microenvironmental, and imaging features of large series of DCIS with long-term follow-up to assess which factors are associated with either harmless or potentially hazardous DCIS, and how these relate to the underlying mechanisms of whether progression might occur or not. Results obtained will be integrated to develop a DCIS risk prediction model. The findings will be prospectively validated in three randomized clinical trials (LORIS, COMET, and LORD) testing the safety of active surveillance for low-risk DCIS. To conquer overtreatment of DCIS, the role of patients cannot be underestimated. They help healthcare providers to focus on the ultimate question distinguishing harmless from potentially hazardous DCIS. This has to be related to their quality of life, including how they cope with uncertainties regarding the risks involved. The comprehensive approach as described above eventually aims to spare many thousands of women needless, but burdensome treatment each year, without compromising the excellent outcomes presently achieved in the management of DCIS.

Resources

[i]https://seer.cancer.gov/

[ii]https://clinicaltrials.gov/ct2/show/NCT02766881

[iii]https://clinicaltrials.gov/ct2/show/NCT02926911

[iv]https://clinicaltrials.gov/ct2/show/NCT02492607

[v]https://www.oncotypeiq.com/en-US/breast-cancer/healthcare-professionals/oncotype-dx-breast-recurrence-score/about-the-test

[vi]https://www.pcori.org/

[vii]https://www.dcisprecision.org/

[viii]https://www.cancerresearchuk.org/

[ix]https://www.kwf.nl/english/pages/default.aspx

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Forum

Glioma EVs Contribute to Immune Privilege in the Brain

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Glioblastoma cells release extracellular vesicles (EVs), sometimes referred to as microvesicles and exosomes, to transfer immune modulating molecules to immune cells, resulting in an immune privileged microenvironment. Here we discuss the potential EV-mediated mechanisms underlying glioma immune modulation, as well as the technical difficulties in studying these interactions.

Glioblastomas are the most common and lethal intracranial primary malignancies in adults. They are heterogeneous tumors with tumor cells and nonmalignant stromal cells [1]. The stromal population consists of resident brain glial cells, including oligodendrocytes, astrocytes, ependymal cells, and microglia; and infiltrating immune cells, such as myeloid-derived monocytes/macrophages and lymphocytes [1]. Together, the stromal and malignant cells form a microenvironment that in general enables the tumor cells to proliferate and infiltrate [1]. Within this microenvironment, cells communicate through secretion of cytokines and other (soluble) proteins, direct cell–cell contact through gap junctions or nanotubes, and extracellular vesicles (EVs) [1]. EVs is the collective term for nanosized and microsized (~50–10 000 nm) membrane-enclosed vesicles that are released by all cell types [2]. As different cellular pathways can result in the release of EVs, different terminology (e.g., exosomes, microvesicles, ectosomes) has been used for the potential subpopulations of EVs (Figure 1) [2,3]. However, since clear markers for these subpopulations are lacking, current consensus is to use the umbrella term 'EVs' [3]. EVs have a similar membrane topology as their cells of origin, and thus (mutant) extracellular domains of transmembrane proteins can be present on the surface of EVs. Simultaneously, donor cell cytosolic components, such as (mutant) proteins, m(i)RNA, and DNA molecules, are contained as cargo inside EVs and can be transferred from donor to recipient cells. This transfer of receptor
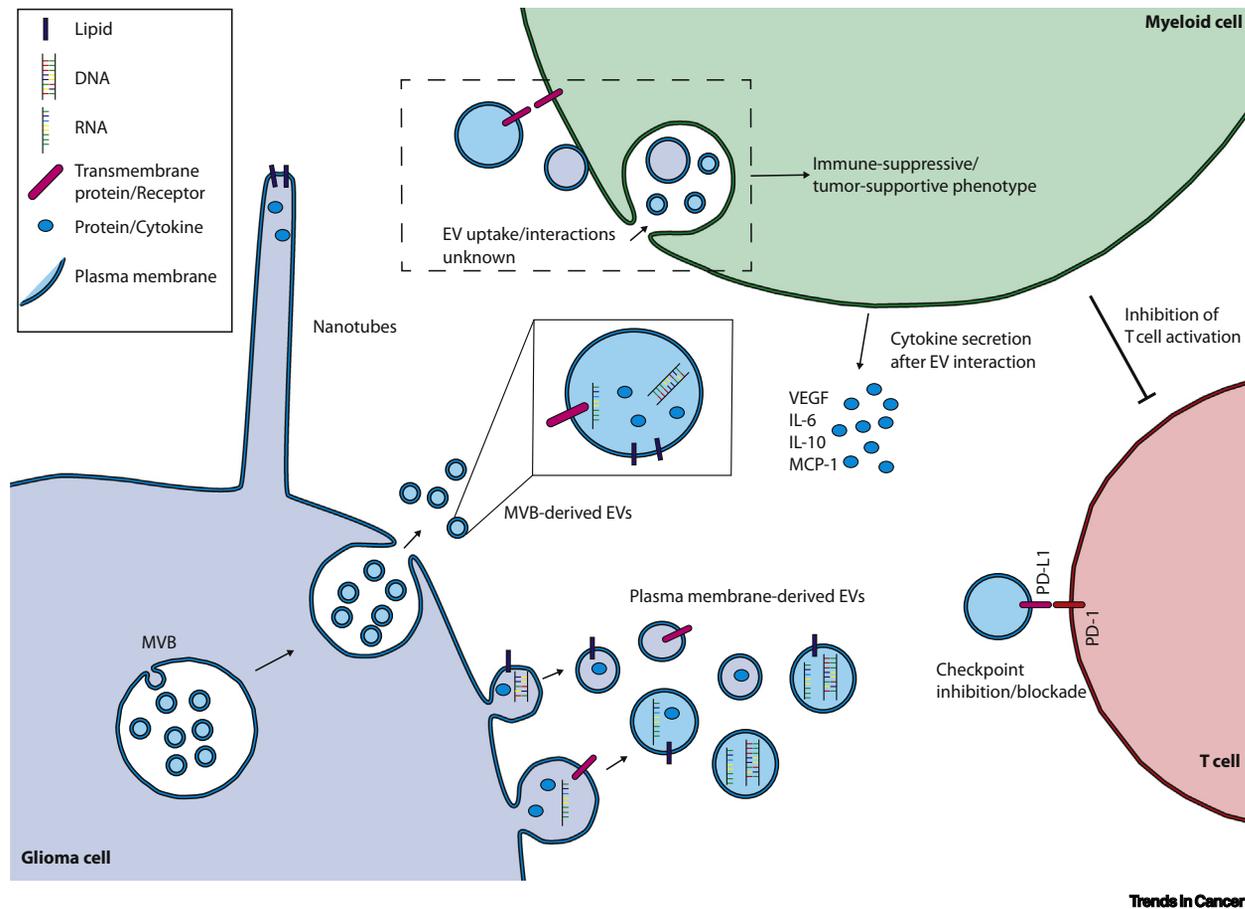
Figure 1. Extracellular Vesicles as a Mode of Intercellular Communication in Glioma Immunity. Extracellular vesicles (EVs) can be formed by both the budding of the plasma membrane or through the fusion of a multivesicular body (MVB) with the plasma membrane. Cell–cell contact and the subsequent exchange of cellular components through nanotubes is an alternative method of (local) intercellular communication. EV uptake by a myeloid-derived innate immune cell can change its phenotype into an immune-suppressive, tumor-supportive effector cell, inhibiting T cell activation and supporting tumor growth by secretion of specific cytokines. Direct interaction between glioma EV surface programmed death-ligand 1 (PD-L1) and programmed cell death-1 (PD-1) expressed on T cells is an alternative direct method for glioma EVs to suppress the T cell response. Abbreviations: IL-6, interleukin 6; IL-10, interleukin 10; MCP-1, monocyte chemoattractant protein-1; VEGF, vascular endothelial growth factor.

and/or cargo molecules can induce intracellular signaling in EV recipient cells [4]. During the past 50 years these concepts have been gradually laid bare, starting with the identification of vesicle-like structures around mammalian cells, to the functional intercellular transfer of mRNAs in 2007 [2]. In different types of tumors, including gliomas, EVs transfer oncogenic messages between malignant cells that enhance their migratory capacities and proliferation, and dampen immunological responses [2]. This forum article first focuses on the role of glioma-derived EVs in the establishment of an immune privileged

microenvironment, and then discusses technical challenges and future prospects for this field of research.

EVs and Glioma Immunity

One of the first indications that brain tumor-derived EVs could influence the (systemic) immune response was the identification of transforming growth factor (TGF)-β1 in EVs isolated from serum of high-grade glioma patients [5]. As TGF-β1 could not be detected in EVs from healthy controls, this finding suggested loading of TGF-β1 into circulating tumor EVs. EVs derived from high-grade gliomas

also contained mutant epidermal growth factor receptor (EGFR); (EGFRvIII). This indicates that at least some of the EVs in the serum from glioma patients are derived from the tumor. TGF-β1 has pleiotropic effects, including stimulation and activation of T cells and monocytes, but in malignancies the effect is mainly immune suppressive [5]. To achieve immune suppression, EV-associated TGF-β1 has to interact with innate and adaptive immune cells. This interaction of glioma EVs with immune cells was identified in subsequent studies. First, proteomic profiling of EVs isolated from glioma cell lines and

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Box 1. Guidelines for Studying Extracellular Vesicles

The interest and number of publications relating to EVs has significantly grown in recent years. However, variability in experimental methods currently impacts progression in this field. A number of factors are responsible for this variability. First, cell culture conditions, including methods to harvest EVs, can heavily impact composition and purity of EVs. For example, the presence of fetal calf/bovine serum in culture can introduce contamination with bovine-derived EVs. Additionally, selection of different centrifugation steps can result in isolation of specific EV subpopulations selected based on size and density. Different storage methods of EVs can affect their function and integrity. Another major obstacle is the lack of standardized methods to quantify EVs and robust markers for EV subtypes. An effort to standardize EV research has been made under the guidelines of 'Minimal Information for Studies of EVs (MISEV)', in which a number of recommendations are listed to guide and structure EV characterization, separation, isolation, and quantification to improve the reproducibility of EV research [3].

glioma stem cell-like cultures identified selective enrichment of proteins involved in recruitment of leukocytes and their focal adhesion [6]. These pathways are required for proliferation, movement, and phagocytosis by monocytic leukocytes, and provide indirect evidence of interaction of glioma EVs with immune cells. Evidence for direct interaction, however, came from culture experiments where glioma EVs were added to peripheral blood mononuclear cells (PBMCs) or purified monocytes. Compared with EVs from nonmalignant cells, addition of glioma EVs resulted in increased survival of PBMCs and purified monocytes, as well as their secretion of multiple cytokines, including interleukin 6 (IL-6), IL-10, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF); [6]. These soluble secreted cytokines have different roles in the tumor microenvironment as IL-6 and IL-10 can both support and reduce tumor growth, MCP-1 attracts myeloid-derived monocytes, and VEGF induces angiogenesis, vital for continued tumor growth [1]. A separate study investigating cytokine release by microglia (brain resident innate immune cells) reported increased levels of cytokines after incubation of microglia with glioma EVs [7]. These studies revealed the potential for direct interaction between glioma EVs and innate immune cells; however, since the spatiotemporal distribution and concentration of EVs in an *in vivo* glioma are unknown, it is unclear to what extent these *in vitro* results represent the true EV/innate immune cell interaction. This challenge was elegantly highlighted in a study that showed different

and even opposite (decreased versus increased) levels of cytokine production when two different EV concentrations were added to PBMC cultures [8]. However, as the studies discussed previously used different donor cells and employed different EV isolation techniques, direct comparisons between studies may not be possible.

In glioblastoma the adaptive T cell response is dependent on the activation state and the composition of different types of T cells [1]. Similar to cells of the innate immune system, glioma EVs can influence T cells both indirectly, through intermediate myeloid-derived innate immune cells, or directly (Figure 1). Factors associated with T-helper (Th)2 immunity (generally assumed to be a tumor-supportive T cell response) found in EVs in the peripheral blood of glioblastoma patients, led to the hypothesis that glioma-derived EVs can suppress the T cell-mediated adaptive immune response [9]. Specifically, the presence of immunoglobulins IgG2 and IgG4 on patient-derived EVs, together with elevated levels of CD14/CD163-positive monocytes, as well as high levels of colony-stimulating factor 2 (CSF2), CSF3, IL-2, IL-4, and IL-13, were considered an indication of Th2 immunity. In addition, it was shown that monocytes after incubation with glioma EVs suppress T cell activation [10]. Although the exact mechanism for the suppression of T cell activation by monocytes after incubation with glioma EV is unknown, it was suggested that glioma EVs induced upregulation of pathways controlled by arginase-1,

increased IL-10 secretion, and decreased human leukocyte antigen-DR isotope (HLA-DR) expression [10]. Contrarily to glioma EV induced effects requiring monocytes as intermediates, a direct effect of glioma EVs on T cells has recently been described [11]. In this study, binding of programmed death-ligand 1 (PD-L1) present on the surface of glioblastoma-derived EVs to the programmed cell death-1 (PD-1) receptor on T cells resulted in inhibition of T cell function, a phenotype that was reversed with the addition of anti-PD-1 receptor blockers. PD-L1/PD-1 inhibition of T cells mediated by glioma EVs does not require intermediate monocytes, as another study failed to detect monocytic PD-L1 expression after incubation with glioma EVs [12].

Together, these results describe capacities for glioma EVs to interfere with the adaptive immune response, however, similar to the findings in innate immune cells, all evidence supporting EV-mediated T cell immune suppression is based on *in vitro* testing, and lacks direct evidence from *in vivo* experiments.

Concluding Remarks and Future Perspectives

As highlighted earlier, challenges in identifying the role of EVs in glioma immunity result from the paucity of results from *in vivo* models and the inability to compare different studies, as virtually every publication uses a different EV isolation technique, yielding varying EV purity, concentration, and sub-population composition (Box 1). To address the lack of standardization, the EV research community has generated a 'Minimal Information for Studies of Extracellular Vesicles (MISEV)' guideline that includes strong recommendations and reporting requirements to improve reproducibility and transferability of published results [3].

Since the immune response in the glioma microenvironment involves malignant and immune cells, including cells from both

the innate and adaptive immune systems, ultimately the effect of EVs needs to be studied *in vivo*. Although different models have been developed to address this situation, setting up proper conditions and controls remains an issue. For example, researchers attempted to investigate the effect of EVs *in vivo* by injecting isolated EVs into (tumor bearing) mice (reviewed in [2]). Since the endogenous concentration and spatiotemporal distribution of EVs are unknowns, these attempts can only partially mimic the interactions between EVs and immune cells. Other *in vivo* strategies have also been developed. For example, optical reporters can be introduced into tumor cells generating EVs *in vivo*, thus avoiding the injection of EVs. One approach is the introduction of tetraspanin-based pH-sensitive CD63 protein reporters. These reporters are fluorescent only after fusion of the multivesicular body with the plasma membrane, and thus generate fluorescent glioma EVs [13]. Alternatively, palmitoylated-GFP/tdTomato reporters expressed in glioma cells label all cellular membranes, including all EVs released from those cells [14]. In addition, a CRE–lox-based system was used to show that CRE is functionally transferred by EVs from tumor to innate immune cells, resulting in activation of reporters that can be used to track EV uptake [15]. These reporters help to visualize the interaction of glioma EVs with immune cells *in vivo* and represent an important development for *in vivo* validation of EV effects observed *in vitro*. Although promising, these models still do not allow for non-EV effects, such as secreted cytokines that may dominate the glioma–immune interaction, making EVs a bystander rather than an instigator. To control for this, a model that allows for the selective and complete knockout of EV release by glioma cells *in vivo* would be invaluable in this research. However, since interference in many of the intracellular pathways involved in EV release affects the vitality of the cell, this may not be feasible [2].

Overall, current yet circumstantial evidence describes a role for glioma-derived EVs in the establishment of an immune privileged tumor microenvironment. This framework of evidence now needs to be built upon using novel reproducible *in vivo* models.

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Forum

New Roles for Glycogen in Tumor Progression

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Glycogen is a high-density glucose polymer, which provides organisms with an immediate source of glucose to support the cell's energy requirements. Epithelial cells primarily store energy as glycogen, but until recently it has not been reported as a major fuel source for cancer growth. Hypoxia, which occurs in many cancers, results in glycogen synthesis and increased survival under stressed conditions. Recently, glycogen mobilization has been shown to play a role in the maturation and immune activity of dendritic cells (DCs) and in the proliferation and metastatic efficiency of cancer cells aided by the tumor microenvironment (TME). These studies indicate that glycogen plays an important role in glucose homeostasis and contributes to key functions related to tumor aggressiveness and the survival of cancer cells.

Introduction

A hallmark of cancer is the metabolic reprogramming of cancer cells for rapid proliferation and growth. It was reported