Intercellular communication between glioma and innate immune cells

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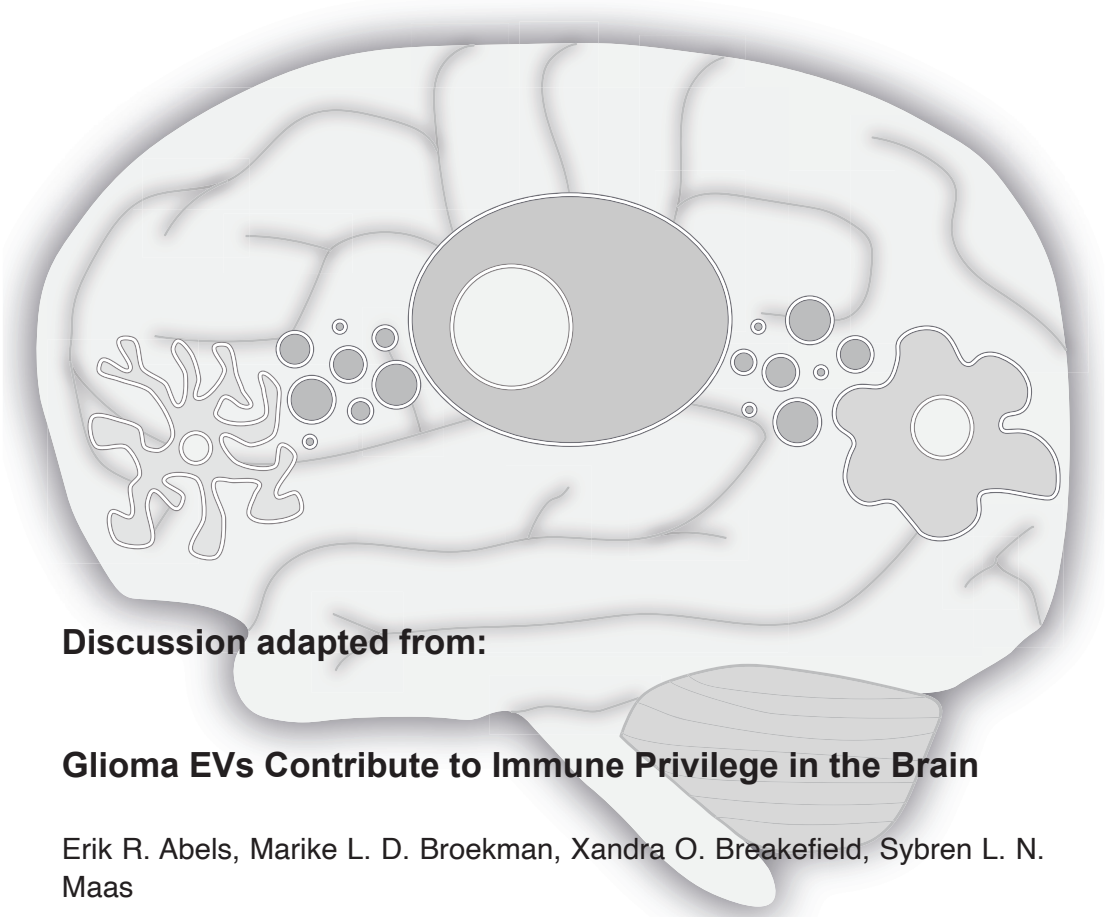
Chapter 7

Summary and Discussion



Discussion adapted from:

Glioma EVs Contribute to Immune Privilege in the Brain

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Summary

Glioblastomas (GBs) are the most common and lethal adult primary brain tumors. They are characterized and defined by their highly aggressive nature involving rapid tumor growth, diffuse invasiveness and resistance to therapy. GBs are made up of a genetically and phenotypically heterogeneous population of tumor cells and various types of stromal cells, which all contribute to tumor progression and resistance to treatment. **Chapter 1** gives an overview of the various cells that make up the tumor microenvironment. A detailed description is given of the innate immune cells, including microglia and infiltrating monocytes and macrophages, and the ways they are recruited to the tumor. In addition, the different modes of intercellular communication between tumor and innate immune cells are discussed. The focus is on the role of EVs in this interaction. To understand this interaction a detailed overview is given on EVs (including exosomes and microvesicles) and how they are be categorized. It captures the biogenesis, release and uptake of EVs in great detail.

In **chapter 2**, the effect of extracellular miRNA transfer is discussed together with the imaging of EV uptake *in vitro* and *in vivo*. A fluorescent reporter that labels EVs that are continuously shed by glioma cells was achieved by stable expression of palmitoylated GFP. By using this reporter, we determined that microglia take up (large numbers of) EVs in culture. This uptake resulted in increased proliferation and shifting of their cytokine profile towards a more immune suppressive one. Moreover, miR451 and miR-21 in glioma EVs was transferred to microglia. The extracellular transfer of this miRNA resulted in a decrease in the miR-21 target *c-Myc* mRNA in both murine and human microglia in culture. *In vivo* analysis allowed direct visualization of release of EVs from glioma cells and their uptake by CX3CR1^{pos} microglia and monocytes/macrophages in the brain. In addition, analysis of isolated microglia and monocytes/macrophages from tumor-bearing brains revealed increased levels of miR-451/miR-21 and reduced levels of *c-Myc* mRNA. This chapter, supports functional effects of glioma EVs following uptake into microglia associated in part with increased miRNA levels, decreased target mRNA and encoded protein. We hypothesize that this could be a means for the tumor to manipulate its environs.

In **chapter 3**, we focus on the *in vivo* extracellular miRNA transfer of miR-21 from glioma to microglia. This consists of examining the uptake of fluorescently labeled glioma EVs by microglia in miR-21-null mice determined by FACS in combination with mRNA sequencing. Mouse glioma cells, stably expressing a palmitoylated GFP to label EVs were implanted intracranially into syngeneic miR-21-null mice.

We demonstrate functional delivery of miR-21, regulating specific downstream mRNA targets in microglia after uptake of tumor-derived EVs. These findings attest to EV-dependent miRNA delivery as studied in an *in vivo* based model and provide insight into the reprograming of microglial cells by tumor cells to create a favorable microenvironment for cancer progression.

In **chapter 4** we discuss the overall transcriptomic changes in microglia upon EV uptake in an intracranial murine glioma model. We show that these microglia have downregulated expression of genes involved in sensing tumor cells and tumor-derived danger signals, as well as genes used for tumor killing and immune-suppression. In contrast, expression of genes involved in facilitating tumor spread were upregulated. These changes appear to be mediated in part by tumor-derived EVs, since intracranial injection of these EVs into normal mouse brain led to similar transcriptional changes in microglia. We observed a similar microglial transcriptomic signature when we analyzed datasets from human patients with glioblastoma. Our data define a $\text{Microglia}_{\text{Glioblastoma}}$ specific phenotype, whereby glioblastomas have hijacked gene expression in the neuroimmune system to avoid tumor sensing, suppress the immune response, clear a path for invasion and enhance tumor propagation. For further exploration we developed an interactive online tool at www.glioma-microglia.com with all expression data and additional functional and pathway information for each gene.

The analysis of the microglial transcriptome in the presence of a tumor was extended to the changes in gene expression occurring in infiltrating monocytes and macrophages in comparison to circulating monocytes, as discussed in **chapter 5**. In this study we analyzed the transcriptomes of eight different monocyte subgroups derived from the brain and the blood of glioma-bearing mice. We compared the expression profile of blood-derived monocytes versus tumor-infiltrating monocytes and found increased expression of both pro- and anti-inflammatory pathways in tumor infiltrating monocytes. To help disseminate these datasets, we created a user-friendly web-based tool accessible at www.glioma-monocytes.com. This tool can be used for validation purposes and to elucidate gene expression profiles of tumor-interacting monocytes and macrophages, as well as blood-derived circulating monocytes. This tool can also be used to identify new markers and targets for therapy in these different cell populations.

The microglial sensome was found to be important for the functioning of microglia and is disrupted in different neuro-pathological settings. In **chapter 6** we discuss the overlap of the murine and human sensome. We analyzed existing

transcriptome dataset from both human and mouse. Here we found an overlap of a number of genes that are share between these species, which we termed "microglial core sensome". Defining these set of genes may help identify changes in microglia in humans and mouse models and can help find therapeutic avenues in diseases where microglia play a key role.

Discussion

Glioblastomas are the most common and lethal intracranial primary malignancies in adults. They are composed of heterogeneous tumor cells and nonmalignant stromal cells(Broekman et al., 2018). 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(Broekman et al., 2018). Together, the stromal and malignant cells form a microenvironment that in general enables the tumor cells to proliferate and infiltrate(Broekman et al., 2018). 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)(Broekman et al., 2018). EVs is the collective term for nanosized and microsized (~50– 10 000 nm) membrane-enclosed vesicles that are released by all cell types(Maas et al., 2017). As different cellular pathways can result in the release of EVs, various terminology (e.g., exosomes, microvesicles, ectosomes) has been used for potential subpopulations of EVs (**Fig. 1**)(Maas et al., 2017; Théry et al., 2018). However, since clear markers for these subpopulations are lacking, current consensus is to use the umbrella term 'EVs'(Théry et al., 2018). EVs have a similar membrane topology as their cells of origin, and thus cell type-specific and 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 and/or cargo molecules can induce intracellular signaling in EV recipient cells(Al-Nedawi et al., 2008). 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(Maas et al., 2017; Valadi et al., 2007). 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(Maas et al., 2017). First, the role of glioma-derived EVs in the establishment of an immune privileged microenvironment will be discussed, followed by the technical challenges and future prospects for this field of research.

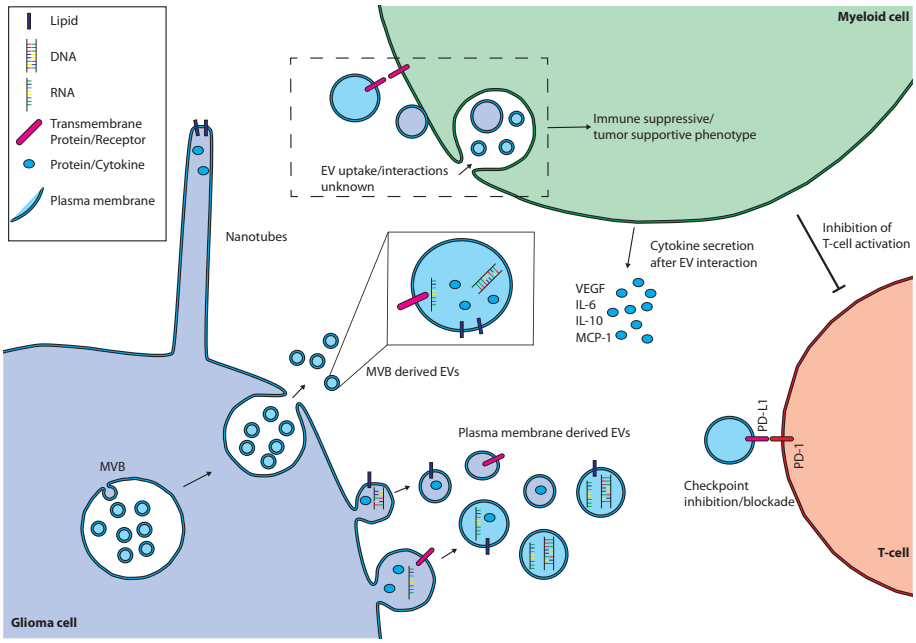

Figure 1. Extracellular Vesicles as a Mode of Intercellular Communication in Glioma Immunity. Extracellular vesicles (EVs) can be formed both by the budding of the plasma membrane or through the fusion of a multivesicular bodies (MVBs) with the plasma membrane. Cell–cell contact and the subsequent exchange 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.

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(Graner et al., 2009). 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(Graner et al., 2009). 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 glioma stem cell-like cultures identified selective enrichment of proteins involved in recruitment of leukocytes (de Vrij et al., 2015). 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 increased secretion of multiple cytokines, including interleukin 6 (IL-6), IL-10, monocyte chemoattractant protein-1 (MCP-1), and vascular endothelial growth factor (VEGF)(de Vrij et al., 2015). 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(Broekman et al., 2018). 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(van der Vos et al., 2016). 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 a glioma in the brain are unknown, it was unclear to what extent these *in vitro* results represented 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(Hellwinkel et al., 2015). However, as the studies discussed previously used different donor cells and employed different EV isolation techniques, direct comparisons between studies is not possible. In glioblastoma, the adaptive T cell response is dependent on the activation state and the composition of different types of T cells(Broekman et al., 2018). 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 (**Fig. 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(Harshyne et al., 2016). 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(Domenis et al., 2017). 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(Domenis et al., 2017). Contrary to glioma EV induced effects requiring monocytes as intermediates, a direct effect of glioma EVs on T cells has recently been described(Ricklefs et al., 2018). 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(Iorgulescu et al., 2016).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.

Technical Challenges and Future Perspectives

As highlighted earlier, challenges in identifying the role of EVs in glioma immunity derive 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 subpopulation 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(Théry et al., 2018).

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 progress 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(Théry et al., 2018).

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 tumor-derived EVs into (tumor-bearing) mice (reviewed in (Maas et al., 2017)). 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(Verweij et al., 2018). Alternatively, palmitoylated-GFP/tdTomato reporters expressed in glioma cells label all cellular membranes, including all EVs released from those cells(Lai et al., 2015). 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(Ridder et al., 2015). 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(Maas et al., 2017).
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. In thesis, data is presented focusing on one specific mode of communication through EVs. The transfer of miRNA is thought to be a powerful tool used by tumor cells to influence their environment. The *in vivo* model and experimental setup used here provides direct evidence of this transfer and the subsequent functional consequence. In addition, uncovering of the transcriptomic changes of innate immune cells in the presence of a tumor using our *in vivo* model has provided the first step to uncover the different mechanisms and gene pathways responsible for establishing an immune privileged tumor microenvironment.

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