive biopsy type analysis and could aid in treatment before large changes can be seen from imaging.

GBM EVs have been shown to participate in modulation of the external environment through a variety of pathways, although specific molecules responsible for the changes are not known. Exogenous brain tumor EVs are capable of enhancing proliferation of GBM cells in a dose-dependent manner, which generate a positive feedback loop *in vivo* [69]. Thus, EVs are partially or largely responsible for rapid propagation of tumor cells. GBM cells often induce their own proliferation through autocrine growth factor signaling, but for cells that have not yet acquired the correct mutations, EVs offer a mechanism to promote proliferation. In addition to proliferation, glioma EVs promote migration of tumor cells as well [70]. Effectively, GBM tumors generate their own chemoattractive gradient away from the core of the tumor towards the external environment. This process is further exacerbated by radiation treatment, as the GBM cells release greater amounts of EVs and exhibit enhanced migration towards their EVs [49]. Extensive proteomic studies have begun to identify material responsible for some of these changes. Profiling all the material con-

tained within tumor-derived EVs and understanding the correct balance of information will help to better characterize targets in the future.

4.2. Tumor-derived EVs and immune response

4.2.1. Cellular decoys

Extracellular vesicles are optimal vehicles for transfer of large amounts of information to amplify signaling without cell-to-cell contact. While this elegant ability can amplify an immune response against pathogens extremely rapidly, it can also be used as a mechanism of immune manipulation by brain tumor cells. The pleiotropic roles of tumor-derived EVs on immune modulation are not fully understood and it is likely the effects in the brain tumor environment may be unique compared to other tumor types. Many of the studies conducted on tumor-derived EVs have occurred *in vitro* or using murine models, but offer great insight about the modulatory capacities and potency of EVs. The immense immunosuppressive roles of tumor-derived EVs reveal the importance of these signaling modalities in the cancer setting. Modulation of both innate and adaptive immune responses undermines the immune system's redundancy against harmful stimuli. Tumor EVs have been shown to inhibit cytotoxic capabilities of natural killer (NK) cells that typically patrol cellular surface antigens for danger signals [71] and presence of MHC. One of the mechanisms by which this occurs is through expression of danger signal MICA on the surface of EVs released into the extracellular space. Binding of MICA on the surface of EVs by NK cells prevents them from identifying the problematic cells and leads to inactivation of NK cells through down regulation of MICA binding protein, NKG2D [72]. This process creates difficulty for future attempts to activate this subset of NK cells in the presence of real tumor and the continued release of tumor EVs as cellular decoys perpetuates inhibition. Similarly, tumor EVs have shown to attenuate humoral immunity by binding antigen-specific antibodies and misguiding antibody-dependent cellular cytotoxicity away from the tumor [73]. Expression of cell surface antigens on the surface of EVs introduces a unique obstacle for treatments targeting surface molecules. Immune cells and pharmacological agents in development will have to circumnavigate the decoy EVs to specifically access tumor cells.

4.2.2. Monocyte transformation

Another worrisome consequence of tumor EVs is their ability to restrict peripheral blood monocytes from fully maturing to dendritic cells [74]. As a central hub bridging innate and adaptive immunity, dendritic cells are critical for developing a specific and adaptable anti-tumor response. The potential of immunotherapy relies heavily on the premise of selective destruction by adaptive cells that have been fully educated by dendritic cells about tumor antigens to target. Further evidence has revealed EVs can not only inhibit, but also promote a transformation of monocytes towards a myeloid-derived suppressor cell (MDSC) phenotype. MDSCs are critically important for facilitation of glioma growth, as discussed above, and can comprise a large portion of glioma mass [29]. Tumor-derived EVs induce release of immuno-

suppressive factors from MDSC including TGFβ and PGE2 and also down regulate expression of MHCII on monocyte cell surfaces, limiting activation potential [75,76].

4.2.3. Migratory capacity

The phenotypic changes of naïve immune cells exposed to tumor-derived EVs dictates the functionality of the immune response to perpetuate tumor growth. One of the most important functional consequences of tumor-derived EVs is the ability to alter migratory capacity of PBMCs. Preliminary data from our group has demonstrated attenuation of migration of mitogen-activated PBMCs moving towards glioma EVs (Figure 2). Migration is a necessary component of activated PBMCs in order to properly activate and then destroy cells of interest. When brain tumor EVs are present, this process is disrupted, which can prevent even the best tumor-specific cells from reaching their targets. Interestingly, studies have demonstrated brain tumor EVs can enhance tumor cell migration as discussed above. If glioma EVs truly facilitate glioma migration while diminishing immune cell migration, it is possible similar pathways are altered in both cell types. Better understanding of the exact mechanisms and pathways involved will lead to pharmacologic targets that could reverse migratory effects. Because EVs are packets of complex information, identification of a switch to reverse both processes simultaneously will be challenging. However, immune cells can still enter the tumor site to promote tumor growth, thus tumor-derived EVs do not entirely eliminate PBMC entry into brain parenchyma. They may, however, select for immune cells that will only aid in tumor progression, such as MDSCs, and limit migration of anti-tumor cells. This selective entry could help explain why resident immune cells at the tumor site do not destroy the tumor and presents a reason for the limited success of immunotherapies that do not address EV presence. Alternatively, brain tumor EVs in peripheral circulation may prevent movement of activated dendritic cells to secondary lymphoid tissue, severely compromising their ability to activate an adaptive immune response.
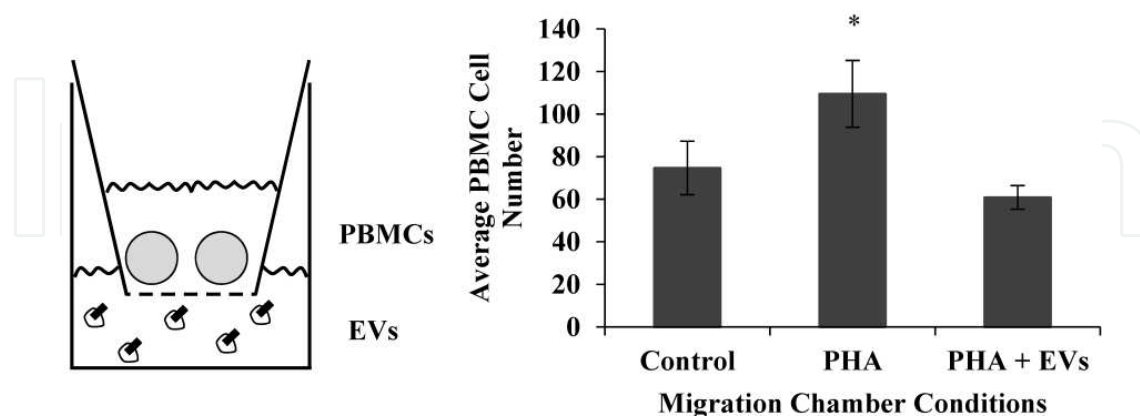
Abbreviations used in this figure. PHA: Phytohemagglutinin; EVs: Extracellular Vesicles; PBMC: Peripheral Blood Mononuclear Cell

Figure 2. Average migration of naïve PBMCs in the presence of mitogenic stimuli with or without extracellular vesicles. PBMCs were placed in the top of a migration chamber with PHA and the bottom chamber contained EVs or media. The number of PBMCs that migrated to the bottom chamber was quantified after 24 hours. *=p < 0.01.

4.3. Altered function of T cells

Aberrations of T cell phenotypes and functional capacities in the cancer setting have been described, but specific mechanisms of alteration are not well understood. T cell deficits induced by tumor EVs are among the most important transformations necessary to protect tumors from immune destruction. T cells cultured with tumor EVs exhibit reduced capacity for interleukin-2 (IL-2) production, thus limiting the proliferative potential for activated T cells [77]. In addition, tumor EVs can direct apoptosis of activated T cells though Fas/Fas-L binding [78]. Expression of the Fas death signal on the surface of activated cells typically is used as a shut off switch to prevent development of autoimmunity during normal pathogenic clearance. Cancer cells are able to take advantage of the molecule expression by releasing EVs containing Fas-L on their surface to kill threatening T cells from afar. Tumor EVs have also proven to be capable of suppressing mitogenic activation abilities of naïve T cells in the ovarian cancer setting, exacerbating the attenuation of immunogenic responses [79]. Although little work has been conducted analyzing the transformation of T cells exposed to glioma exosomes, evidence exists that brain tumor EVs decrease quantities of cytotoxic CD8 cells and inhibit interferon gamma release from remaining cells [80]. Our studies analyzing intracellular signaling cascades within naïve T cells exposed to glioma EVs reveal aberrations of many signaling pathways seen in other cancer types, including T cell receptor signaling (Figure 3). Analysis of just a few members of the signaling cascade can predict the overall activation status of this pathway and isolate particular proteins of interest to better understand how inhibition is occurring. Inhibition of the T cell receptor cascade by EVs is of particular importance for immunotherapies, which rely on generation of antigen-specific anti-tumor cells. Although many other factors in the tumor microenvironment can alter T cell signaling, EVs appear to play a substantial role in creating this change. Much like the effects of tumor EVs on monocytes, T cells can be inhibited and converted to a suppressive phenotype [81]. Induction of regulatory T cells by tumor EVs cultivates immunosuppressive factors in the tumor microenvironment to further prevent activation of cells that may threaten tumor survival. Elevated levels of regulatory T cells are common across many types of cancer and soluble factors pushing naïve cells towards a regulatory phenotype have been characterized from gliomas [36]. Tumor EVs by themselves are capable of inducing this transformation and may be an important factor to target in order to prevent transformation.

4.4. Transformation of T cells over time

To date, no studies have analyzed the transformation of T cells in GBM over time or over the course of treatment. Understanding the process of cellular transformation can offer information about response to treatment or act as a prognostic predictor before changes can be seen on radiological imaging. Other research in the brain tumor EV field has been able to correlate EV contents to the constitution of tumor cells, which will allow EVs to act as a liquid biopsy to track treatment. Perhaps as important are the downstream consequences of changing EV contents released from GBM and the amount of time needed to produce
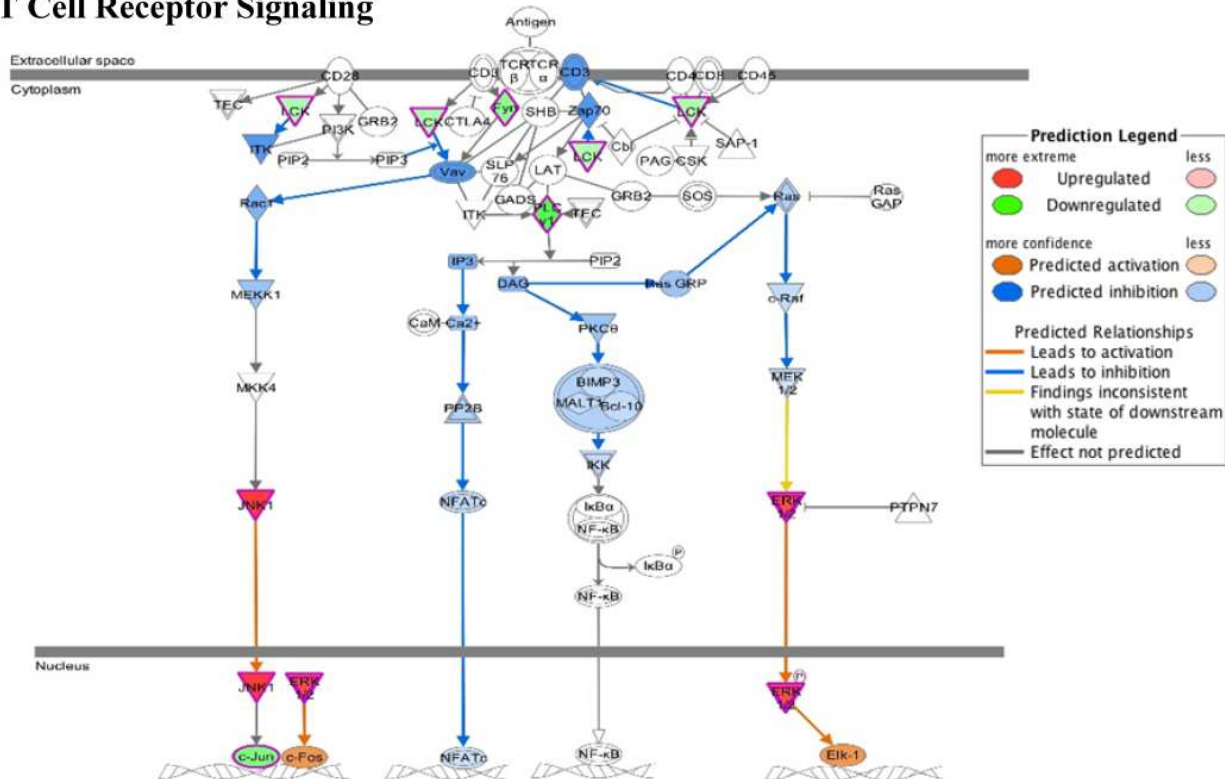
Figure 3. PBMCs were incubated with PHA and EVs for 72 hours and intracellular signaling molecules were quantified using protein arrays. Output from Ingenuity Pathway Analysis revealed pathways predicted to change based on EV exposure.

functional immune consequences. Better understanding of pathways affected earliest could offer insight about which functions the tumors consider a priority to eliminate. Transformation of stimulated T cells after exposure to brain tumor-derived EVs occurs quickly and we have been able to characterize some of the important changes that occur in the first 72 hours of glioma EV presence (Table 1). By analyzing protein expression profiles, we observed the transformation of intracellular signaling pathways within naïve T-cells exposed to tumor-derived extracellular vesicles at 72 hours compared to 2 hours. Apoptotic and developmental cascades were affected in the early stages, whereas migratory pathways become increasingly dysregulated during later time points. These data parallel previous studies in other tumor types, but here we are able to identify early activation of apoptotic pathways that do not persist for a prolonged period of time in GBM. Instead, the EVs continue to transform T cells without killing them, with a focus on modification of migratory capacity and cytotoxicity of cells. Modification of invasive capacity of T cells within tumor is an important functional deficit induced by glioma EVs. Lymphocyte entry into brain parenchyma is loosely restricted by the blood-brain barrier and EVs may offer another obstacle preventing activated T cells from accessing tumor cells.

Function	Predicted Activation State	Z-Score
Binding of blood cells	Decreased	-2.000
Proliferation of T lymphocytes	Decreased	-2.028
Formation of cytoskeleton	Decreased	-2.043
Chemotaxis of cells	Decreased	-2.227
Adhesion of immune cells	Decreased	-2.323
Cytotoxicity of leukocytes	Decreased	-2.414
Migration of cells	Decreased	-3.469

Table 1. PBMCs were incubated with PHA and EVs for 2 or 72 hours, and then purified for T cells and analyzed for intracellular signaling changes. Output from Ingenuity Pathway Analysis revealed functions predicted to change based on protein expression. Z-score represents confidence of associated functions change.

5. Manipulation of EVs in the tumor setting

5.1. T cell rescue

GBM EVs utilize a variety of mechanisms to enhance tumor malignancy as discussed above. However, the functional impact they have on naïve immune cells is still under investigation. Disruption of function of naive T cells in the circulation of GBM patients may reveal a significant barrier in production of anti-tumor responses in immunotherapy. Our data reveal EVs alter migration pathways in T cells that have not seen tumor before, and EVs can functionally inhibit and convert naïve T-cells. However, GBM patients are not significantly globally immune compromised and are able to mount healthy responses against communicable diseases as well as grafted organs. This suggests specific immune tolerance to cancerous cells, but it is not known whether cells that have been transformed by extracellular vesicles can be rescued when challenged with a pathogen or if this is the job of truly naïve T cells. Determining activation abilities of GBM tolerant T cells will be necessary in order to understand how to develop new therapies to compensate for vesicle-induced modulation and to reactivate or ablate affected cells.

5.2. EV release

Because of the known roles of EVs in propagating tumor survival, disruption of EV genesis, release, or fusion may all offer viable solutions to lessen negative consequences of EV exposure. In recent years, better understanding of packaging and release mechanisms have been discovered. Some studies have been able to effectively inhibit release of EVs by inhibition of neutral sphingomyelinases, which are necessary for EV formation [82]. Other inhibitors of vesicle release have exhibited reduction in tumor growth *in vivo*, which further underlines the importance of tumor EVs in the microenvironment [83]. Many other targets aimed to reduce EV release are under investigation, but an obstacle in this approach is the removal of healthy EVs as well. EVs are known to maintain homeostasis by communicating with every cell in the

body and generating an effective immune response in a noncancerous setting. The conse‐
quences of nonselective EV removal are not understood, thus the safest inhibitor will need to
access the tumor site and selectively prevent EV release in a controlled manner. As more
evidence emerges concerning packaging and release of EVs, better targets will become
available to prevent the negative effects of tumor EV on supporting cells.

5.3. Therapeutic EVs

Engineered EVs represent a new mechanism for drug delivery and therapeutic potential in the
GBM setting. They maintain stability in circulation, protect cargo from metabolism and can
penetrate the blood-brain barrier to deliver cargo to a specific cell [41]. Their potential has not
been fully realized due to the complexity of specific binding, but it is possible in the future that
EVs will be engineered with high affinity for cellular targets. This delivery method would
provide a wide variety of benefits over antibody delivery, which has been the predominant
method of specific targeting in recent years. However, even the best targeted EV will not have
the dynamic capabilities of the natural immune system, thus EVs are under investigation as a
means to enhance the immune response against tumors. Because tumor-derived EVs contain
tumor antigen, EVs have been explored as an antigen delivery mechanism from dendritic cells
in order to prime an adaptive antitumor response. One approach that has been explored is
loading of tumor antigens onto MHC molecules on EV surfaces by pulsing dendritic cells with
tumor antigen [43]. EVs released from dendritic cells in a nontumor setting are potent
immunostimulatory agents because of their ability to express antigen, costimulatory mole‐
cules, and adhesion molecules. Artificial activation of dendritic cells must be sufficient to
produce mature cells, which express these molecules on their cell surface, in order to be
incorporated on to EV surface [84]. Full maturation of dendritic cells prior to antigen pulsing
is necessary to generate optimal antitumor responses. Once cells are loaded with antigen, EVs
can be isolated and reintroduced to the patient, or the activated dendritic cells can be injected
directly into the patient to elicit a response *in vivo* [69]. Studies are still under investigation to
determine the best method of transfer and optimal conditions for incubation of stimulated cells
to produce effective antitumor responses. Another approach to utilize tumor antigens within
EVs is by direct vaccination of tumor exosomes to generate specific immunity. Interestingly,
GBM EVs are not immunosuppressive in a tumor naïve setting. Prophylactic glioma EV
injection before tumor implantation in mice leads to rejection of tumor [85]. These mice exhibit
no evidence of tumor growth over time and when challenged with a second tumor implanta‐
tion, mice successfully rejected the graft again. However, EV delivered after tumor implanta‐
tion has no effect on tumor rejection. This evidence reveals potential immunostimulatory
capacity of GBM EVs in the correct setting. However, the tremendous obstacle in utilizing EVs
is that treatment for tumors must come after the tumor is established. Understanding and
mimicking the optimal environment in patients may allow for self-vaccination against their
own tumor.

5.4. Gene therapy

Gene therapy is gaining recognition as a possible avenue for future GBM treatment because
of its ability to recruit the immune system for a targeted therapy approach. It has many forms,

ranging from virotherapy with a conditionally replicating virus to genetic immunotherapy. By altering the genetics of target (or attack) cells, gene therapy is efficient while minimizing detrimental systemic effects. Gene therapy employs a vector, either viral or non-viral, to deliver genes to the cancer cells. Viral vectors are the most frequently used [86], and are being explored in multiple glioblastoma clinical trials. An example is using a retroviral vector that codes for an artificial molecule against a tumor-specific antigen to genetically modify T cells. The artificial molecules are chimeric antigen receptors (CARs). Fusing an extracellular variable domain from a high affinity monoclonal antibody specific for a tumor-associated antigen with an intercellular signaling domain from CD3ξ of an antigen specific T cell receptor creates a CAR. When the target antigen activates the extracellular domain, downstream signaling is initiated to activate the T cell [87]. The survivability of viral vectors has been an issue, and there are safety concerns with ensuring viruses are rendered non-replicative. To minimize risk of this therapy, non-viral vectors, including EVs, are currently being explored. EVs have been shown to be inherently capable of transferring genetic material, so using them for targeted gene therapy is logical. Their small size, bi-lipid membrane for protecting cargo, natural, non-viral state, capacity to be taken up by target cells, and stability during laboratory work are all advantages [88]. It is also possible that EVs could reduce non-specific delivery and immunogenicity issues. One group has been able to deliver short interfering RNA (siRNA) to disrupt gene expression in the brain using EVs as a vehicle in order to achieve targeted gene knockdown. In this study, dendritic cells altered to express an EV membrane protein, Lamp2b, were fused to neuron-specific rabies virus glycoprotein peptide 3 (RVG peptide-3). The EVs isolated from the culture media of these cells were loaded with siRNA via electroporation, and they were targeted to neurons with RVG-p3. Although administered systemically, the EVs were able to specifically deliver siRNA to neurons, microglia, and oligodendrocytes in the brain and produce gene knockdown. Moreover, the mRNA and protein levels of the gene were strongly knocked down (approximately 60%) [89]. Recently, a group studied a non-toxic peptide-based carrier loaded with VEGF-siRNA and BCNU, an approved chemotherapy for GBM treatment, to target GBM cells [90]. The VEGF-siRNA and BCNU were both efficiently delivered to cultured GBM cells, and VEGF expression was successfully reduced. The combination of gene therapy and drugs is an exciting possibility and EVs offer an ideal mechanism of transfer as newer therapies become available. Gene therapy is promising because it offers the opportunity deliver novel therapies directly and efficiently into the brain. It also holds the potential for combination therapy, such as gene therapy/engineered drug delivery with immunotherapy, which is the most probable method for successful treatment in this complicated and unpredictable disease.

6. Conclusion

The GBM microenvironment is a complex and heterogeneous conglomeration of many factors working in concert to ensure survival of the tumor. Extracellular vesicles represent a component that has not been well studied and may offer a way for GBM to exhibit dynamic adaptation. Current clinical trials investigate neutralization or blockage of

individual molecules, but a single driver does not define the heterogeneous nature of GBM. The complexity of the tumor is likely to parallel the complexity of its signaling modalities. Extracellular vesicles provide transfer of discrete packets of information as a means of influencing countless pathways in facilitation of tumor progression. Here we discuss paracrine, autocrine, and immune modulatory effects of GBM-derived extracellular vesicles. Environmental modulation is necessary for GBM progression and extracellular vesicles influence many of the necessary changes needed for tumor survival. The adaptability of GBM to tolerate toxic insults is characteristic of the tumor's vigor. We are just now beginning to understand the complexity of extracellular vesicle packaging to produce a tumorigenic environment. As we understand more about the role of each signaling moiety contained within the vesicles, more information dictating treatment can be generated based on their overall composition. Appropriate treatment regimens will then be catered to each patient to more efficiently target GBM cells for destruction.

Acknowledgements

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