Advances in Microglia Cellular Models: Focus on Extracellular Vesicle production

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Abstract

Microglia are the major component of the innate immune system in the central nervous system. They promote the maintenance of brain homeostasis as well as support inflammatory processes that are often related to pathological conditions such as neurodegenerative diseases. Depending on the stimulus received, microglia cells dynamically change their phenotype releasing specific soluble factors and largely modify the cargo of their secreted extracellular vesicles (EVs). Despite the mechanisms at the basis of microglia actions have not been completely clarified, the recognized functions exerted by their EVs in patho-physiological conditions represent the proof of the crucial role of these organelles in tuning cell-to-cell communication, promoting either protective or harmful effects. Consistently, *in vitro* cell models to better elucidate microglia EV production and mechanisms of their release have been increased in the last years. In this review, the main microglial cellular models that have been developed and validated will be described and discussed, with particular focus on those used to produce and derive EVs. The advantages and disadvantages of their use will be evidenced too. Finally, given the wide interest in applying EVs in diagnosis and therapy too, the heterogeneity of available models for producing microglia EVs is here underlined, to prompt a cross-check or comparison among them.

Introduction

Microglia is nowadays recognized as the tissue-resident population of macrophages of the CNS, where it constitutes the first line of defense from invading pathogens, endogenous misfolded proteins, and other harmful cells, either cancerous or just inflamed [1–4]. The origin of microglia during brain development has been elegantly fate-mapped in the developing mouse embryo, from the yolk sac-derived erythromyeloid progenitors to its migration into the neuroepithelium [5,6]. Since its first identification in 1919 [7], microglia have been studied almost exclusively in relation to pathological processes; in fact, it has been immediately recognized as a rapidly reacting kind of phagocytic cells in injuries site and, consequently, in brain pathology.

Although the research field on microglia has tremendously grown in the last years [8,9], there are still a lot of controversies about microglia function, state, and activity, especially regarding its role in inflammatory pathologies of the CNS. Classical examples of those pathologies are neurodegenerative diseases such as Alzheimer's Disease (AD), Parkinson's Disease (PD), and Multiple Sclerosis (MS).

One of the most exemplifying topics is probably the definition of the activation state of microglia from a ‘resting’ phenotype, which is to date quite controversial [10–13]. The original paradigm describes three distinct microglia states: M0, M1, and M2. The M0 phenotype refers to the homeostatic microglia as found in CNS during normal function [14–18]. The M1 phenotype is associated with high neurotoxicity and the development of neurodegenerative diseases, characterized by secretion of pro-inflammatory chemokines and cytokines such as TNF-α, IL-6, IL-1β [4]. Conversely, the polarization to the M2 phenotype is characterized by the secretion of IL-10 and TGF-β [4], which promote an anti-inflammatory and regenerative activity of microglia. Nonetheless, it is more and more accepted that this paradigm does not adequately recapitulate microglia activation *in vivo*. As a matter of fact, *in vivo* microglia rarely displays a significant bias toward either the M1 or M2 phenotype [4,14,19]. Interestingly, it has been reported that M2 shift is not always beneficial and M1 can also promote axonal regeneration [20]. Moreover, the M2 phenotype seems to be favored by aging as well [4,21], adding further evidence that the M1 and M2 states are not always easy to discriminate. The scenario is further complicated when translated *in vitro*, the obtainment of a distinct phenotype is challenging and cultured cells can be already in an activated state.

Historically, microglia phenotypes have been related to their morphological changes. The ramified and amoeboid morphologies have been often referred to as ‘resting’ and ‘activated/reactive’ microglia respectively, it has become clear, however, that this nomenclature is quite deviating and the term

'activation' refers to a shift between activated states rather than to an awakening [8,9,22]. Thus, it is evident that associating a molecular phenotype (M0, M1, or M2) to a precise morphological state is not trivial especially for microglia cells. Of note, it is still unclear to what extent the morphological alterations of microglia reflect the molecular and functional changes that these cells undergo during physiological and pathological conditions, given that in the human cortex the simultaneous presence of the aforementioned morphologies has been identified [8,23].

The microglia phenotype is primarily driven by the microenvironment, and based on the received stimuli, they secrete cytokines, free radicals, and soluble lipids to mediate the inflammatory response [24,25]. Such response is not always beneficial, especially in the context of neurodegeneration where prolonged exposure to inflammatory factors can become neurotoxic [2,26–29]. However, the number of physiologic or positive roles ascribed to microglia is also constantly increasing and go from its role in supporting neurogenesis in the adult [30–33] to the remodeling of axons and synaptic connection in developing brain [34–36], all functions which have in common the trophic support to neurons and the efficient maturation of neural circuits in physiological and pathological conditions, and in case of brain injuries [37–44].

Microglia cells communicate with the other cells in the CNS via cell-to-cell contact, secreted molecules, and release of Extracellular Vesicles (EVs); this last one, in particular, allows regulation across distant brain regions [2,45–49]. EVs are divided into large and small EVs based on their dimension and biogenesis. Large EVs originate from the outward budding of the plasma membrane and typically have a mean diameter spanning from 150 to 800 nm. Conversely, small EVs derive by the exocytosis of an organelle of the endocytic pathway, the multivesicular body, and have a diameter spanning from 30 to 250 nm [50]. The experimental workflow to obtain EVs from microglia and the techniques that can be used to validate them, are reported respectively in **Fig. 1** and **Fig. 2**.

Small and Large EVs, being cell-derived membrane vesicles, carry both membrane-associated material and cytosol components such as mRNA, miRNA, proteins, lipids, and pro-peptides. In the last decade, several studies have demonstrated the modulation of exosome composition based on the phenotype of microglia-releasing cells. Notably, the important role of EVs in the regulation of intercellular communication has been widely proposed [2,51,52]. Indeed, recent studies have identified functional mRNA and miRNA as EVs cargoes able to modify gene expression and metabolism in the recipient cells [2,53–57]. Microglial EVs have been proposed to act as both a propagation means of toxic proteins, while other studies indicated their positive impact on protein aggregates clearance and regulation of neuronal viability [58–63] further corroborating the implication of EVs in cell-to-cell communication.

Microglia cells can produce EVs under different stimulation conditions (MI, M2 stimuli) leading to a variety of EV cargos. A detailed overview of the morphological characteristics, content composition, and functions of microglia will be dealt with elsewhere [50]. Herein we describe in detail the most widely used cellular models to produce microglia EVs.

Cellular Models of microglia: Focus on EV Production

Several microglial *in vitro* cellular models have been reported in the literature [64], most of them deriving from *mus musculus*; however, not all of them are used for EVs-related studies. We will discuss the experimental demands for each of these models and highlight the potential advantages and disadvantages of their use whenever EVs isolation is the goal of the study. Most of this information is also summarized in **Table 1,** along with the list of the experimental methods that have been used for EVs derivation and, where available, the relative yields**.**

Murine and Rat Models

The BV2 and N9 microglia cell lines are the most used murine cell lines in the field of EV research; they were both created and reported for the first time by Italian research groups in 1990 [65] and 1993 [66], respectively.

The BV2 cell line has been obtained through transformation by the recombinant retrovirus J2 of neonatal primary microglia from C57BL/6 mice [65], and has been validated to have morphological, phenotypical, and functional markers of macrophages by transcriptomic analysis and proteomic analysis. BV2 microglial cells have been the most used model for *in vitro* studies of neuroinflammation for more than a decade because of their capability of reacting to LPS, mimicking an inflammatory response, and because they reduce the requirement of continuously maintaining primary cell preparations from animals [67]. BV2 cells express macrophage markers (MAC1 and MAC2) but no astrocyte (GFAP) or oligodendrocytes (Gal-C) ones. MAC2 in particular is important when this line is used to model neuroinflammatory processes due to its capability, in its secreted form, of activating neighboring microglial cells [68].

The N9 cell line also comes from adult primary microglia of CD-1 mice [66,69] transformed with the recombinant retrovirus 3RV. As the BV2 line, they were recognized as a good model for CNS macrophages expressing Immunoglobulin G receptors, glycoprotein F4/80 and MAC1, and being

negative for GFAP and Gal-C. Like BV2 cells, N9 cells produce and secrete pro-inflammatory cytokines after LPS stimulation and can phagocytize Aβ fibrils [66].

There is also the MG6 cell line coming from C57BL/6 mice [70]. This line was obtained by primary microglial cultures infected with a retroviral vector containing the human c-myc and neomycin-resistant gene. This line has very few citations in the literature comparing to BV2 and N9 [64]. Even if it was established that MG6 cells have characteristics similar to those of BV2 [70], the presence of a wider knowledge on the latter is probably the reason they are so less diffused.

Alternatively, primary microglia are also commonly used in this field of research, although they are mostly used as recipient cell models from EVs gathered from immortalized cell lines after various treatments. The main source for primary microglia come from post-natal extraction from the C57BL/6 inbred strain of laboratory mouse.

There are also two microglia cell lines derived from *Rattus norvegicus*, HAPI [71], and MLS-9 [72], but in the EV research field, most studies make use of primary rat microglia isolated from the Sprague-Dawley rats. Indeed, we did not find any study using these two immortalized cell lines for the investigation on EVs.

Primary microglia may be considered a more reliable source of information than immortalized cell lines [73] but its isolation is not easy and, most importantly, obtaining a sufficient amount of cells for EVs recovery is not trivial and would require sacrifice of many animals. Also, as described in detail by Lian et al. and Ni et al. [74,75], establishing a stable culture of pure primary microglia, with no contaminating oligodendrocyte or astrocyte, may take several days, or even weeks. Finally, considering the ease by which microglia can switch from the M0 phenotype into the inflammatory M1, major stresses derived from the isolation procedure may not be the best condition to work with.

The Leech Model

The medicinal leech (*Hirudo medicinalis*) has arisen as an interesting animal model to study microglia behavior. This is due to three main features of its CNS: i) its spatial anatomic organization; ii) its regenerative properties; iii) the lack of the adaptive immune response. First, the interactions between microglia and neurons represent a key process for the maintenance of CNS integrity and neuroinflammatory regulations. In leech CNS, neuronal cell bodies are concentrated in ganglia and project the axons into the connective tissues [76,77]. Microglia are located in ganglia as well as in connective tissue, allowing a distinct interaction with neuronal somata or axons. For this reason, it is possible to artificially injure just the axons and study the role of microglia in the different neuronal

compartments. Moreover, the leech has a CNS with regenerative capacity. If cut or damaged, the leech nerve cord can regenerate within a few days and this regeneration is increased in septic conditions than in sterile ones, which suggests the involvement of a controlled immune response [78].

In the CNS of mammals, the combination of innate and adaptive immune responses makes it difficult to analyze and dissect the mechanism at the basis of the shift from pro-regenerative to neurodegenerative microglia phenotype. Interestingly, the infiltration of blood cells is very low in leech CNS specifically favoring the study of resident microglia activation [77].

Issues about the use of this model could be raised, considering its evolutionary distance from the human. However, an extensive proteomic analysis of primary leech microglia-derived EVs gathered after LPS or ATP stimulation, showed that leech-EVs bear most of the cargos of mammal EVs identifying this model as very accurate for such studies [79,80]. It has been shown, by the same research group, that leech microglia-derived EVs have a big effect on the differentiation of rat PC12 cells. Even though differentiated PC12 cells are an established model for peripheral and not central neurons, the treatment with leech-EVs significantly increased their neurite varicosity compared to an EV-free control, suggesting that leech microglia-derived EVs could share a similar molecular dialog in the CNS [76]. Together with the fact that this crossing-species effect was also observed between rat and human cells [81], and also between plant cells and murine macrophages [82], it has been suggested that communication between cells can be more a matter of molecule and secreted factor than of species.

What about Human Models?

It is worth noticing that relatively few studies have so far used human microglia in EVs studies, either primary microglia from post-mortem donors or immortalized human microglial cell lines (**Fig. 1**). We found few reports that described the use of human primary microglia from post-mortem specimens as recipient cells for glioblastoma-derived EVs [83], and also from cryopreserved human brain tissue [84]. This may be explained by the fact that for precise characterization of an EVs population, there is the need for a large number of parental cells. This cannot be achieved easily with primary cells. On the other hand, some human microglia cell lines have been reported recently [85,86], but so far a thorough characterization of their released EVs to allow their use as the standard model in this field has remained elusive.

The HMC3 line, the most widely used, was established from the immortalization of human embryonic brain-derived macrophages and validated for several markers, resulting to express most of macrophagic

markers but none of the monocyte ones, such as CD14, CD4, CD68/Ki-M6 and CD11c [87]. Their main characteristics have been detailed by Dello Russo et al. [86]. Notably, this cell line has been recently used for the preparation of EVs to investigate uncovered effects of cocaine [88].

Furthermore, another human microglia cell line was established, coming from adult brain tissue, the C20 line [85]. Primary cells were transformed with lentiviral vectors expressing SV40 T antigen or a combination of SVR40 T antigen and hTERT and the resulting line was tested for key microglial surface markers, such as TGFβR, P2RY12 and CD11b. Interestingly, there is evidence that some human microglia cell lines, such as the C20 cell line, cannot respond in the same way as murine microglia to LPS [85,89,90]. This is particularly relevant for the EVs field, because of their proven implication in the immune response following LPS stimulation.

An increasingly interesting possibility is to derive microglia cells from patient-derived stem cells [91]. This method is based on the differentiation of induced pluripotent stem cells (iPSC) and presents clear advantages in terms of generating a large number of cells and increasing the replicates with respect to the collection of new tissue, one of the main ethical and practical drawbacks of working with human cells. Specifics extraction and differentiation protocols and relative advantages and drawbacks are detailed by Hasselmann et al. [92]. The differentiation of iPSC to various cell types has been a diffused practice over the last years but, only recently, protocols for generating human microglia have been reported. This has been made possible by recent studies on the development of microglia which traced it from mesodermal primitive yolk sack to erythromyeloid progenitors (EMPs), yolk sack macrophages and lastly to brain microglia [5,6,92]. All the available protocols, while unique in their specific details [92–94], share an experimental workflow that replicates *in vitro* the variation in the expression of developmental factors that is driving the development *in vivo*. The starting point can be either iPSC or Embryonic Stem Cells (ESCs) directly extracted from a blastocyst [95] (**Fig. 1**); once a batch of pluripotent stem cells is established, the first step is to differentiate them to primitive hematopoietic precursors to EMPs and later to microglia [92]. These cells can be also used in the formation of organoids and xenotransplantation. In the *in vivo* context, there is an interesting report indicating the possibility to develop a human pluripotent stem cell (hPSC)-based microglial chimeric mouse brain model by transplanting hPSC-derived primitive macrophage progenitors into neonatal mouse brains [96]. The emerging and growing use of iPSC derived microglia can, at least in part, solve the inaccessibility to actual human primary microglia *in vivo* and *in vitro* [97]. Even though iPSC-derived microglia is an interesting model, there are a few drawbacks at present that limit their use for the study of EV production. The main limitation is probably the need of a large quantity of parental cells to produce EVs and this,

summed with the time needed for the differentiation, may cause an excessive increase of the handling costs. These reasons explain and justify the still vast use of immortalized cell lines for these experimental purposes.

Conclusion

Research in the field of microglia EVs has originated several years ago. Since then, most studies have been carried out using murine microglia lines and testing the effects of the secretion of these lines on the corresponding primary cultures. For obvious practical and ethical reasons, this approach is not reproducible for the human species. Nevertheless, there is increasing evidence that the immune system, particularly in the CNS, responds to the same stimuli in very different ways between species [98,99].
Unveiling and characterizing the activity of microglia in physiological and pathological conditions is critical for a complete understanding of neurodegenerative diseases and brain traumas, e.g., TBI or ischemic stroke. The availability of new models for studying microglia and related EVs, as summarized in **Fig. 1**, is enriching the panorama of studies that can be performed and thus the molecular information that can be obtained. However, a cross-check between the different cell models used to study microglia-derived EVs is currently needed. This aspect is particularly important given that the nature of microglia is to be restless cells, constantly scanning, reacting and adapting to their environment in physiological as well in pathological conditions and modifying their morphology, metabolism, and gene expression according to every change [100–102]. This behavior makes these cells quite demanding to study, the main experimental problem being the ease of repeatability. Importantly, the new microglia cell lines and the use of iPSC-derived human microglia hold the promise to expand the study of microglia-derived EVs to human samples. For what concerns the *in vitro* studies, the newest and advanced 3D cellular cultures could offer a reliable model [103]. The 3D environment of tissues is critical for the correct behavior of a specific cell population and the mechanosensing and mechanotransduction, being relevant in the physiological and pathological brain [104], could likely change the composition of the secretome of the CNS subpopulation. Brain organoids, 3D cultures, and xenotransplantation are all techniques that could allow a better understanding of how environmental factors influence cell-to-cell communication and CNS development, maintenance and diseases [8,96,97,105,106]. This is particularly true for microglia-derived EVs, whose content and biological effect are highly influenced by the CNS area where microglia is localized and by the microenvironmental stimuli [107].

Perspectives

1. Microglial cells play fundamental roles in the homeostatic maintenance of the brain responding to insults also via the release of EVs; therefore, the comprehension of the mechanism of EV production and release is critical for neurodegenerative and brain damage research.
2. The low accessibility of human microglia tissue has forced scientists in the past to work with non-human models. The advent of new methods to obtain human models for microglia is exciting and could turn out to be particularly important in the EVs field.
3. The importance of cross-checking the characteristics of all the available models is undeniable. There are a variety of omics disciplines that are currently making this feasible. This could lead to reduced variability in the results proposed in this field and to better reproducibility of experimental data.

Abbreviations

AD	Alzheimer's Disease
Aβ	Amyloid-B
CNS	Central Nervous System
EMPs	Erythromyeloid Progenitors
ESCs	Embryonic Stem Cells
EVs	Extracellular Vesicles
hPSC	Human Pluripotent Stem Cell
iPSC	Induced Pluripotent Stem Cells
LPS	Lipopolysaccharide
MS	Multiple Sclerosis
PD	Parkinson's Disease
TBI	Traumatin Brain Injury

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Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

L.C. and L.M. conceived the area of focus for this review. L.C., C.G. and L.M. wrote the main text. C.M. and C.G. edited.

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Figures and Tables

Table 1 *Summarized characteristics of the main microglia cell model used for EVs production.*

Specie	***Name***	***Model and Line Establishment***	***Time needed before EVs extraction***	***Used Protocol for EVs Extraction***	***Increase of EV production per stimulus***	***Yield***
Homo Sapiens	**HMC3**	Embryonic, SV-40 [87]	Line established, ready to use	Differential Ultracentrifugation + 0.2µm filtration [88]	100 µM cocaine→reduction but not significant [88]	10^8 EV/mL [88]
	C20	Adult, VSVG SV40 [85]	Line established, ready to use	n/d	n/d	n/d
	Primary	Biopsy and autopsy [108,109]	Up to 7 days of culture after extraction [108,109]	Immunoprecipitation with CD11b [84]	n/d	n/d
	iPSC-derived	Human iPSC	30-50 days of differentiation [92,97,110]	n/d	n/d	n/d
Mus Musculus	**BV2**	C57BL/6, Neonatal brain, v-raf, v-myc retrovirus [65]	Line established, ready to use	Differential Ultracentrifugation [111–113] Differential Ultracentrifugation + 0.2µm filtration [114] SBI Exo-Quick [115]	0.2 µM α-Syn→increased [114] 20 ng/mL LPS→increased MP production [111] 25 µM Serotonin→increased > 200% [112] 10 nM Wnt3→increased [113] 25 ng/mL IL-4→increased [44,115]	n/d n/d n/d n/d n/d n/d
	N9	CD-1, Embryonic brain, 3RV retrovirus [66]	Line established, ready to use	Differential Ultracentrifugation [116,117] Biot-Annexin-V + Streptavidin beads [118]	1-3 mM ATP→increased [116–118]	10^7-10^8 EV/mL [116,117]
	MG6	C57BL/6, Neonatal brain, c-myc retrovirus [70]	Line established, ready to use	Exosome Isolation Reagent (Life Technologies) [119]	1 µg/mL LPS + 2-4 mM ATP→ 100% increased [119]	n/d
	Primary	C57BL/6 [111,119] CD-1 [120]	neonatal mice, 10-20 day mixed glia culture [119] 0 day pups, 7 day after separation from mixed glia culture [120]	Exosome Isolation Reagent (Life Technologies) [119] Sucrose step gradient Ultracentrifugation [120] Differential Ultracentrifugation [120]	1 µg/mL LPS + 2-4 mM ATP→ 100% increased [119] LPS + ATP→increased [120]	n/d n/d
Rattus Norvegicus	**HAPI**	Neonatal brain [71]	Line established, ready to use	n/d	n/d	n/d
	MLS-9	Wistar, Neonatal brain,	Line established, ready to use	n/d	n/d	n/d

		Cultured with CSF-1-containing medium [72]				
	Primary	Sprague-Dawley [116,121,122] Wistar [107]	18-19 day embryo, 3 week mixed glia culture [118] 2 day rat pups, 7-10-14 days mixed glia culture [116,121,122] 3 day rat pups, 4 days in vitro, 7 days after separation from mixed glia culture[107,123]	Annexin-V streptavidin beads [118] Differential Ultracentrifugation [107,116,121–123]	1-3 mM ATP→increased [116,118] Th1 citockine cocktail + 1 mM ATP→increased [121,122] 500 ng/mL LPS→increased [107,123]	10^7-10^8 EV/mL [116] 2-3*10^8 EV/mL [121] 10^6-10^7 EV/mL [107,123]
Hirudo Medicinalis	**Primary Leech**	/	Adult leech, 4 day after dissection [76,79,80]	Differential Ultracentrifugation + 0.2 µm filtration [76,79,80] Optiprep™ density gradient Ultracentrifugation [80]	Resting	10^8-10^9 EV/mL [79]

Figures

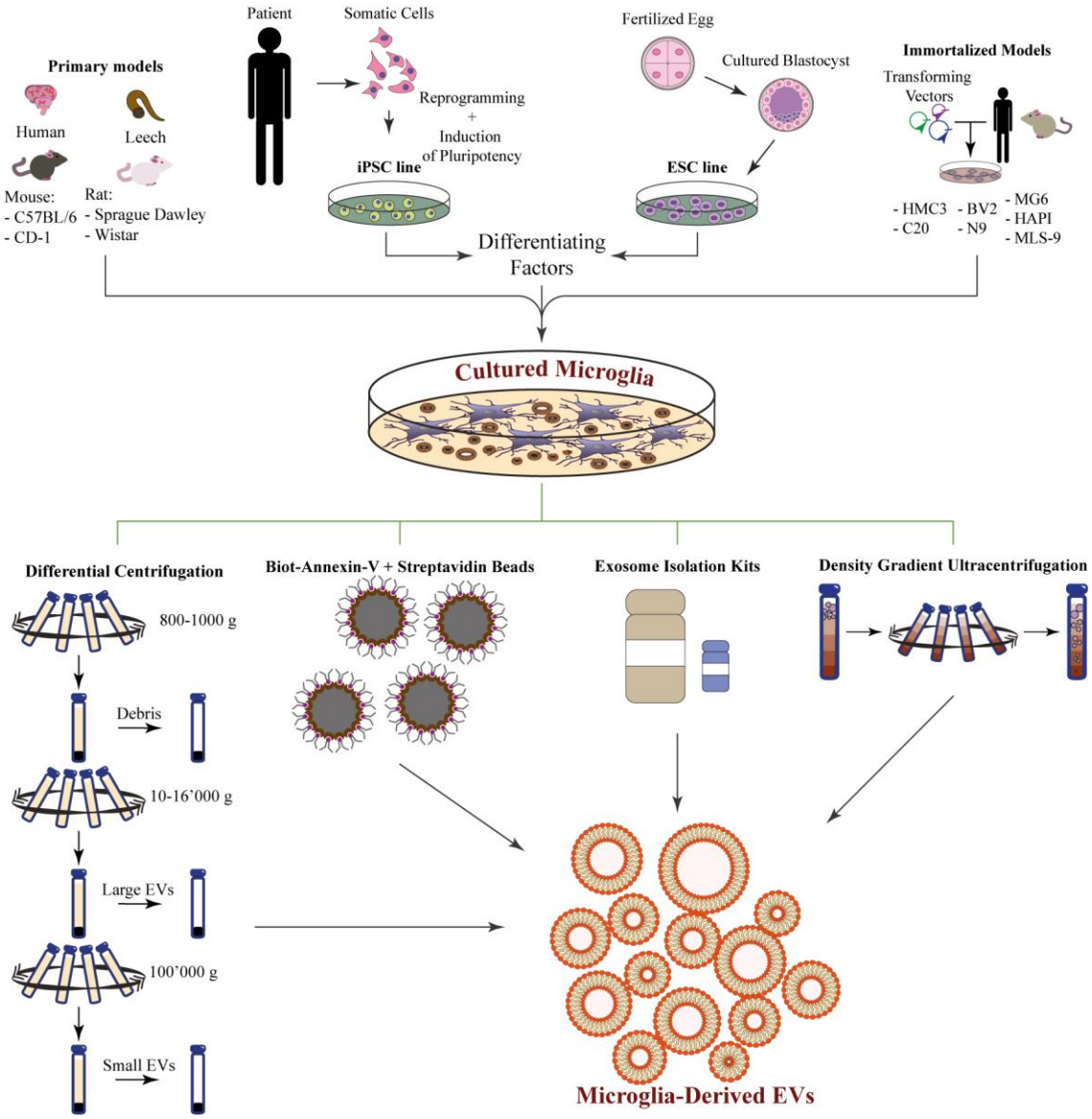

Figure 1 Methods for EVs derivation from Cultured Microglia. Top: scheme of the four main types of parental cells (primary cells, iPSC lines, ESC lines, and immortalized cell lines) that can be used to obtain microglia cell cultures. Bottom: scheme of the four main types of EV extraction and isolation methods used to gather microglia-derived EVs.

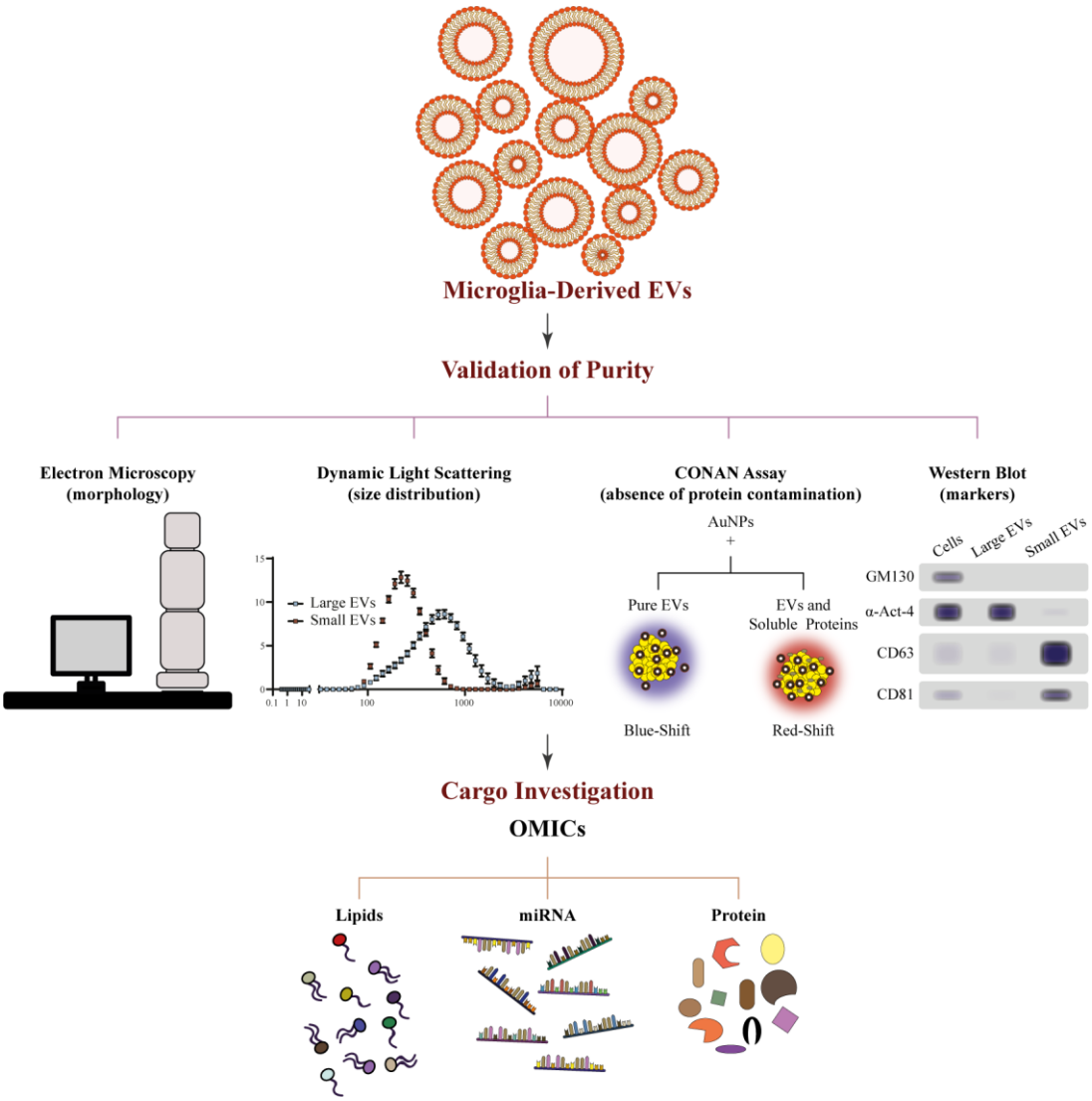

Figure 2 Methods for validation and investigation of extracted microglia-derived EVs. Middle: scheme of four main methods to validate a successful separation of large and small EVs and to assess the absence of soluble protein contamination (for more detail, see Ref. 124). Bottom: scheme of the most used methods to investigate the cargo of EVs preparation with validated purity. For more details, see Refs. 76-103).