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Using microglia-derived extracellular vesicles to capture diversity of microglial activation phenotypes following neurological injury

Austyn D. Roseborough, Nikita Ollen-Bittle, Shawn N. Whitehead*

Microglia are one of the three glial cell populations in the central nervous system (CNS), along with astrocytes and oligodendrocytes. While microglia are unique among brain cells due to their hematologic origin and perform immune functions similar to peripheral macrophages, they are not simply macrophages of the CNS. Microglia are crucial for many brain-specific functions such as synaptic pruning, facilitation of myelin turnover and communication with both neurons and astrocytes (Saijo and Glass, 2011). They are a highly active cell type, constantly surveying their environment and responding to both exogenous and endogenous danger signals which can include reactive oxygen species, cytosolic DNA, heat shock proteins, plasma proteins and microbial components.

Microglia are particularly dynamic in the setting of acute neurological injury. In response to stroke, hemorrhage, or traumatic brain injury, their adaptive nature allows them to shift between a variety of phenotypes. At any given time, the microglia population in the brain is a heterogeneous collection of phenotypes that vary by region, tissue type (gray matter *vs*. white matter) and proximity to pathology (Dubbelaar et al., 2018). Our understanding of microglia diversity is constantly evolving thanks to advances in proteomic and single-cell RNA sequencing techniques. Well-defined microglial phenotypes, including damage-associated microglia, white matter aging microglia and pro-inflammatory microglia (among others), are differentiated by cell surface markers, upregulation of signaling pathways, cytokine release and levels of phagocytic activity (Dubbelaar et al., 2018). More importantly, microglial phenotype in response to neurological injury is highly context dependent and varies with age, sex, brain region, history of prior neurological injury, and chronicity of the current insult.

Unfortunately, microglia activity is not uniformly beneficial to brain homeostasis. In response to acute injury, microglia initially work to clear debris from the injury site and promote tissue repair. However, when microglial activity becomes dysregulated, it has detrimental effects on synaptic integrity and myelin degradation, induces neurotoxicity and promotes the prolongation of pro-inflammatory cytokine signaling. In studies of both stroke and traumatic brain injury, the maintenance of chronic (> 4 weeks) pro-inflammatory activity by microglia is associated with worse outcomes (Hu et al., 2012; Saba et al., 2021).

Even though the harmful nature of dysregulated microglia activity has been well reported, there remain significant limitations in our ability to modulate the microglia response to neurological injury. Firstly, we lack techniques for rapid and accurate measurement of microglial phenotypes *in vivo*. Secondly, therapeutic modulation of microglia has been largely unsuccessful. This is likely due to inaccurate timing of intervention and/or the inability to appropriately target the correct microglial phenotype. Studies attempting to measure and modulate microglia after stroke exemplify both limitations. Positron emission tomography imaging using ligands designed to bind microglia receptors is not cell specific and cannot adequately capture pro-inflammatory microglia, whilst attempts to deplete microglia populations after stroke have not improved outcomes (Jin et al., 2017; Al-Khishman et al., 2020). Broad depletion of the microglia population overlooks the beneficial roles that microglia play in the immediate stage after injury and future strategies should instead involve targeted manipulation of phenotype alone. Agents such as MCC950 have shown promise in targeting inflammasome activity, thereby affecting primarily pro-inflammatory microglia signaling (Ismael et al., 2018), but it remains difficult to determine the best time window for pharmacological intervention. Furthermore, since an individual's response to acute brain injury is highly variable, it is difficult to identify when chronic dysregulation of microglia signaling is present and therefore targetable.

To address these limitations, we propose the use of circulating microglia-derived extracellular vesicles (MEVs) as indicators of microglial phenotypes. Extracellular vesicles (EVs) are membrane-bound vesicles 40–1000 nm in diameter that are released from all cells in the body (Raposo and Stoorvogel, 2013). EVs encompass a variety of vesicles including but not limited to exosomes derived from the endo-lysosomal system, microvesicles (or ectosomes) that bud directly from the cell-surface membrane and apoptotic bodies which are remnants from dying cells (Raposo and Stoorvogel, 2013). Given that the subtypes of EVs are difficult to reliably distinguish from one another using commonly reported technical approaches, this article will refer to the EV population as a whole.

EVs are attractive biomarkers for several reasons: they are relatively stable and can be detected in biofluids, they cross the blood-brain barrier meaning that EVs being released from brain cells can be detected in the peripheral circulation, and they bear surface markers and carry cargo reflective of their cell of origin. This means that EVs released from microglia have surface markers that correspond to the phenotype of the cell and carry cargo that can be indicative of cell function. The EV cargo profile includes miRNA, mRNA, cytosolic proteins, cytokines, and extracellular proteins/debris phagocytosed by microglia.

With respect to neurological diseases, EVs released from neurons and astrocytes have been used for cell-specific biomarker development, primarily in studies of neurodegenerative diseases. Using antibodies against neural cell adhesion marker (neurons) or glial fibrillary acidic protein (astrocytes), EV subpopulations can be isolated and characterized in biofluids from a variety of disease populations or preclinical models. Unfortunately, MEV biomarker identification has lagged, because microglia do not express many cell-specific proteins in a stable manner and share significant expression profiles with peripheral macrophages. Although the heterogeneity of microglia makes EV identification challenging, it also represents an important opportunity to develop biomarkers specific to disease states. Our work and that of others has shown that surface proteins can be detected on MEVs, including TMEM119, CD14 and CD68, with expression levels that vary according to the phenotype of the cell (Drago et al., 2017; Roseborough et al., 2023a). Excitingly, these surface proteins can be used to detect MEVs in plasma using novel high throughput methods such as nanoflow cytometry or nanoimaging (Roseborough et al., 2023a). Using these approaches, we have reported that MEVs released from pro-inflammatory microglia can be detected and are elevated in circulating plasma after stroke in a preclinical model. Temporal profiling of post-stroke MEV release could be used to identify fluctuations of microglial phenotypes after injury and for more accurate pharmacological targeting. This highlights the need to further validate phenotype-specific MEV surface markers and to develop reliable tools for their measurement in biofluids.

While MEV surface markers are attractive targets for quantifying or imaging intact EVs, cargo can provide more biological information about the specific stimuli to which microglia are responding. Therefore, isolating MEVs and characterizing cargo using proteomics, sequencing, or other single molecule assays, can offer insight into cellular function in ways that previously would have only been possible with histopathology. For example, blood-brain barrier dysfunction is a common post-stroke sequela involving the extravasation of serum proteins such as fibrinogen into the brain parenchyma. This provokes a pro-inflammatory microglia response and the uptake of fibrinogen into microglia which can be confirmed immunohistochemically. Interestingly,

MEVs released from microglia after stroke reflect this blood-brain barrier damage and carry fibrinogen as cargo. Importantly, this cargo can be detected in circulating plasma MEVs, supporting their utility as peripheral biomarkers of microglia activity (Roseborough et al., 2023b). Other cargo that may be reflective of microglia activity in response to injury could include synaptic proteins, myelin associated proteins, plasma proteins and cytokines. miRNA molecules implicated in pro- or anti-inflammatory signaling cascades after injury have been the most widely studied MEV cargo and are known to vary based on cellular phenotype (Panaro et al., 2020). Future studies incorporating cargo analysis of MEVs following neurological injury will help better understand the microglia response and direct intervention.

Significant challenges remain in the implementation of EV-based biomarker approaches. The technology required to measure EVs is not widely available and consistent measurements across centers require protocol harmonization and technical proficiency. Cell specificity remains a challenge with any blood-based assay of EVs. The implementation of MEV biomarker profiles using multiple surface proteins in combination with cargo analysis is likely required to achieve specificity. If a MEV bears microglia markers that can also be expressed by peripheral cells, but carries CNS-derived cargo (synaptic proteins, myelin, etc.), our confidence in its microglia origin is increased. Despite these challenges, we believe that the benefits of MEVs outweigh their limitations. This includes the accessibility of MEV measurement in blood versus CSF-based assays, the long-term stability of MEVs when stored at –80°C, and the ability of MEVs to reflect CNS-specific inflammatory signaling. "Omics" approaches are needed to improve the identification of MEV profiles specific to age and disease states (**Figure 1**). Ideally, MEVs will be measured in combination with other populations of brain-derived EVs including neuronal, astrocytic, and brain-endothelial subpopulations.

In conclusion, MEVs represent an exciting opportunity to develop novel indicators of brain injury that can be used to select populations with aberrant microglia signaling, identify precise time points for intervention and implement phenotype-specific pharmacological approaches. While great strides have been made in our understanding of neuronal and astrocytic EVs in neurological diseases, studies on MEV have lagged. Future studies should continue to delineate MEV phenotypes while refining existing methods for their measurement to better enable their use in preclinical and clinical studies.

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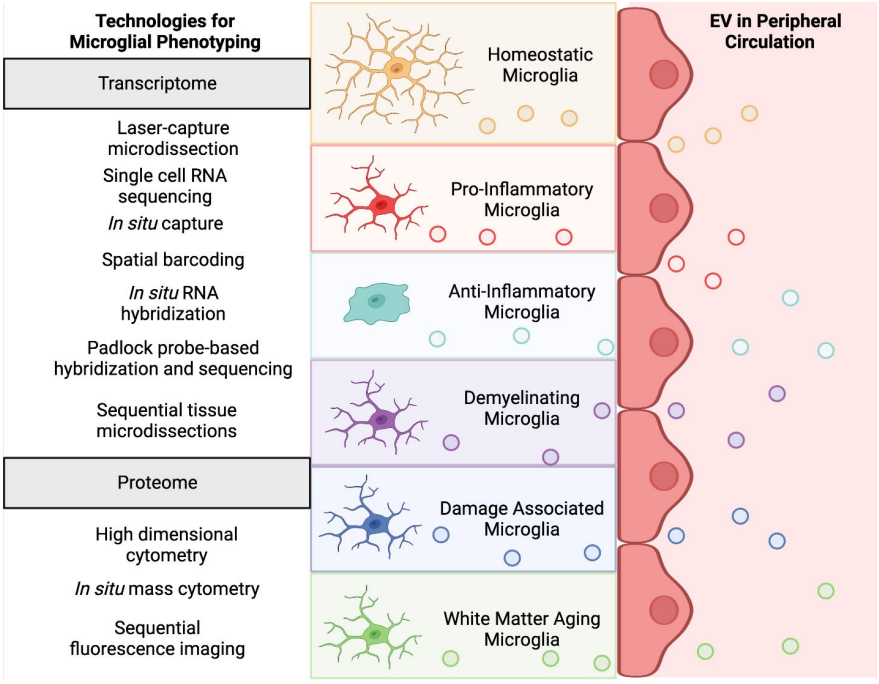

Figure 1 | **Approaches to the identification of novel MEV targets.**
Examples of transcriptomic and proteomic strategies are listed that can be used to define microglial phenotypes along with corresponding MEV profiles that can be tested as peripheral biomarkers. EV: Extracellular vesicles; MEV: microglia-derived extracellular vesicles. Created with BioRender.com.

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