Intercellular communication between glioma and innate immune cells

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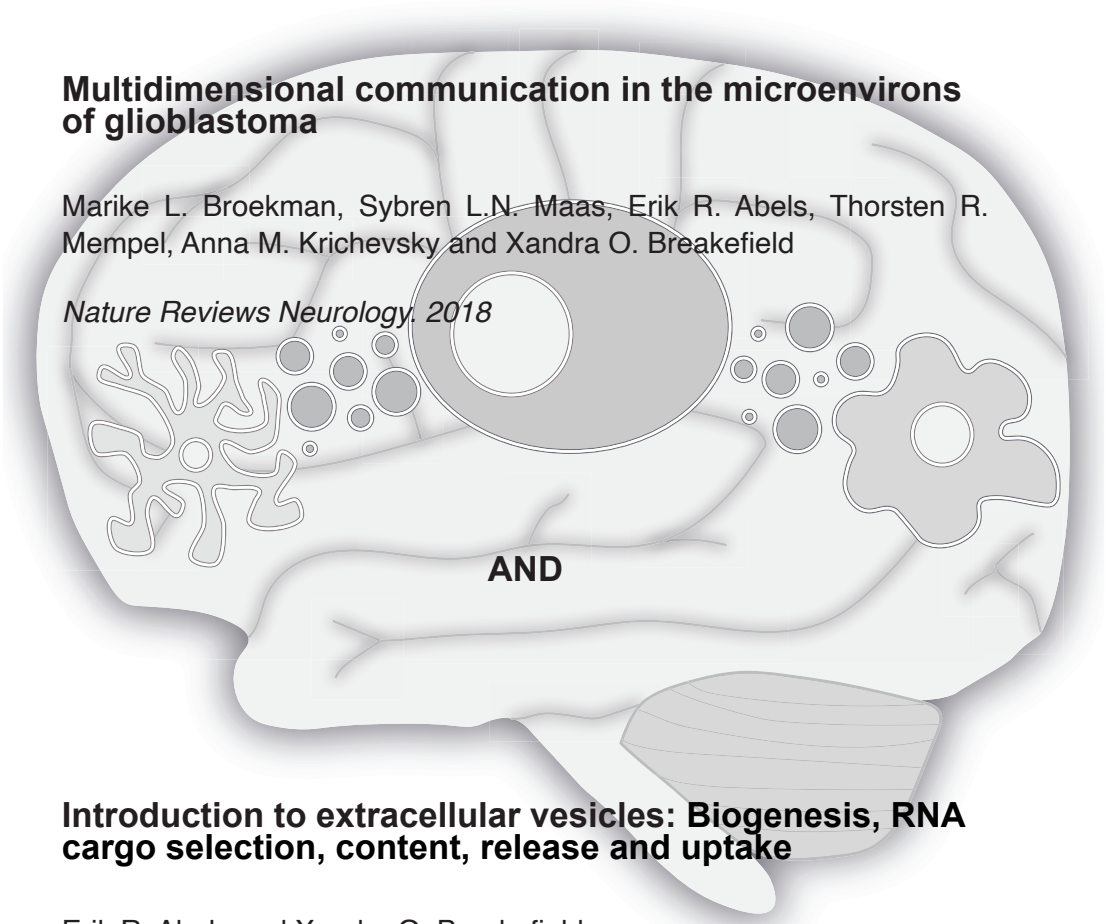
Chapter 1

General Introduction



Multidimensional communication in the microenvirons of glioblastoma

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Introduction to extracellular vesicles: Biogenesis, RNA cargo selection, content, release and uptake

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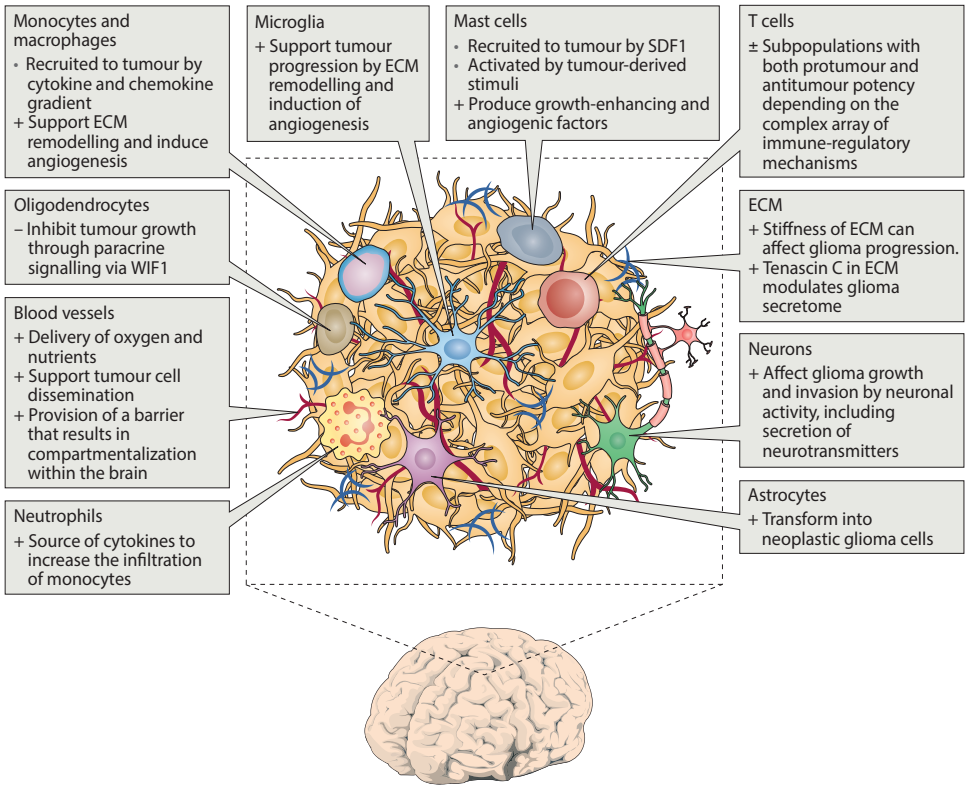
Glioblastoma

Glioblastomas remain one of the most aggressive malignancies, with no change in the standard of care for almost 20 years and a median lifespan from time of diagnosis to death of about 15 months (Stupp et al., 2009). This bleak outcome has stimulated ongoing efforts to reveal new insights into these tumors and the surrounding cells to facilitate development of new treatment strategies. New studies and technologies have deepened our understanding of the factors that make these tumors so formidable but have highlighted two major challenges. First, a lack of models that can authentically reproduce the genetic and phenotypic properties of human glioblastoma, especially regarding the analysis of glioblastoma microenvironmental communication, is hampering progress into the development of new therapies for the condition. Second, as underlined by the 2016 WHO classification system, evidence increasingly demonstrates that glioblastoma is genetically heterogeneous and thus will probably require combinatorial approaches for different subtypes of tumor cells even within a single glioblastoma tumor. In addition to this deepening understanding of the genetic and phenotypic variability within glioblastoma, the field has gained increasing awareness of the ability of these tumors to manipulate and exploit normal brain cells. Almost all cell types in the tumor environs are affected: the tumor is able to stimulate angiogenesis and co-opt existing vasculature(Jhaveri et al., 2016), disarm microglia and macrophages that should recognize and fight foreign elements in the brain(Roesch et al., 2018), coerce astrocytes into supporting tumor progression(Okolie et al., 2016) and even change the extracellular matrix (ECM) to facilitate invasion(Pencheva et al., 2017). Conversely, new insights into the presence of adaptive immune cells in the brain and the presence of a CNS lymphatic system(Aspelund et al., 2015; Louveau et al., 2015) may give rise to therapeutic opportunities that manipulate this system to recognize tumor neoantigens(Boussiotis and Charest, 2018), similarly to the immune strategies currently being clinically applied for some melanoma and lung cancer patients (**Fig. 1**).

Figure 1. Glioblastoma microenvironment. The glioblastoma environ consists of tumor cells, extracellular matrix (ECM), blood vessels, innate immune cells (monocytes, macrophages, mast cells, microglia and neutrophils), T cells and non-tumorous neurons, astrocytes and oligodendrocytes. +, protumor function; −, antitumor function; ±, mixed protumor and antitumor functions; SDF1, stromal cell-derived factor 1; WIF1, WNT inhibitory factor 1



Innate immune system and glioblastoma

Interaction between glioblastoma and microglia, monocytes or macrophages.

The glioblastoma microenvironment contains brain-resident microglia and infiltrating monocytes. Once monocytes have infiltrated the tumor, they can differentiate into macrophages(Bowman et al., 2016; Chen et al., 2017). Although often grouped together under the term tumor-associated macrophages or myeloid cells (TAMs), these cells represent distinctly different populations(Bowman et al., 2016). Microglia are derived from immature yolk sac progenitors during early embryonic development and maintain themselves in the brain through self-renewal(Ajami et al., 2007; Ginhoux et al., 2010). In non-pathological settings, microglia are the main innate immune cells in the brain and are important in the defence against pathogens and noxious stimuli(Hickman et al., 2013). Glioblastoma leads to some disruption of the blood–brain barrier (BBB), which enables bone marrow haematopoietic stem cell-derived monocytes and macrophages to

infiltrate the tumor(Bowman et al., 2016; Chen et al., 2017; Müller et al., 2015). Studies have shown that in specific cases up to 50% of the glioblastoma mass can consist of TAMs(Hambardzumyan et al., 2016). Chimeric and cell lineage models have shown that the exact composition of the different types of TAMs changes over time(Bowman et al., 2016; Müller et al., 2015). One study examined the infiltration of peripheral immune cells in a syngeneic GL261 mouse glioma model that received head protected irradiation, in which BBB disruption due to irradiation is avoided. Fluorescently tagged myeloid-derived monocytes and macrophages transplanted by intravenous injections into these mice constituted up to 25% of TAMs in the glioblastoma tumor after 21 days, with lower percentages of myeloid-derived TAMs observed at earlier time points(Müller et al., 2015). The influx of myeloid-derived monocytes in mouse glioblastoma tumors was confirmed in a haematopoietic stem cell lineage tracing model, in which >35% of TAMs were myeloid-derived(Bowman et al., 2016). As such, the population of glioblastoma TAMs can progress from strictly microglial in early phases to a mixture of microglia and infiltrating monocytes and macrophages in later phases of tumor progression. In mice, accurate separation of microglia and macrophages can be achieved by fluorescence-activated cell sorting using αM integrin (also known as CD11b) and receptor-type tyrosine-protein phosphatase C (also known as CD45) markers, with microglia expressing CD11b to a high degree and CD45 to an intermediate level (Bowman et al., 2016). In humans, α4 integrin (also known as CD49D) can accurately separate these two cell types in tumors, as it is exclusively expressed in macrophages as compared to microglia(Bowman et al., 2016). Here, when studies used these specific markers for separation of microglia and myeloid-derived cells we refer to the cellular subpopulation studied, otherwise the generic term 'TAMs' is used to include both.

TAM recruitment.

The recruitment of TAMs to glioma is mostly mediated by cytokine and chemokine gradients released by glioblastoma cells (**Fig. 2**). These factors have been extensively reviewed elsewhere and include CC-chemokine ligand 2 (CCL2; also known as MCP1) and CCL7 (also known as MCP3), glial-derived neurotrophic factor (GDNF), hepatocyte growth factor (HGF), SDF1, tumor necrosis factor (TNF), VEGF, ATP, macrophage colony-stimulating factor 1 (CSF1) and granulocyte–macrophage colony-stimulating factor (GM–CSF)(Hambardzumyan et al., 2016; Li and Graeber, 2012). TAMs can also be recruited to a specific subset of glioblastoma cells, such as oligodendrocyte transcription factor 2 (OLIG2)-expressing and transcription factor SOX2-expressing tumor-initiating cells, which secrete periostin to recruit TAMs(Zhou et al., 2015). Medical interventions can also stimulate TAM recruitment; for example, intracranial biopsies can increase infiltration of circulating monocytes into the tumor in a CCL2-dependent manner(Alieva et al., 2017). Microglia and

macrophages themselves also secrete CCL2 to increase infiltration of $CCR2^{+}Ly6C^{+}$ monocytes, thus creating a positive feedback loop for the continued infiltration of myeloid cells(Chang et al., 2016).

TAM activation state.

Interaction between glioblastoma cells and TAMs is multifactorial and occurs both in close proximity by direct cell–cell contact and distantly by the release of factors either as solubles or carried in extracellular vesicles (EVs). The secretome consists of a multitude of molecules, including soluble lipids, cytokines and chemokines(Li and Graeber, 2012; Wurdinger et al., 2014). Glioblastomas also release EVs that contain a cargo of many types of molecules that have been shown to influence TAM status in a combinatorial way in culture and *in vivo*(de Vrij et al., 2015; van der Vos et al., 2016). However, no techniques currently are available to specifically suppress extracellular vesicle release from glioblastomas; therefore, the overall relevance of the interaction between glioblastoma extracellular vesicles and TAMs remains to be elucidated. Ultimately, the combination and timing of all glioblastoma-released factors determine the activation state and function of TAMs.

The traditional model of the activation states of TAMs describes a binary system of either tumor-suppressive (M1) or tumor-supportive (M2) macrophages(Ransohoff, 2016). This model was based on stimulation of cells in culture by IFNγ, lipopolysaccharide (LPS) or IL-4 and was later extended to include M2 subtypes activated by other types of stimulation, comprising M2a (IL-4 and IL-13), M2b (immune complexes, Toll-like receptor (TLR) or IL-1R) and M2c (IL-10) (Mantovani et al., 2002). However, RNA sequencing in response to different stimuli has extended the number to a combination of 28 known factors and revealed that a wide spectrum of activation states can be induced. These findings have demonstrated that macrophage differentiation is much more complex than the binary M1– M2 model(Xue et al., 2014), even when stimulated in culture. This complexity became more apparent when microglia, monocytes and macrophages were isolated from glioblastoma *in vivo* and analyzed by RNA sequencing. The most upregulated genes were found to be shared between traditional M1, M2a, M2b and M2c transcriptomes, suggesting that the activation state *in vivo* is very different from that in culture(Bowman et al., 2016; Gabrusiewicz et al., 2011; Szulzewsky et al., 2015). Single-cell sequencing confirmed that activation of both M1 and M2 signatures can be observed even in individual cells in an *in vivo* brain trauma model(Kim et al., 2016). Consequently, the M1 and M2 designations are being replaced by more precise situation-specific models(Ransohoff, 2016). Altogether, these findings suggest that TAMs express gene sets *in vivo* that are associated with stimulation by different factors and pathologic conditions, highlighting the

variety of information transfer in the tumor microenvironment.

TAMs contribute to tumor proliferation.

The role of secreted molecules on TAM function and, subsequently, on tumor growth has been studied extensively(Hambardzumyan et al., 2016). This interplay between glioblastoma cells and TAMs is especially apparent in tissue remodeling and is necessary for glioblastoma cells to infiltrate the brain (**Fig. 2**). One group of proteins that is crucial in tissue remodeling is matrix metalloproteinases (MMPs) (Kessenbrock et al., 2010). In glioblastoma, MMP2 has an important role in ECM degradation, which facilitates glioblastoma cell migration and invasion(Du et al., 2008). MMP2 is released in a precursor form (pro-MMP2) that is cleaved by MMP14 to an active state(Hambardzumyan et al., 2016). However, glioblastoma cells secrete pro-MMP2, but not MMP14. Conversely, microglia in the tumor microenvironment are a major source of MMP14. Two different glioblastoma-derived factors act to increase microglial MMP14 release(de Vrij et al., 2015; Hu et al., 2015). First, the ECM protein versican is released from glioma and induces MMP14 release by TAMs through its upstream receptor TLR2(Hu et al., 2015). Second, studies of cell-culture models have shown that glioblastoma-derived extracellular vesicles can also induce microglial expression of MMP14 RNA, although the mechanism and *in vivo* relevance remain to be elucidated(de Vrij et al., 2015). Owing to their rapid growth, glioblastomas are in constant need of neovascularization and release angiogenic factors, such as EGF and VEGF(Li and Graeber, 2012). Additionally, in glioblastoma, microglia and macrophages accumulate around blood vessels and also produce the pro-angiogenic chemokines VEGF and CXC-chemokine ligand 2 (CXCL2)(Brandenburg et al., 2016). Furthermore, glioblastoma cells may promote angiogenesis indirectly through microglial cells, as CSF1 secreted by glioblastoma cells *in vitro* induces microglia cells to release insulin-like growth factor-binding protein 1 (IGFBP1), which can induce angiogenesis(Nijaguna et al., 2015). RAGE (receptor for advanced glycation end products; also known as AGER) is thought to play a part in a number of diseases, including tumors. In tumor-bearing mice, RAGE ablation increases survival by reducing the levels of VEGFA secreted by infiltrating TAMs, which results in leaky (rather than fully developed) vasculature and disturbed tumor perfusion(Chen et al., 2014). However, these effects were reported in syngeneic GL261 mouse tumors (a frequently used cellular model of glioma), which do not represent the invasive growth pattern observed in glioblastoma patients. Thus, TAMs have a crucial role in tumor angiogenesis through multiple signaling mechanisms. Overall, the interaction between glioblastoma and TAMs is bidirectional and multifactorial. This plethora of paracrine loops can determine the ultimate effects of TAMs on tumor growth and can differ depending on local variables such as hypoxia, the extent of necrosis, TAM infiltration density and/or

TAM activation state.

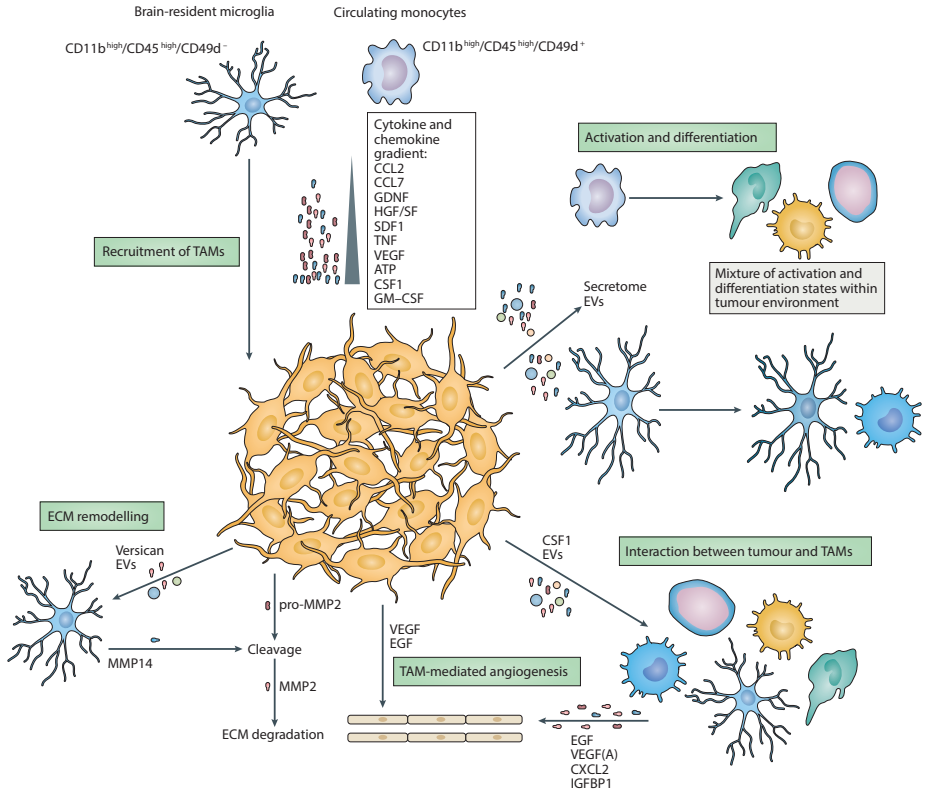


Figure 2. Interactions between glioma and TAMs. Recruitment of tumor-associated macrophages or myeloid cells (TAMs), including blood monocytes and brain-resident microglia, is based on the gradient of chemokines and cytokines released by the glioblastoma cells. Once recruited, TAMs can be activated and differentiated under the influence of the secretome and extracellular vesicles (EVs) released by the tumor. The various recruited and activated TAMs can affect tumor growth by promoting angiogenesis through secretion of epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), CXC-chemokine ligand 2 (CXCL2) and insulin-like growth factor-binding protein 1 (IGFBP1). This process is further promoted by the release of tumor-derived VEGF and EGF. Invasion and growth of the tumor are accomplished by remodeling the extracellular matrix (ECM) surrounding the tumor. For example, versican and EVs from the tumor induce the release of matrix metalloproteinase 14 (MMP14) by microglia. The release will facilitate the cleavage of tumor-derived pro-MMP2 following extracellular degradation by the active enzyme MMP2. CCL2, CC-chemokine ligand 2 (also known as MCP1); CSF1, macrophage colony-stimulating factor 1; GDNF, glial cell line-derived neurotrophic factor; HGF, hepatocyte growth factor; SDF1, stromal cell-derived factor 1; TNF, tumor necrosis factor.

This glioblastoma 'takeover' of the brain involves multiple types of communication and directive exchanged between tumor cells and surrounding cells. Cell-secreted soluble factors, including transforming growth factor-β (TGFβ), IL-6, Notch,

platelet-derived growth factor (PDGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 (SDF1; also known as CXCL12), are well known to serve as signaling molecules by binding to receptors on target cells, but the importance of other routes of communication — such as gap junctions, extracellular vesicles and nanotubes — are now being recognized. A distinguishing feature of glioblastomas is their ability to form a virtual nuclear and cytoplasmic continuum with neighboring cells, whereby they can introduce not only inorganic elements but also genetic elements and proteins into normal cells to change their phenotype and rescue fellow tumor cells that are in trouble, for example, as a result of radiotherapy or chemotherapy. These newly recognized transit routes can transmit non-secretable molecules, including transcription factors, directive RNAs and DNA and even mitochondria and nuclei. Small molecules such as Ca^{2+}, ATP, metabolites and microRNAs (miRNAs) can be transferred between adjacent cells through gap junctions(Hong et al., 2015; Thuringer et al., 2016). Connexins, which form a structural component of these junctions, are upregulated in tumor-initiating cells(Balça-Silva et al., 2017) and are associated with increased invasiveness of gliomas(Hong et al., 2015). Non-secretable proteins (including transcription factors), RNA, DNA, lipids and metabolites can be transferred through tumor-derived extracellular vesicles released from cells via fusion of multivesicular bodies with the cell membrane (which yields exosomes), budding from the plasma membrane (giving rise to microvesicles and large oncosomes)(Maas et al., 2017; Minciacchi et al., 2015; Tkach and Théry, 2016) or budding off of the tips of nanotubes that extend out from the cells(Lai et al., 2014; Rilla et al., 2013). These tumor-derived extracellular vesicles can change the phenotype of normal cells to promote angiogenesis, immune suppression, tumor cell invasion and metabolic regulation(D'Asti et al., 2016; Fonseca et al., 2016; Redzic et al., 2014). Tumor cells can also be linked by 'tunneling' nanotubes and microtubes that form gap junctions or a cytoplasmic continuum between cells to enable transport of molecules and organelles(Osswald et al., 2015; Vignais et al., 2017; Wang et al., 2010). The involvement of microtubes has been indicated in the regrowth of tumors after surgery and in conferring resistance to chemotherapy(Weil et al., 2017), although they are not apparent in some glioma models(van der Vos et al., 2016).These different modes of physical support among tumor cells, and the two-way crosstalk between tumor cells and normal cells in their vicinity, together with the epigenetic flexibility of cells, enable the tumor to create a pluripotent environment that can adapt to changes and thus give the tumor many options to survive therapeutic assault. Here, we focused on the intercellular communication between tumor cells and their microenvironment through EVs.

Extracellular vesicles

EVs are a heterogeneous family of membrane-limited vesicles originating from the endosome or plasma membrane. Pan and Johnstone (1983) were among the first to describe EVs(Pan and Johnstone, 1983). Initially, it was shown that the release of EVs was part of a disposal mechanism to discard unwanted materials from cells. Subsequent research has shown that the release of EVs is also an important mediator of intercellular communication that is involved in normal physiological process as well as in pathological progression(Barteneva et al., 2013; Fruhbeis et al., 2012; Fruhbeis et al., 2013; Luga et al., 2012; Marcilla et al., 2012; Regev-Rudzki et al., 2013).

EVs are currently classified based on their mode of release or size. EVs can be released by "donor" cells either through the outward budding of the plasma membrane, termed shedding microvesicles (MVs) or ectosomes(Minciacchi et al., 2015). Another release process involves the inward budding of the endosomal membrane, resulting in the formation of multivesicular bodies (MVBs), with exosomes released by fusion of the outer MVB membrane to the plasma membrane (Denzer et al., 2000; Thery et al., 2009). Vesicles may also be released from nanotubular structures extending from the plasma membrane(Rilla et al., 2013; Rilla et al., 2014). In addition to the differences in the mode of release, the size of the vesicles is also used for characterization. Although different scales are used, MVs range from 50 nm to 10,000 nm, and exosomes are smaller with a diameter of 30 to 150 nm(Baietti et al., 2012; Colombo et al., 2013; Gyorgy et al., 2011). Overall EVs are comprised of a wide variety of different type of vesicles ranging from 30 nm to 1000 nm in size with a variety of cargos, and the different types of vesicles overlap in their size distribution. It must be emphasized that there is some controversy on nomenclature and sizes of the different types of vesicles(Gould and Raposo, 2013; Witwer et al., 2013), however basic requirements of criteria for EVs have been established(Lotvall et al., 2014; Mathieu et al., 2018). So far, no real standards have been set to classify the different types of vesicles, so one should be careful with the use of size alone in defining different types of vesicles. In the future, the mode of biogenesis, means of isolation, lipid components and cargo may turn out to be far more important criteria. Given how the different isolation methods may influence the nature of EVs, methods should be compared in order to develop a gold standard for the different protocols and measurements (Momen-Heravi et al., 2012). To be able to compare results it must be stressed that publications on EVs need to clarify their isolation methods in detail, and the general term, EVs should be used unless there are specific markers defined to classify the different types of vesicles(Théry et al., 2018).

So far, extensive evidence on all these different types of vesicles indicates that EVs are a key player in the intercellular communication between cells, along with secretion of small soluble molecules (the secretome) and cell-cell contact (Cocucci et al., 2009; Raposo and Stoorvogel, 2013). Once released the EVs can be internalized via endocytosis or membrane fusion, releasing their contents into "recipient" cells (Mulcahy et al., 2014). Recent studies have shown that these EVs contain various proteins, sugars, lipids and a wide variety of genetic materials, such as DNA, mRNA and non-coding (nc)RNAs with the content protected from proteases and nucleases in the extracellular space by the limiting membrane (Henderson and Azorsa, 2012; Thery et al., 2002). EVs have the potential to deliver combinatorial information to multiple cells in their tissue microenvironment and throughout the body (Baj-Krzyworzeka et al., 2006; Ratajczak et al., 2006; Skog et al., 2008).

The ins and outs of EVs

Vesicle biogenesis

As EVs have traditionally been classified based on differences in biogenesis, we will focus on the different molecular mechanisms resulting in either the release of vesicles upon the fusion of the MVBs with the plasma membrane or the release via the outward budding and fission of the plasma membrane(Akers et al., 2013).

Exosome biogenesis

Exosomes are derived from the endosomal system and are formed as intraluminal vesicles (ILVs) in the MVBs. This network of ILVs is used to degrade, recycle or exocytose proteins, lipids and nucleic acids. Within the endosomal system or endocytic pathway, the endosomes are divided into different compartments - early endosomes, late endosomes and recycling endosomes(Grant and Donaldson, 2009). Endosomes form by invagination of the plasma membrane. The early endosomes can fuse with endocytic vesicles in the cytoplasm, at which point the content is destined for degradation, recycling or secretion. Contents to be recycled are sorted into recycling endosomes(Morelli et al., 2004). The remaining early endosomes transform into late endosomes(Stoorvogel et al., 1991). The late endosomes accumulate ILVs formed by inward budding of the endosomal membrane. During this process cytosolic proteins, nucleic acids and lipids are sorted into these small vesicles. Late endosomes containing a multitude of small vesicles are termed MVBs. These MVBs can either fuse with the lysosome if the content is fated for degradation or fuse with the cellular membrane releasing the ILVs as exosomes into the extracellular space(Grant and Donaldson, 2009).

The formation of the ILVs within MVBs is the start of the biogenesis of exosomes. ILV formation can be achieved through two different mechanisms. First, the endosomal sorting complexes required for transport (ESCRT) dependent formation of ILV which requires a combination of ESCRT protein working in sequence together with ESCRT associated proteins ALIX, TSG101, CHMP4 and SKD1(Babst et al., 2002; Bache et al., 2003; Baietti et al., 2012; Colombo et al., 2013; Fernandez-Borja et al., 1999; Henne et al., 2011; Henne et al., 2013; Katzmann et al., 2001; Matsuo et al., 2004; McCullough et al., 2008; Raiborg and Stenmark, 2009; Razi and Futter, 2006; Shields et al., 2009; Tamai et al., 2010; Wollert and Hurley, 2010)(**Fig. 3A**). Second, the alternative ESCRT pathway, or syndecan-syntenin-ALIX pathway, is dependent on heparanase, syndecan heparan sulphate proteoglycans, ADP ribosylation factor 6 (ARF6), phospholipase D2 (PLD2) and syntenin to mediate exosome biogenesis, including vesicle formation and loading of proteins is outlined in Figure 3B(Baietti et al., 2012)(**Fig. 3B**).

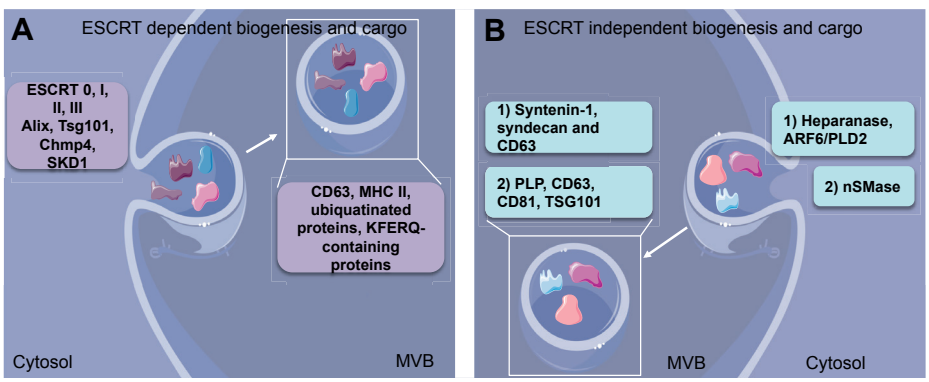


Figure 3. **Molecular mechanisms of ESCRT dependent and independent MVB biogenesis.** Multiple biogenesis machineries have been described for generating ILVs in MVBs. (**A**) ESCRT dependent MVB biogenesis requires the ESCRT protein and ESCRT associated proteins (ALIX, TSG101, CHMP4 and SKD1) to form MVBs containing CD63, MHC II, ubiquitinated proteins and KFERQ-containing proteins. (**B**) Two ESCRT independent pathways are controlled by different proteins: 1) heparanase and ARF6/PLD2, associated with the presence of syntenin-1, syndecan and CD63 in exosomes; 2) nSMase, in which the exosomes are enriched with PLP, CD63, CD81 and TSG101.

Exosome release

Release of exosomes into the extracellular space is facilitated by the fusion of the MVB limiting membrane with the plasma membrane. Similar to the different mechanisms proposed for the biogenesis of exosomes, a variety of mechanisms have also been proposed for the release of exosomes. As is shown in figure 4A a number of Rab GTPases, including RAB11 and RAB35, or RAB27A and RAB27B, are recognized to play an important role (Hsu et al., 2010; Laulagnier et al., 2004; Savina et al., 2003). A summary of the different proteins involved in exosome

release are shown in figure 4A (Alonso et al., 2007; Alonso et al., 2011; Fader et al., 2009; Logan et al., 2006; Ostrowski et al., 2010; Puri and Roche, 2008; Rao et al., 2004; Stenmark, 2009; Tiwari et al., 2008). Overall, exosomes can be generated and released from different subtypes of endosomes by various mechanisms and harbor different cargo as a function of cell type and probably physiologic state (**Fig. 4A**).

Microvesicle biogenesis and release

The biogenesis of the MVs is far less defined as compared to exosomes. Biogenesis and release of MVs has been investigated in several cellular model systems. Different mechanisms are found to be responsible for the shedding of MVs. In general, these types of vesicles appear to be formed though the outward budding and fission of the plasma membrane. A combination of factors will result in the formation of MVs such as the redistribution of phospholipids, including the repositioning of phosphatidylserine to the outer leaflet, and contraction of the actin-myosin machinery(Akers et al., 2013). The detailed process is shown in figure 4B (Bucki et al., 1998; Muralidharan-Chari et al., 2009; Nabhan et al., 2012; Pasquet et al., 1996; Tauro et al., 2012; Wang et al., 2014). The different mechanisms underlining the release of MV from the plasma membrane can be distinguished based on the content of the released MVs (**Fig. 4B**). Some of these mechanisms are similar to those described for extracellular budding of virus particles, such as retroviruses(Gould et al., 2003), and, in fact, a substantial portion of EVs released from cancer cells are retrovirus-like particles(Akers et al., 2013; Balaj et al., 2011).

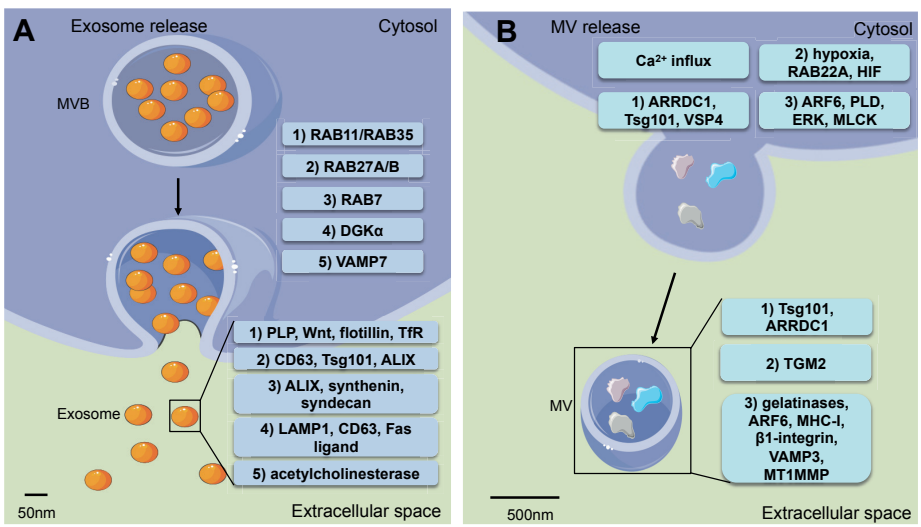


Figure 4. **Molecular machineries of EV release**. (**A**) Proteins involved in controlling the fusion of MVBs with the outer membrane to the plasma membrane, resulting in release of

exosomes. Five different machineries have been described so far; 1) RAB11 and RAB35 facilitate the fusion of MVBs to the plasma membrane, releasing exosomes containing PLP, Wnt, flotillin and TfR; 2) RAB27A and RAB27B promote release of exosomes loaded with CD63, TSG101 and ALIX; 3) RAB7 dependent release yields release of exosomes harboring ALIX, synthenin and syndecan; 4) DGKα protein is implicated in release of exosomes carrying LAMP1, CD63 and Fas ligand; and 5) VAMP7 regulates the membrane fusion associated with release of acetylcholinesterase-containing exosomes release. (**B**) EV released via the outward budding and fission of the plasma membrane controlled by different proteins and extracellular signaling results in release of MVs with a distinct protein profile. Three pathways have been described including markers found in released MVs: A) ARRDC1, TSG101 and VSP4 are responsible for the shedding of MVs containing TSG101 and ARRDC1; B) hypoxia following expression of RAB22A via HIF, characterizes the secretion of EVs carrying TGM2; and C) the ARF6, PLD, ERK and MLCK cascade induces release of EVs containing gelatinases, ARF6, MHC-I, β1-integrin, VAMP3, and MT1MMP.

Uptake of EVs

So far, it has been proposed that the cells internalize EVs either by fusion with the plasma membrane or via endocytosis(Mulcahy et al., 2014). Uptake via endocytosis can be categorized into the different types of endocytotic processes, including clathrin-mediated endocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, macropinocytosis, and phagocytosis. The uptake mode of EVs may be dependent on the type of cell and its physiologic state, and whether ligands on the surface of the EV recognize receptors on the surface of the cell or vice-versa. Different mechanisms of internalization have been described for different cell types. For example, clathrin-dependent endocytosis or phagocytosis in neurons, macropinocytosis by microglia, phagocytosis or receptor-mediated endocytosis by dendritic cells, caveolin-mediated endocytosis in epithelial cells, and cholesterol- and lipid raft-dependent endocytosis in tumor cells(Barres et al., 2010; Feng et al., 2010; Fitzner et al., 2011; Fruhbeis et al., 2013; Montecalvo et al., 2012; Morelli et al., 2004; Nanbo et al., 2013; Svensson et al., 2013).

The mode of EV interaction with and/or entry into cells determines their functional effects. The EV membrane surface can trigger signaling through interaction with receptors/ligands on the cell surface without EV entry (Al-Nedawi et al., 2008; Cossetti et al., 2014; Patel et al., 2016). In many cases functionality of the EV contents depends on entry into the cytoplasm, and potentially even into the nucleus. Direct entry into the cytoplasm can be achieved by fusion of EVs to the plasma membrane of the recipient cells, but some form or endocytosis seems to be the most common mode of entry(Mulcahy et al., 2014). If the EVs enter by endocytosis, their cargo must exit that inherently degradative pathway, as endosomes mature into lysosomes, or be ejected out again through the MVB-plasma membrane fusion pathway. There must be a way through this maze, as so far, the functional transfer of nucleic acids has been described both in

culture as well as *in vivo* (Lai et al., 2015; Pegtel et al., 2010; Ridder et al., 2014). The mechanism of effective transfer out of the endosomal compartment is still unclear. This process has been visualized using fluorescent probes labeling EVs in tumor and dendritic cells(Montecalvo et al., 2012; Parolini et al., 2009). A different approach utilized luciferin-loaded EVs internalization into cytosol containing luciferase which allowed monitoring of the fate of the cargo(Abrami et al., 2013). To conclude, different cell types are able to take up EV using various mechanisms resulting in either functional transfer of cargo or degradation of the EV content. The fate may be determined by cell specific ligands/receptors that "direct the conversation".

Outline of this thesis

This thesis will focus on the interaction between glioma and innate immune cells, including microglia and infiltrating monocytes and macrophages. Here, the main focus is on the intercellular communication from tumor to innate immune cells through EVs. Various reports have shown that miRNA is one of the most abundant RNA species found in EVs. These RNA molecules are potent regulators and involved in maintaining cellular homeostasis and when dysregulated play a major role in pathology, such as oncogenesis. In **chapter 2**, the effect of extracellular miRNA transfer is determined together with the imaging of this exchange *in vitro* and *in vivo*. Here, a reporter to fluorescently label EV continuously shed by tumor cells was used to show EV uptake by microglia *in vivo*. In **chapter 3**, the focus is on the *in vivo* extracellular miRNA transfer of miR-21 from glioma to microglia. This consist of examining the uptake of fluorescently labeled glioma EVs by microglia in miR-21 null mice determined by FACS in combination with mRNA sequencing. A similar *in vivo* model is used to investigate the overall gene expression changes microglia undergo in the presence of a glioma. In **chapter 4** these transcriptome changes occurring in microglia exposed to glioma and glioma EVs studied in a glioma murine model will be discussed. This is extended to the changes in gene expression upon exposure to a glioma and the subsequent EV uptake by infiltrating monocytes and macrophages in comparison to circulating monocytes, as will be discussed in **chapter 5**. In **chapter 6**, a comprehensive analysis of publicly available microglia specific RNAseq data is used to identify a core set of gene in the microglia sensome which is shared between species. This finding is important to be able to translate the changes we have detected in murine models to humans. In conclusion, the limitation and future prospective for EV research will be presented in **the discussion**

References

Abrami, L., Brandi, L., Moayeri, M., Brown, M.J., Krantz, B.A., Leppla, S.H., and van der Goot, F.G. (2013). Hijacking multivesicular bodies enables long-term and exosome-mediated long-distance action of anthrax toxin. Cell Rep *5*, 986-996.

Ajami, B., Bennett, J.L., Krieger, C., Tetzlaff, W., and Rossi, F.M.V. (2007). Local self-renewal can sustain CNS microglia maintenance and function throughout adult life. Nature Neuroscience *10*, 1538-1543.

Akers, J.C., Gonda, D., Kim, R., Carter, B.S., and Chen, C.C. (2013). Biogenesis of extracellular vesicles (EV): exosomes, microvesicles, retrovirus-like vesicles, and apoptotic bodies. J Neurooncol *113*, 1-11.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., and Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. Nat Cell Biol *10*, 619-624.

Alieva, M., Margarido, A.S., Wieles, T., Abels, E.R., Colak, B., Boquetale, C., Jan Noordmans, H., Snijders, T.J., Broekman, M.L., and van Rheenen, J. (2017). Preventing inflammation inhibits biopsy-mediated changes in tumor cell behavior. Sci Rep *7*, 7529-7514.

Alonso, R., Mazzeo, C., Merida, I., and Izquierdo, M. (2007). A new role of diacylglycerol kinase alpha on the secretion of lethal exosomes bearing Fas ligand during activation-induced cell death of T lymphocytes. Biochimie *89*, 213-221.

Alonso, R., Mazzeo, C., Rodriguez, M.C., Marsh, M., Fraile-Ramos, A., Calvo, V., Avila-Flores, A., Merida, I., and Izquierdo, M. (2011). Diacylglycerol kinase alpha regulates the formation and polarisation of mature multivesicular bodies involved in the secretion of Fas ligand-containing exosomes in T lymphocytes. Cell Death Differ *18*, 1161-1173.

Aspelund, A., Antila, S., Proulx, S.T., Karlsen, T.V., Karaman, S., Detmar, M., Wiig, H., and Alitalo, K. (2015). A dural lymphatic vascular system that drains brain interstitial fluid and macromolecules. The Journal of experimental medicine *212*, 991-999.

Babst, M., Katzmann, D.J., Estepa-Sabal, E.J., Meerloo, T., and Emr, S.D. (2002). Escrt-III: an endosome-associated heterooligomeric protein complex required for mvb sorting. Dev Cell *3*, 271-282.

Bache, K.G., Brech, A., Mehlum, A., and Stenmark, H. (2003). Hrs regulates multivesicular body formation via ESCRT recruitment to endosomes. J Cell Biol *162*, 435-442.

Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E.*, et al.* (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. Nat Cell Biol *14*, 677-685.

Baj-Krzyworzeka, M., Szatanek, R., Weglarczyk, K., Baran, J., Urbanowicz, B., Branski, P., Ratajczak, M.Z., and Zembala, M. (2006). Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes. Cancer Immunol Immunother *55*, 808-818.

Balaj, L., Lessard, R., Dai, L., Cho, Y.-J., Pomeroy, S.L., Breakefield, X.O., and Skog, J. (2011). Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. Nature Communications *2*, 180.

Balça-Silva, J., Matias, D., Dubois, L.G., Carneiro, B., do Carmo, A., Girão, H., Ferreira, F., Ferrer, V.P., Chimelli, L., Filho, P.N.*, et al.* (2017). The Expression of Connexins and SOX2 Reflects the Plasticity of Glioma Stem-Like Cells. Transl Oncol *10*, 555-569.

Barres, C., Blanc, L., Bette-Bobillo, P., Andre, S., Mamoun, R., Gabius, H.J., and Vidal, M. (2010). Galectin-5 is bound onto the surface of rat reticulocyte exosomes and modulates vesicle uptake by macrophages. Blood *115*, 696-705.

Barteneva, N.S., Maltsev, N., and Vorobjev, I.A. (2013). Microvesicles and intercellular communication in the context of parasitism. Front Cell Infect Microbiol *3*, 49.

Boussiotis, V.A., and Charest, A. (2018). Immunotherapies for malignant glioma. Oncogene *37*, 1121-1141.

Bowman, R.L., Klemm, F., Akkari, L., Pyonteck, S.M., Sevenich, L., Quail, D.F., Dhara, S., Simpson, K., Gardner, E.E., Iacobuzio-Donahue, C.A.*, et al.* (2016). Macrophage Ontogeny Underlies Differences in Tumor-Specific Edu-

cation in Brain Malignancies. Cell reports *17*, 2445-2459.

Brandenburg, S., Müller, A., Turkowski, K., Radev, Y.T., Rot, S., Schmidt, C., Bungert, A.D., Acker, G., Schorr, A., Hippe, A.*, et al.* (2016). Resident microglia rather than peripheral macrophages promote vascularization in brain tumors and are source of alternative pro-angiogenic factors. Acta Neuropathol *131*, 365-378.

Bucki, R., Bachelot-Loza, C., Zachowski, A., Giraud, F., and Sulpice, J.C. (1998). Calcium induces phospholipid redistribution and microvesicle release in human erythrocyte membranes by independent pathways. Biochemistry *37*, 15383-15391.

Chang, A.L., Miska, J., Wainwright, D.A., Dey, M., Rivetta, C.V., Yu, D., Kanojia, D., Pituch, K.C., Qiao, J., Pytel, P.*, et al.* (2016). CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells. Cancer Res *76*, 5671-5682.

Chen, X., Zhang, L., Zhang, I.Y., Liang, J., Wang, H., Ouyang, M., Wu, S., da Fonseca, A.C.C., Weng, L., Yamamoto, Y.*, et al.* (2014). RAGE expression in tumor-associated macrophages promotes angiogenesis in glioma. Cancer Res *74*, 7285-7297.

Chen, Z., Feng, X., Herting, C.J., Garcia, V.A., Nie, K., Pong, W.W., Rasmussen, R., Dwivedi, B., Seby, S., Wolf, S.A.*, et al.* (2017). Cellular and Molecular Identity of Tumor-Associated Macrophages in Glioblastoma. Cancer Res *77*, 2266-2278.

Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. Trends Cell Biol *19*, 43-51.

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L.F., Thery, C., and Raposo, G. (2013). Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. J Cell Sci *126*, 5553-5565.

Cossetti, C., Iraci, N., Mercer, T.R., Leonardi, T., Alpi, E., Drago, D., Alfaro-Cervello, C., Saini, H.K., Davis, M.P., Schaeffer, J.*, et al.* (2014). Extracellular vesicles from neural stem cells transfer IFN-gamma via Ifngr1 to activate Stat1 signaling in target cells. Mol Cell *56*, 193-204.

D'Asti, E., Chennakrishnaiah, S., Lee, T.H., and Rak, J. (2016). Extracellular Vesicles in Brain Tumor Progression. Cell Mol Neurobiol *36*, 383-407.

de Vrij, J., Maas, S.L., Kwappenberg, K.M., Schnoor, R., Kleijn, A., Dekker, L., Luider, T.M., de Witte, L.D., Litjens, M., van Strien, M.E.*, et al.* (2015). Glioblastoma-derived extracellular vesicles modify the phenotype of monocytic cells. Int J Cancer *137*, 1630-1642.

Denzer, K., Kleijmeer, M.J., Heijnen, H.F., Stoorvogel, W., and Geuze, H.J. (2000). Exosome: from internal vesicle of the multivesicular body to intercellular signaling device. J Cell Sci *113 Pt 19*, 3365-3374.

Du, R., Petritsch, C., Lu, K., Liu, P., Haller, A., Ganss, R., Song, H., Vandenberg, S., and Bergers, G. (2008). Matrix metalloproteinase-2 regulates vascular patterning and growth affecting tumor cell survival and invasion in GBM. Neuro-oncology *10*, 254-264.

Fader, C.M., Sanchez, D.G., Mestre, M.B., and Colombo, M.I. (2009). TI-VAMP/VAMP7 and VAMP3/cellubrevin: two v-SNARE proteins involved in specific steps of the autophagy/multivesicular body pathways. Biochim Biophys Acta *1793*, 1901-1916.

Feng, D., Zhao, W.L., Ye, Y.Y., Bai, X.C., Liu, R.Q., Chang, L.F., Zhou, Q., and Sui, S.F. (2010). Cellular internalization of exosomes occurs through phagocytosis. Traffic *11*, 675-687.

Fernandez-Borja, M., Wubbolts, R., Calafat, J., Janssen, H., Divecha, N., Dusseljee, S., and Neefjes, J. (1999). Multivesicular body morphogenesis requires phosphatidyl-inositol 3-kinase activity. Curr Biol *9*, 55-58.

Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hanisch, U.-K., and Simons, M. (2011). Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J Cell Sci *124*, 447-458.

Fonseca, P., Vardaki, I., Occhionero, A., and Panaretakis, T. (2016). Metabolic and Signaling Functions of Cancer Cell-Derived Extracellular Vesicles. Int Rev Cell Mol Biol *326*, 175-199.

Fruhbeis, C., Frohlich, D., and Kramer-Albers, E.M. (2012). Emerging roles of exosomes in neuron-glia communi-

cation. Front Physiol *3*, 119.

Fruhbeis, C., Frohlich, D., Kuo, W.P., Amphornrat, J., Thilemann, S., Saab, A.S., Kirchhoff, F., Mobius, W., Goebbels, S., Nave, K.A.*, et al.* (2013). Neurotransmitter-triggered transfer of exosomes mediates oligodendrocyte-neuron communication. PLoS Biol *11*, e1001604.

Gabrusiewicz, K., Ellert-Miklaszewska, A., Lipko, M., Sielska, M., Frankowska, M., and Kaminska, B. (2011). Characteristics of the alternative phenotype of microglia/macrophages and its modulation in experimental gliomas. PloS one *6*, e23902.

Ginhoux, F., Greter, M., Leboeuf, M., Nandi, S., See, P., Gokhan, S., Mehler, M.F., Conway, S.J., Ng, L.G., Stanley, E.R.*, et al.* (2010). Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science *330*, 841-845.

Gould, S.J., Booth, A.M., and Hildreth, J.E. (2003). The Trojan exosome hypothesis. Proc Natl Acad Sci U S A *100*, 10592-10597.

Gould, S.J., and Raposo, G. (2013). As we wait: coping with an imperfect nomenclature for extracellular vesicles. J Extracell Vesicles *2*.

Grant, B.D., and Donaldson, J.G. (2009). Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol *10*, 597-608.

Gyorgy, B., Szabo, T.G., Pasztoi, M., Pal, Z., Misjak, P., Aradi, B., Laszlo, V., Pallinger, E., Pap, E., Kittel, A.*, et al.* (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. Cell Mol Life Sci *68*, 2667-2688.

Hambardzumyan, D., Gutmann, D.H., and Kettenmann, H. (2016). The role of microglia and macrophages in glioma maintenance and progression. Nature Neuroscience *19*, 20-27.

Henderson, M.C., and Azorsa, D.O. (2012). The genomic and proteomic content of cancer cell-derived exosomes. Front Oncol *2*, 38.

Henne, W.M., Buchkovich, N.J., and Emr, S.D. (2011). The ESCRT pathway. Dev Cell *21*, 77-91.

Henne, W.M., Stenmark, H., and Emr, S.D. (2013). Molecular mechanisms of the membrane sculpting ESCRT pathway. Cold Spring Harb Perspect Biol *5*.

Hickman, S.E., Kingery, N.D., Ohsumi, T.K., Borowsky, M.L., Wang, L.-c., Means, T.K., and El Khoury, J. (2013). The microglial sensome revealed by direct RNA sequencing. Nature Neuroscience *16*, 1896-1905.

Hong, X., Sin, W.C., Harris, A.L., and Naus, C.C. (2015). Gap junctions modulate glioma invasion by direct transfer of microRNA. Oncotarget *6*, 15566-15577.

Hsu, C., Morohashi, Y., Yoshimura, S., Manrique-Hoyos, N., Jung, S., Lauterbach, M.A., Bakhti, M., Gronborg, M., Mobius, W., Rhee, J.*, et al.* (2010). Regulation of exosome secretion by Rab35 and its GTPase-activating proteins TBC1D10A-C. J Cell Biol *189*, 223-232.

Hu, F., Dzaye, O.D., Hahn, A., Yu, Y., Scavetta, R.J., Dittmar, G., Kaczmarek, A.K., Dunning, K.R., Ricciardelli, C., Rinnenthal, J.L.*, et al.* (2015). Glioma-derived versican promotes tumor expansion via glioma-associated microglial/macrophages Toll-like receptor 2 signaling. Neuro-oncology *17*, 200-210.

Jhaveri, N., Chen, T.C., and Hofman, F.M. (2016). Tumor vasculature and glioma stem cells: Contributions to glioma progression. Cancer Lett *380*, 545-551.

Katzmann, D.J., Babst, M., and Emr, S.D. (2001). Ubiquitin-dependent sorting into the multivesicular body pathway requires the function of a conserved endosomal protein sorting complex, ESCRT-I. Cell *106*, 145-155.

Kessenbrock, K., Plaks, V., and Werb, Z. (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell *141*, 52-67.

Kim, C.C., Nakamura, M.C., and Hsieh, C.L. (2016). Brain trauma elicits non-canonical macrophage activation states. J Neuroinflammation *13*, 117.

Lai, C.P., Kim, E.Y., Badr, C.E., Weissleder, R., Mempel, T.R., Tannous, B.A., and Breakefield, X.O. (2015). Visualization and tracking of tumour extracellular vesicle delivery and RNA translation using multiplexed reporters. Nature Communications *6*, 7029.

Lai, C.P., Mardini, O., Ericsson, M., Prabhakar, S., Maguire, C.A., Chen, J.W., Tannous, B.A., and Breakefield, X.O. (2014). Dynamic Biodistribution of Extracellular Vesicles in Vivo Using a Multimodal Imaging Reporter. ACS Nano *8*, 483-494.

Laulagnier, K., Grand, D., Dujardin, A., Hamdi, S., Vincent-Schneider, H., Lankar, D., Salles, J.P., Bonnerot, C., Perret, B., and Record, M. (2004). PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. FEBS Lett *572*, 11-14.

Li, W., and Graeber, M.B. (2012). The molecular profile of microglia under the influence of glioma. Neuro-oncology *14*, 958-978.

Logan, M.R., Lacy, P., Odemuyiwa, S.O., Steward, M., Davoine, F., Kita, H., and Moqbel, R. (2006). A critical role for vesicle-associated membrane protein-7 in exocytosis from human eosinophils and neutrophils. Allergy *61*, 777-784.

Lotvall, J., Hill, A.F., Hochberg, F., Buzas, E.I., Di Vizio, D., Gardiner, C., Gho, Y.S., Kurochkin, I.V., Mathivanan, S., Quesenberry, P.*, et al.* (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. J Extracell Vesicles *3*, 26913.

Louveau, A., Smirnov, I., Keyes, T.J., Eccles, J.D., Rouhani, S.J., Peske, J.D., Derecki, N.C., Castle, D., Mandell, J.W., Lee, K.S.*, et al.* (2015). Structural and functional features of central nervous system lymphatic vessels. Nature *523*, 337-341.

Luga, V., Zhang, L., Viloria-Petit, A.M., Ogunjimi, A.A., Inanlou, M.R., Chiu, E., Buchanan, M., Hosein, A.N., Basik, M., and Wrana, J.L. (2012). Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. Cell *151*, 1542-1556.

Maas, S.L.N., Breakefield, X.O., and Weaver, A.M. (2017). Extracellular Vesicles: Unique Intercellular Delivery Vehicles. Trends in Cell Biology *27*, 172-188.

Marcilla, A., Trelis, M., Cortes, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L., Sanchez del Pino, M.M., Munoz-Antoli, C., Toledo, R.*, et al.* (2012). Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS One *7*, e45974.

Mathieu, M., Martin-Jaular, L., Lavieu, G., and Théry, C. (2018). Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. Nat Cell Biol, 1-9.

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Faure, J., Blanc, N.S., Matile, S., Dubochet, J., Sadoul, R.*, et al.* (2004). Role of LBPA and Alix in multivesicular liposome formation and endosome organization. Science *303*, 531-534.

McCullough, J., Fisher, R.D., Whitby, F.G., Sundquist, W.I., and Hill, C.P. (2008). ALIX-CHMP4 interactions in the human ESCRT pathway. Proc Natl Acad Sci U S A *105*, 7687-7691.

Minciacchi, V.R., Freeman, M.R., and Di Vizio, D. (2015). Extracellular vesicles in cancer: exosomes, microvesicles and the emerging role of large oncosomes. Seminars in Cell & Developmental Biology *40*, 41-51.

Momen-Heravi, F., Balaj, L., Alian, S., Tigges, J., Toxavidis, V., Ericsson, M., Distel, R.J., Ivanov, A.R., Skog, J., and Kuo, W.P. (2012). Alternative methods for characterization of extracellular vesicles. Front Physiol *3*, 354.

Montecalvo, A., Larregina, A.T., Shufesky, W.J., Stolz, D.B., Sullivan, M.L., Karlsson, J.M., Baty, C.J., Gibson, G.A., Erdos, G., Wang, Z.*, et al.* (2012). Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. Blood *119*, 756-766.

Morelli, A.E., Larregina, A.T., Shufesky, W.J., Sullivan, M.L., Stolz, D.B., Papworth, G.D., Zahorchak, A.F., Logar, A.J., Wang, Z., Watkins, S.C.*, et al.* (2004). Endocytosis, intracellular sorting, and processing of exosomes by dendritic cells. Blood *104*, 3257-3266.

Mulcahy, L.A., Pink, R.C., and Carter, D.R. (2014). Routes and mechanisms of extracellular vesicle uptake. J Extracell Vesicles *3*.

Müller, A., Brandenburg, S., Turkowski, K., Müller, S., and Vajkoczy, P. (2015). Resident microglia, and not peripheral macrophages, are the main source of brain tumor mononuclear cells. International journal of cancer *137*,

278-288.

Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., and D'Souza-Schorey, C. (2009). ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. Curr Biol *19*, 1875-1885.

Nabhan, J.F., Hu, R., Oh, R.S., Cohen, S.N., and Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proc Natl Acad Sci U S A *109*, 4146-4151.

Nanbo, A., Kawanishi, E., Yoshida, R., and Yoshiyama, H. (2013). Exosomes derived from Epstein-Barr virus-infected cells are internalized via caveola-dependent endocytosis and promote phenotypic modulation in target cells. J Virol *87*, 10334-10347.

Nijaguna, M.B., Patil, V., Urbach, S., Shwetha, S.D., Sravani, K., Hegde, A.S., Chandramouli, B.A., Arivazhagan, A., Marin, P., Santosh, V*., et al.* (2015). Glioblastoma-derived Macrophage Colony-stimulating Factor (MCSF) Induces Microglial Release of Insulin-like Growth Factor-binding Protein 1 (IGFBP1) to Promote Angiogenesis. J Biol Chem *290*, 23401-23415.

Okolie, O., Bago, J.R., Schmid, R.S., Irvin, D.M., Bash, R.E., Miller, C.R., and Hingtgen, S.D. (2016). Reactive astrocytes potentiate tumor aggressiveness in a murine glioma resection and recurrence model. Neuro-oncology *18*, 1622-1633.

Osswald, M., Jung, E., Sahm, F., Solecki, G., Venkataramani, V., Blaes, J., Weil, S., Horstmann, H., Wiestler, B., Syed, M*., et al.* (2015). Brain tumour cells interconnect to a functional and resistant network. Nature *528*, 93-98.

Ostrowski, M., Carmo, N.B., Krumeich, S., Fanget, I., Raposo, G., Savina, A., Moita, C.F., Schauer, K., Hume, A.N., Freitas, R.P*., et al.* (2010). Rab27a and Rab27b control different steps of the exosome secretion pathway. Nat Cell Biol *12*, 19-30; sup pp 11-13.

Pan, B.T., and Johnstone, R.M. (1983). Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. Cell *33*, 967-978.

Parolini, I., Federici, C., Raggi, C., Lugini, L., Palleschi, S., De Milito, A., Coscia, C., Iessi, E., Logozzi, M., Molinari, A*., et al.* (2009). Microenvironmental pH is a key factor for exosome traffic in tumor cells. J Biol Chem *284*, 34211-34222.

Pasquet, J.M., Dachary-Prigent, J., and Nurden, A.T. (1996). Calcium influx is a determining factor of calpain activation and microparticle formation in platelets. Eur J Biochem *239*, 647-654.

Patel, B., Patel, J., Cho, J.H., Manne, S., Bonala, S., Henske, E., Roegiers, F., Markiewski, M., and Karbowniczek, M. (2016). Exosomes mediate the acquisition of the disease phenotypes by cells with normal genome in tuberous sclerosis complex. Oncogene *35*, 3027-3036.

Pegtel, D.M., Cosmopoulos, K., Thorley-Lawson, D.A., van Eijndhoven, M.A., Hopmans, E.S., Lindenberg, J.L., de Gruijl, T.D., Wurdinger, T., and Middeldorp, J.M. (2010). Functional delivery of viral miRNAs via exosomes. Proc Natl Acad Sci U S A *107*, 6328-6333.

Pencheva, N., de Gooijer, M.C., Vis, D.J., Wessels, L.F.A., Würdinger, T., van Tellingen, O., and Bernards, R. (2017). Identification of a Druggable Pathway Controlling Glioblastoma Invasiveness. Cell reports *20*, 48-60.

Puri, N., and Roche, P.A. (2008). Mast cells possess distinct secretory granule subsets whose exocytosis is regulated by different SNARE isoforms. Proc Natl Acad Sci U S A *105*, 2580-2585.

Raiborg, C., and Stenmark, H. (2009). The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. Nature *458*, 445-452.

Ransohoff, R.M. (2016). A polarizing question: do M1 and M2 microglia exist? Nature Neuroscience *19*, 987-991.

Rao, S.K., Huynh, C., Proux-Gillardeaux, V., Galli, T., and Andrews, N.W. (2004). Identification of SNAREs involved in synaptotagmin VII-regulated lysosomal exocytosis. J Biol Chem *279*, 20471-20479.

Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol *200*, 373-383.

Ratajczak, J., Miekus, K., Kucia, M., Zhang, J., Reca, R., Dvorak, P., and Ratajczak, M.Z. (2006). Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and

protein delivery. Leukemia *20*, 847-856.
Razi, M., and Futter, C.E. (2006). Distinct roles for Tsg101 and Hrs in multivesicular body formation and inward vesiculation. Mol Biol Cell *17*, 3469-3483.
Redzic, J.S., Balaj, L., van der Vos, K.E., and Breakefield, X.O. (2014). Extracellular RNA mediates and marks cancer progression. Semin Cancer Biol *28*, 14-23.
Regev-Rudzki, N., Wilson, D.W., Carvalho, T.G., Sisquella, X., Coleman, B.M., Rug, M., Bursac, D., Angrisano, F., Gee, M., Hill, A.F.*, et al.* (2013). Cell-cell communication between malaria-infected red blood cells via exosome-like vesicles. Cell *153*, 1120-1133.
Ridder, K., Keller, S., Dams, M., Rupp, A.K., Schlaudraff, J., Del Turco, D., Starmann, J., Macas, J., Karpova, D., Devraj, K.*, et al.* (2014). Extracellular vesicle-mediated transfer of genetic information between the hematopoietic system and the brain in response to inflammation. PLoS Biol *12*, e1001874.
Rilla, K., Pasonen-Seppanen, S., Deen, A.J., Koistinen, V.V., Wojciechowski, S., Oikari, S., Karna, R., Bart, G., Torronen, K., Tammi, R.H.*, et al.* (2013). Hyaluronan production enhances shedding of plasma membrane-derived microvesicles. Exp Cell Res *319*, 2006-2018.
Rilla, K., Siiskonen, H., Tammi, M., and Tammi, R. (2014). Hyaluronan-coated extracellular vesicles--a novel link between hyaluronan and cancer. Adv Cancer Res *123*, 121-148.
Roesch, S., Rapp, C., Dettling, S., and Herold-Mende, C. (2018). When Immune Cells Turn Bad-Tumor-Associated Microglia/Macrophages in Glioma. Int J Mol Sci *19*, 436.
Savina, A., Furlan, M., Vidal, M., and Colombo, M.I. (2003). Exosome release is regulated by a calcium-dependent mechanism in K562 cells. J Biol Chem *278*, 20083-20090.
Shields, S.B., Oestreich, A.J., Winistorfer, S., Nguyen, D., Payne, J.A., Katzmann, D.J., and Piper, R. (2009). ESCRT ubiquitin-binding domains function cooperatively during MVB cargo sorting. J Cell Biol *185*, 213-224.
Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T., Carter, B.S., Krichevsky, A.M., and Breakefield, X.O. (2008). Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. Nat Cell Biol *10*, 1470-1476.
Stenmark, H. (2009). Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol *10*, 513-525.
Stoorvogel, W., Strous, G.J., Geuze, H.J., Oorschot, V., and Schwartz, A.L. (1991). Late endosomes derive from early endosomes by maturation. Cell *65*, 417-427.
Stupp, R., Hegi, M.E., Mason, W.P., van den Bent, M.J., Taphoorn, M.J.B., Janzer, R.C., Ludwin, S.K., Allgeier, A., Fisher, B., Belanger, K.*, et al.* (2009). Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. Lancet Oncol *10*, 459-466.
Svensson, K.J., Christianson, H.C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L.M., Morgelin, M., and Belting, M. (2013). Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid Raft-mediated endocytosis negatively regulated by caveolin-1. J Biol Chem *288*, 17713-17724.
Szulzewsky, F., Pelz, A., Feng, X., Synowitz, M., Markovic, D., Langmann, T., Holtman, I.R., Wang, X., Eggen, B.J.L., Boddeke, H.W.G.M.*, et al.* (2015). Glioma-associated microglia/macrophages display an expression profile different from M1 and M2 polarization and highly express Gpnmb and Spp1. PloS one *10*, e0116644.
Tamai, K., Tanaka, N., Nakano, T., Kakazu, E., Kondo, Y., Inoue, J., Shiina, M., Fukushima, K., Hoshino, T., Sano, K.*, et al.* (2010). Exosome secretion of dendritic cells is regulated by Hrs, an ESCRT-0 protein. Biochem Biophys Res Commun *399*, 384-390.
Tauro, B.J., Greening, D.W., Mathias, R.A., Ji, H., Mathivanan, S., Scott, A.M., and Simpson, R.J. (2012). Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. Methods *56*, 293-304.
Thery, C., Ostrowski, M., and Segura, E. (2009). Membrane vesicles as conveyors of immune responses. Nat Rev Immunol *9*, 581-593.
Théry, C., Witwer, K.W., Aikawa, E., Alcaraz, M.J., Anderson, J.D., Andriantsitohaina, R., Antoniou, A., Arab, T.,

Archer, F., Atkin-Smith, G.K., *et al.* (2018). Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. J Extracell Vesicles *7*, 1535750.

Thery, C., Zitvogel, L., and Amigorena, S. (2002). Exosomes: composition, biogenesis and function. Nat Rev Immunol *2*, 569-579.

Thuringer, D., Boucher, J., Jego, G., Pernet, N., Cronier, L., Hammann, A., Solary, E., and Garrido, C. (2016). Transfer of functional microRNAs between glioblastoma and microvascular endothelial cells through gap junctions. Oncotarget *7*, 73925-73934.

Tiwari, N., Wang, C.C., Brochetta, C., Ke, G., Vita, F., Qi, Z., Rivera, J., Soranzo, M.R., Zabucchi, G., Hong, W., *et al.* (2008). VAMP-8 segregates mast cell-preformed mediator exocytosis from cytokine trafficking pathways. Blood *111*, 3665-3674.

Tkach, M., and Théry, C. (2016). Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. Cell *164*, 1226-1232.

van der Vos, K.E., Abels, E.R., Zhang, X., Lai, C., Carrizosa, E., Oakley, D., Prabhakar, S., Mardini, O., Crommentuijn, M.H.W., Skog, J., *et al.* (2016). Directly visualized glioblastoma-derived extracellular vesicles transfer RNA to microglia/macrophages in the brain. Neuro-oncology *18*, 58-69.

Vignais, M.-L., Caicedo, A., Brondello, J.-M., and Jorgensen, C. (2017). Cell Connections by Tunneling Nanotubes: Effects of Mitochondrial Trafficking on Target Cell Metabolism, Homeostasis, and Response to Therapy. Stem Cells Int *2017*, 6917941-6917914.

Wang, T., Gilkes, D.M., Takano, N., Xiang, L., Luo, W., Bishop, C.J., Chaturvedi, P., Green, J.J., and Semenza, G.L. (2014). Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. Proc Natl Acad Sci U S A *111*, E3234-3242.

Wang, X., Veruki, M.L., Bukoreshtliev, N.V., Hartveit, E., and Gerdes, H.-H. (2010). Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. Proceedings of the National Academy of Sciences of the United States of America *107*, 17194-17199.

Weil, S., Osswald, M., Solecki, G., Grosch, J., Jung, E., Lemke, D., Ratliff, M., Hänggi, D., Wick, W., and Winkler, F. (2017). Tumor microtubes convey resistance to surgical lesions and chemotherapy in gliomas. Neuro-oncology *19*, 1316-1326.

Witwer, K.W., Buzas, E.I., Bemis, L.T., Bora, A., Lasser, C., Lotvall, J., Nolte-'t Hoen, E.N., Piper, M.G., Sivaraman, S., Skog, J., *et al.* (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. J Extracell Vesicles *2*.

Wollert, T., and Hurley, J.H. (2010). Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature *464*, 864-869.

Wurdinger, T., Deumelandt, K., van der Vliet, H.J., Wesseling, P., and de Gruijl, T.D. (2014). Mechanisms of intimate and long-distance cross-talk between glioma and myeloid cells: how to break a vicious cycle. Biochim Biophys Acta *1846*, 560-575.

Xue, J., Schmidt, S.V., Sander, J., Draffehn, A., Krebs, W., Quester, I., De Nardo, D., Gohel, T.D., Emde, M., Schmidleithner, L., *et al.* (2014). Transcriptome-based network analysis reveals a spectrum model of human macrophage activation. Immunity *40*, 274-288.

Zhou, W., Ke, S.Q., Huang, Z., Flavahan, W., Fang, X., Paul, J., Wu, L., Sloan, A.E., McLendon, R.E., Li, X., *et al.* (2015). Periostin secreted by glioblastoma stem cells recruits M2 tumour-associated macrophages and promotes malignant growth. Nat Cell Biol *17*, 170-182.