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The Messenger Apps of the cell: Extracellular Vesicles as Regulatory Messengers of Microglial Function in the CNS

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Abstract

The intense effort of investigators, in particular during the past decade, has highlighted the importance of extracellular vesicles (EVs) such as exosomes in regulating both innate and adaptive immunity in the course of a variety of infections, with clear implications for development of novel vaccines, therapeutics, and diagnostics. Current and future efforts now need to focus strongly on teasing apart the intricate and complex molecular mechanisms that operate during EV regulation of immunity. In this review, we discuss recent advances that bear on our current understanding of how EVs, including exosomes, can contribute to the innate immune functions of microglia within the central nervous system (CNS), and we also highlight future important mechanistic questions that need to be addressed. In particular, recent findings that highlight the crosstalk between autophagy and exosome pathways and their implications for innate immune functions of microglia will be presented. Microglial activation has been shown to play a key role in neuroAIDS, a neuro-infectious disease for which the importance of exosome functions, including exosome-autophagy interplay, has been reported. The importance of exosomes and exosome-autophagy crosstalk involving microglia has also been shown for the Parkinson's disease (PD), a neurodegenerative disease that is thought to be linked with immune dysfunction and involve infectious agents as trigger. Considering the accumulation of recent findings and the vibrancy of the EV field, we anticipate that future studies will continue to have a deep impact on our understanding of the CNS pathologies that are influenced by the functions of microglia and of the infectious disease mechanisms in general.

Graphical Abstract

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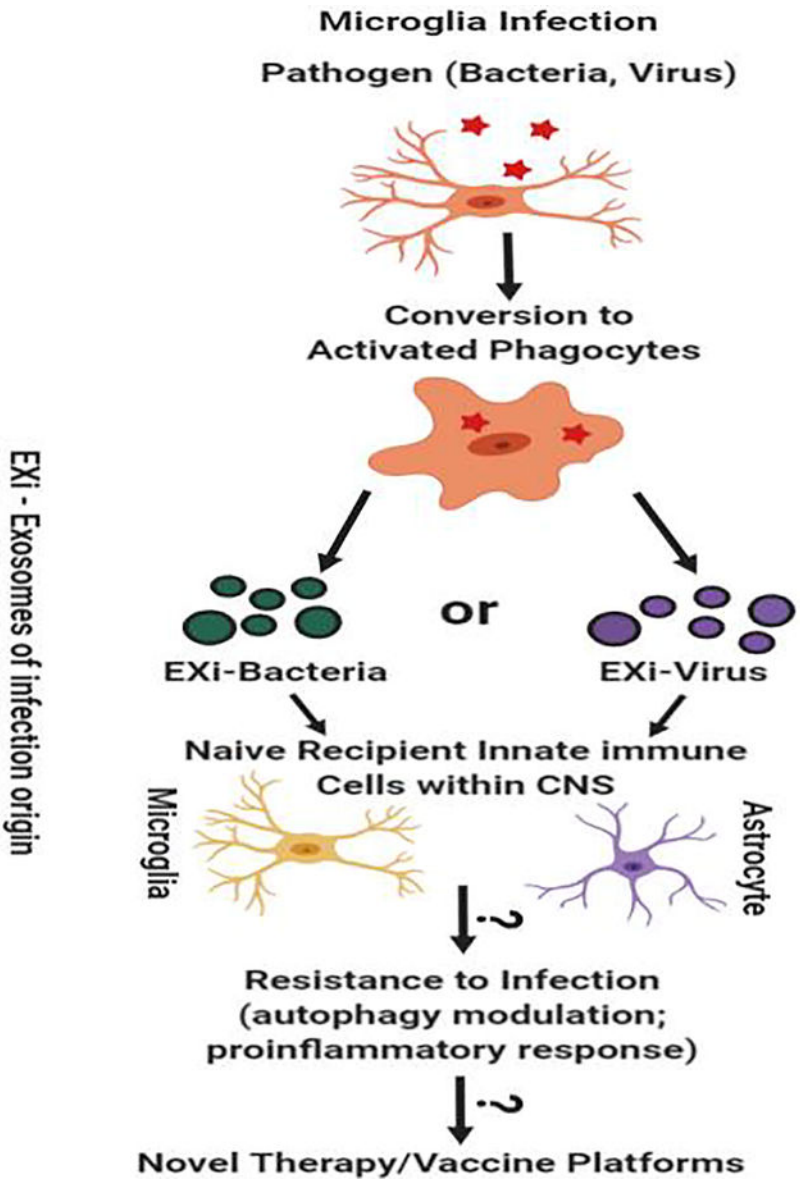

BRIEF INTRODUCTION

Extracellular vesicles (EV), including exosomes (EX), have attracted significant attention among scientists during the last decade as the influence of their communicative effects in a number of different human pathologies, including infectious diseases, have become apparent (Delorme-Axford et al. 2013). Their role in regulating immunity first became a subject of interest when it was discovered that certain cells secretes EVs harboring major histocompatibility complex class II (MHC class II), which can induce antigen specific MHC class II T cell responses (Raposo et al. 1996; Schorey and Harding 2016). Since this discovery, many studies have highlighted the ability of EVs to influence both innate and adaptive immune responses. However, a detailed understanding of the complexity involved remains to be attained. In this regard, the unfolding knowledge of the EV regulation of

innate immune responses, including the exosome-autophagy interplay as part of this regulation, has opened new windows of understanding in the field and is therefore of intense current interest. In this review, we present a synthesis of the emerging evidence that bears on EV/EX regulation of innate immune responses in microglia and its influence on central nervous system (CNS) interactions, with a particular emphasis on the latest findings for exosome-autophagy crosstalk mechanisms as a component of this response. We also discuss some challenges that lie ahead, and propose areas of investigation to advance our understanding of the mechanisms by which EV modulation of innate immune response, including microglial response, occurs. From the accumulation of evidence discussed here, including work from our own laboratory, a central hypothesis emerges that EVs, including exosomes, released from infected microglia may induce specific innate immune responses in target naïve recipient microglia and astrocytes to fight off infection efficiently when they encounter the invading pathogen. Such responses may include induction of proinflammatory cytokine release and modulation of major signaling pathways such as p38 MAPK to allow improved pathogen clearance. We also hypothesize that autophagy mechanisms may influence this process by affecting the number of released vesicles as well as their content, or through EV regulation of autophagy responses in recipient cells. In this review, we begin by providing a brief introduction to the general properties of exosomes and other EVs, including some of the known mechanisms that govern their cargo selection that in turn determines the nature and magnitude of recipient cell responses. We next describe the state of knowledge about how EV release regulates microglial immune functions in the CNS. As part of this, studies of the closely related macrophage/monocyte populations are also informative to consider and are discussed. Finally, we highlight an emerging aspect of how the EV regulation of microglial response to infection, including microbial clearance, may be influenced; namely, the contributions of exosome-autophagy interactions. We conclude the review by a discussion of important mechanistic questions that remain to be thoroughly investigated in future studies and some of the challenges and unique opportunities that lie ahead.

PROPERTIES OF EXOSOMES AND OTHER EV SUBTYPES

EVs are nanosized vesicles released from cells into extracellular milieu (Liao et al. 2017). EVs consist of a mixed population of various vesicles, which include microvesicles (ectosomes), exosomes, and apoptotic bodies (Raposo and Stoorvogel 2013). These vesicles are distinguished mainly based on their size/density, biogenesis, and mode of release (Zaborowski et al. 2015), although due to overlap in size, composition, and protein markers, making absolute assignment of vesicle identities remains a challenge (Harties et al. 2019). The purification schemes designed for isolation of macrovesicles and exosomes have been discussed in detail in other review articles (Konoshenko et al. 2018; Zhang et al. 2019). Apoptotic bodies are released from dying cells, with sizes ranging from 50 nanometer (nm) to 5000 nm in diameter (Hartjes et al. 2019). Microvesicles are released directly from the plasma membrane, with sizes ranging from 100 nm to 1000 nm (Heijnen et al. 1999). Exosomes, the third common subtype of EVs, are endocytic in origin and have a diameter range of approximately 30 nm to 150 nm (Hessvik and Llorente 2018). They are released from the cell by the fusion of multivesicular bodies (MVBs) with the plasma membrane. As

with microvesicles, exosomes elicit specific responses in recipient cells, such as changes in cytokine release profile, changes in signaling activities of immune cells, or alteration of susceptibility to infection (Fleming et al. 2014 Barclay et al. 2017; Madison and Okeoma 2015; Sampey et al. 2016). Although they were first identified in reticulocyte culture media (Johnstone et al.1987), exosomes are released from almost all eukaryotic cells in different organisms and can be isolated from different biological fluids such as plasma, urine, saliva, seminal fluid, nasal secretions, breast milk, and amniotic fluid (Caby et al. 2005; Keller et al. 2011; Lasser et al. 2011; Pisitkun et al. 2004; Poliakov et al. 2009). Overall, much still remains to be uncovered regarding the functions of exosomes, and a significantly more in-depth understanding of the signaling pathways and external stimuli that regulate their packaging and secretion is required (Zhang et al. 2019). For the studies discussed in this review, we will use the general term “EV” unless the authors have provided evidence for working with exosome-enriched materials based on a combination of size analysis (e.g., electron microscopy, or nanoparticle tracking assay), protein marker analysis (e.g., Western blot, or immunostaining), and use of appropriate purification schemes for exosome recovery.

The specific content of exosomes and other EVs is influenced by environmental factors, as well as the types of cells that produce them (Pallet et al. 2013; Tauro et al. 2013). In addition, the pathological and physiological states of the cell affect EV content, including protein cargo (Fleming et al. 2014; Sampey et al. 2014; Schwab et al. 2015). As such, EV-associated proteins can serve as indicators of disease, in addition to contributing to EV formation and trafficking (Andreu and Yanez 2014; Raposo and Stoorvogel 2013; Zhang et al. 2014). Work from different laboratories, including studies from our laboratory, have demonstrated that specific pathogen components get packaged within host EVs such as exosomes during infection (Ahsan et al. 2016; Chen et al. 2018; Fleming et al. 2014; Fleming et al. under review). For instance, we have shown the presence of viral RNA genome and the viral nucleocapsid protein in exosomes derived from cells infected with the Rift Valley fever virus, and the presence of specific bacterial proteins in exosomes of infection origin has been shown for different bacterial infections. Some of the mechanisms by which pathogenic components are packaged into exosomes have been described although much remains to be discovered. Evidence suggests that trafficking of EVs, including exosomes, shares many properties with viral assembly processes (Meckes 2015). For instance, MVBs can contain both EVs and virions (retroviruses) that are released to the extracellular milieu after fusion of MVBs to the plasma membrane, reflecting similarities in the biogenesis of EVs and retroviruses (Nolte-’t Hoen et al. 2016). Therefore, the intersection of the EV pathways with viruses is one mechanism contributing to sorting viral components into EVs (Lenassi et al. 2010; Nolte-’t Hoen et al. 2016). In support of this notion, the HIV virus Gag protein associates with CD81 and CD63 tetraspanins in endosome-like domains of the plasma membrane and is released in EVs (Booth et al. 2006). Another example is the involvement of the endosomal sorting complex (ESCRT) machinery and annexin A2 in packaging HCV RNA into EVs (Dreux et al. 2012). Some of the signals involved in incorporation of nucleic acids into EVs have been identified although a great deal is still unknown. For instance, it has been proposed that miRNA strands are transported into the MVBs by RNA binding proteins (RBPs), enabling their secretion via exosome release (Janas et al. 2015). More recently, possible RNA-loading motifs that allow miRNA

packaging into EVs have also been proposed (Gao et al. 2018). Some of the mechanisms for incorporation of bacterial components have also been identified. Studies have shown that host E3 ligase-dependent ubiquitination machinery is involved in trafficking mycobacterial proteins into EVs (Smith et al. 2015). Besides ubiquitination, other specific posttranslational modifications (PTMs) have also been shown to serve as selective protein sorting mechanisms (Villarroya-Beltri et al. 2014), and therefore merit detailed investigation of their contributions. These provide some examples of the rules governing cargo loading of EV/EX; for an extensive coverage of this topic, the readers are referred to a recent review article by Anand and colleagues (Anand et al. 2019).

In recent years, exosomes have become the most investigated extracellular vesicles due to their diagnostic and therapeutic potential for a variety of human pathologies, including infectious diseases. Aside from being pivotal for physiological crosstalk between cells (Pant et al. 2012), exosomes may also affect the progression of different disease conditions through the function of components that get specifically packaged into them (Cobb et al. 2018; Huang et al. 2019; Smith et al., 2017). These range from various neurological disorders to cancer, infectious diseases, and cardiovascular diseases (Ailawadi et al., 2015; Janas et al. 2016). In the context of infectious diseases, exosomes can either inhibit or activate the immune system depending on the type of pathogen, hence providing potential therapeutic and diagnostic opportunities (Kruh-Garcia et al. 2014). However, the specific signaling pathways altered by these vesicles during innate immune response remain largely unknown and significantly more work is required in this area. Furthermore, future experiments should also aim at determining more extensively the pleiotropic effects of exosomes versus those that are immune cell specific.

MICROGLIA AND EV REGULATION OF CELLULAR INNATE IMMUNITY IN THE CNS

EV regulation of microglial immune functions

The function of EVs in relation to immunity became a subject of interest in 1996 when it was discovered that B lymphoblastoid cells secrete EVs harboring MHC class II molecules and that EVs derived from B lymphocytes induce antigen-specific MHC class II-restricted T cell responses (Raposo et al. 1996). Since this seminal report, many studies have demonstrated the ability of EVs to mediate both activation and suppression of innate and adaptive immune responses (Bobrie et al. 2011; Obregon et al. 2009; Schorey and Harding 2016; Thery et al. 2002 Zitvogel et al. 1998; Smyth et al. 2013). As resident macrophage cells, microglia provide critical innate immune function in the CNS. Similar to the oligodendrocytes (OLs), astrocytes, and neurons within the CNS, microglia secret EVs, including exosomes, with distinct characteristics and functional properties that include immune-related activities. Functional relationships between components of the innate immune system and microglial EVs have been documented. For instance, microglia transfected with the Nef protein (Negative Factor) of HIV secrete EVs that contain Nef and promote secretion of Toll-like receptor-induced cytokines and chemokines in recipient microglia (Raymond et al. 2016). This is consistent with our stated hypothesis that exosomes derived from infected microglia may induce protective immune responses in recipient CNS

innate immune cells. It has also been shown that stimulation of microglia with interferon gamma (IFN-γ) leads to release of EVs that express elevated levels of MHC class II (Potolicchio et al. 2005). Interestingly, IFN-γ can also induce formation of extracellular traps (ETs) in Listeria-infected microglia, which release ET carrying EVs in response to Listeria infection (Wang et al. 2019). These microglial extracellular traps (MiET) consist mainly of extracellular DNA (eDNA), matrix metallopeptidases, citrullinated histone H3, and peptidyl arginine deiminase 2, and serve to protect the CNS against Listeria infection by arresting or killing the bacteria (Wang et al. 2019). These findings are consistent with our hypothesis that exosomes from infected microglia can prompt naïve recipient cells to fight off an invading pathogen more effectively in the event of becoming infected. The inflammatory cytokine IL-1β is also released in association with microglial EVs (Bianco et al. 2005). Intriguingly, it has been demonstrated that multitudes of cytokines can become packaged into EVs (Fitzgerald et al. 2018). Therefore, it is conceivable that EVs derived from microglia, including exosomes, may serve as delivery vehicles for cytokines that regulate inflammatory response or neurotransmission (Prada et al. 2013; Turola et al. 2012). Recent studies indicate that RNA components of microglial exosomes can also carry out important functions, including effects that are part of response to infection. It has been shown that let-7a and let-7b miRNAs associated with exosomes released from microglial cells that are infected with the Japanese encephalitis virus (JEV) cause caspase activation in recipient neuronal cells (Mukherjee et al. 2019). Additionally, it has been demonstrated that microglia secrete exosomes that are highly enriched in miR-124–3p, providing protection to the brain by inhibiting neuronal inflammation (Huang et al. 2017). This effect occurs through miR-124–3p inhibition of phosphodiesterase 4B (PDE4B) gene expression and thus suppression of the mTOR signaling activity (Huang et al. 2017). As inhibition of PDE4B is known to suppresses inflammation in response to bacterial infections (Komatsu et al. 2013), miR-124–3p delivery by microglial EVs may be one of the mechanisms by which inflammatory response to bacterial infections of the CNS is regulated. Again, these support our stated hypothesis of immune protection by exosomes released from infected microglia. The demonstration of microglial EVs regulating inflammatory response points to their potential utility as markers of brain inflammation. In a related note, a fascinating, albeit challenging, aspect that is critical to address in future work is how the complex microenvironment of the CNS impacts both the packaging and release of microglial EVs and how that in turn influences their functions in recipient cells. Factors such as the enrichment of particular CNS cell types that interact with microglia in various regions and the influence of external stimuli such as invading pathogens need to be extensively studied.

Several mechanisms that stimulate EV release from microglia have been identified. The dependence of EV release from immune-activated microglia on glutamine metabolism has been reported, as the inhibition of glutaminase, an enzyme involved in the metabolism of glutamine, reduces EV release from immune-activated microglia while α-ketoglutarate is associated with an opposite effect (Wu et al. 2018). Neuroinflammatory cytokines such as TNF-α, IFN-γ, and IL1β have also been shown to enhance vesicle shedding from microglia (Verderio et al. 2012). In another study, ATP activation of the P2X7R receptor has been shown to induce the release of both exosomes and MVs from microglia, likely as a means of transporting GAPDH to the outside of the cell (Takenouchi et al. 2015). These findings are

consistent with previous observations that ATP-activated P2X7R induces exosome release from macrophages through exocytosis of multivesicular endosomes (Qu et al. 2007), and that P2X7R receptor activation by astrocyte-derived ATP induces MV release from microglial cells (Bianco et al. 2005). ATP not only acts as a stimulant for MV/exosome shedding but also modifies the content of these microglial EVs, enriching for proteins involved in extracellular matrix organization, cell adhesion, cellular metabolism, and autophagy lysosomal pathway, thus influencing microglial EV signaling to astrocytes that also serve innate immune functions within the CNS (Drago et al. 2017). The lipopolysaccharide (LPS) of gram-negative bacteria also stimulate microglial EV release (Kumar et al. 2017). The LPS-induced microvesicles are enriched in miR-155 and IL-1β, both of which are proinflammatory in function (Kumar et al. 2017). Furthermore, it has been demonstrated that serotonin can stimulate vesicle release from microglial cells, mediated by microglia 5-HTRs and involving the elevation of calcium levels (Glebov et al. 2014). While the functions of these vesicles were not analyzed, the presence of neurotrophic factors in exosomes has been demonstrated by proteomic analysis (Mathivanan et al. 2012), suggesting that microglia might use vesicle release as another means of promoting neuronal health and differentiation. How these stimulatory factors may play a role during response to infection in the CNS remains to be investigated; such studies will provide a wealth of information on how EV release from microglia is regulated as part of the infection process

Besides secreting EVs such as exosomes to carry out specific functions, microglia also respond to EVs secreted by other cell types within the CNS, including astrocytes. In the context of infection, HIV Tat protein has been shown to induce the release of astrocyte exosomes that contain miR-9 and promote microglial migration (Yang et al. 2018). Recent findings show that astrocyte-derived exosomes can also regulate phagocytotic activity in microglial cells that receive them (Hu et al. 2018), an effect that could influence cellular response to infection. Modulation of microglial phagocytic activity has also been observed in response to exosomes derived from motor neuronal cells that are transfected with mutant superoxide dismutase 1 (mSOD1) (Pinto et al. 2017). Additionally, alterations in microglial polarization were observed. The findings suggest that inflammatory-associated miR-124 packaged in motor neuron-derived exosomes determines these phenotypic alterations in the recipient microglial cells (Pinto et al. 2017).

EV regulation of monocyte/macrophage immune functions

According to the mononuclear phagocyte system (MPS) model, committed marrow progenitors give rise to blood monocytes that upon migration to tissues differentiate to macrophages (Hume 2006). Microglia are considered to represent the resident macrophage population within the CNS. Similar to macrophages, they migrate to the site of injury or infection where they may proliferate and become phagocytic (Gonzalez-Scarano and Baltuch 1999). Furthermore, they release inflammatory cytokines that induce the recruitment of cells to the site of infection or trauma, and similar to macrophages they can release tumor necrosis factor alpha and other potential neurotoxins that are associated with infectious diseases such as AIDS (Gonzalez-Scarano and Baltuch 1999). The macrophage population, which includes microglia, and the precursor monocyte population are members of the mononuclear phagocyte system (MPS) that share many overlapping characteristics

(Guilliams et al. 2014). Therefore, for assessing EV contributions to microglial functions, and for considering areas of exploration in future EV studies of microglia, it is informative and relevant to also discuss EV regulatory aspects for monocytes/macrophages.

Several EV studies of macrophages infected with *Mycobacterium tuberculosis* (M.tb) have been reported to date. Macrophages infected with M.tb release exosomes that promote innate and acquired immunity both *in vitro* and *in vivo* (Singh et al. 2015; Smith et al. 2017). The M.tb RNA packaged into these exosomes can be directly translated into protein upon delivery into host cells (Singh et al. 2015). In addition, the mycobacterial RNA and pathogen-associated molecular patterns (PAMPs) packaged within these exosomes can promote production of proinflammatory cytokines (Singh et al. 2015; Smith et al. 2017), an effect that also supports our hypothesis that exosomes from infected microglia provide protection against infection in part by inducing proinflammatory cytokine release. Several additional mechanisms of regulating innate immunity by exosomes released from macrophages have also been reported to date. Exosome trafficking of IL-1β, which is a major driver of innate immune response, has been proposed as an important transport mode by which IL-1β is released by macrophages and DCs through the non-classical secretion pathway (Qu et al. 2007), reminiscent of the findings by Bianco and colleagues discussed above that microglial EVs can carry IL-1β. Another example is the finding that in the presence of oxidized low-density lipoprotein-containing immune complexes, macrophages release exosomes carrying IL-1β, in addition to HSP70 and sphingomyelinase, that can drive the induction of atherosclerotic plaques (Truman et al. 2012). Studies of macrophage response to EVs from infected cells has also been reported. It has been shown that EVs released from HBV-infected hepatocytes package viral RNA within them that are sensed by macrophages and dendritic cells via TLRs and play an important role in innate immune response against HBV (Kouwaki et al. 2016). Our own laboratory has been investigating the mechanisms by which monocytes respond to exosomes of infection origin, which we designate as EXi for short. Our focus has been investigation of highly pathogenic agents that are classified by the CDC and NIAID as Category A pathogens, or pathogens of highest concern. Specifically, we have been studying EXi that are derived from cells infected with either *Yersinia pestis* (Yp) bacteria or Rift Valley fever virus (RVFV). In both cases, we have found that the EXi are packaged with specific protein and nucleic acid components from the infecting pathogen (Ahsan et al. 2016; Fleming et al. 2014; Fleming et al. 2018; Fleming et al. manuscript under review; Alem et al. manuscript under review). In both infection models, the EXi activate recipient monocytes, inducing strong anti-viral response in monocytes treated with EXi-RVFV and significant reduction of intracellular bacterial load following pretreatment with EXi-Yp (Fleming et al. 2018; Fleming *et al*. manuscript under review; Hobbs *et al*. manuscript under review). These studies demonstrate that the EXi can serve a protective role for the host during infection, highlighting the potential for these exosomes to serve as novel vaccination vehicles. In concurrence with several studies alluded to above, our observations further support our stated hypothesis for how exosomes derived from infected microglia may influence their recipient cells; namely, activation of upstream signaling pathways that induce proinflammatory cytokine release, which could in turn enable the cells to reduce pathogen load in the event of becoming infected. Interestingly, in contrast to our RVFV findings, for a number of other virus infections the EVs released from infected cells

produce the opposite effect and contribute instead to facilitate viral spread (Arakelyan et al. 2017; Margolis and Sadovsky 2019). Future work is needed to tease apart the mechanisms that determine whether EVs released during a particular infection aid the host or instead assist viral amplification or spread. Questions such as whether this is primarily determined by the type of cargo packaged into EVs, or whether other factors such as the type or quantities of EVs delivered to recipient cells or multiplicity of infection (MOI) are also main determinants, should be investigated. Due to the highlighted similarities between tissue macrophages and microglia, it is very likely that at least some of the attributes observed for exosomes derived from infected macrophages are also relevant for microglial response to infection. Therefore, future studies such as whether exosomes from infected microglia also package PAMPs that can promote the production of proinflammatory cytokines, or carry pathogen-derived RNA that can be translated in recipient cells, will be quite informative.

IMPLICATIONS OF AUTOPHAGY-EXOSOME INTERPALY FOR MICROGLIAL RESPONSE TO INFECTION

Signaling mechanisms regulating autophagy

Autophagy is a well-established mechanism used by phagocytic innate immune cells such as microglia to defend against infection by eliminating pathogens (Plaza-Zabala et al. 2017). Of direct relevance to this review article are the recent findings that this important defense mechanism shows cross regulatory interactions with the exosome biogenesis and release pathways (Figure 1). As we stated earlier, we have found that pretreatment of naïve recipient monocytes with exosomes of infection origin can lower pathogen burden in these cells during a subsequent infection, and hypothesize that a similar mechanism may be involved for microglia. Clearly, this effect may be due to EV-associated modulation of autophagy in the recipient cells, which has been demonstrated for microglia by several recent studies that we discuss in the next section. To provide the necessary background for full appreciation of this crosstalk, in this section we describe the autophagy pathway and signaling network interactions that regulate it. There are three major pathways for mammalian autophagy: microautophagy, macroautophagy, and chaperone-mediated autophagy (Ke 2018; Parzych and Klionsky 2014). Although each of these three pathways differ in the way their functions are carried out, the endpoint for all three is fusion with lysosomes in order to break down and recycle materials for cellular use or destroy engulfed pathogens (Ke 2018; Parzych and Klionsky 2014). The focus of this review relates to macroautophagy (referred to simply as autophagy here), which is involved in the engulfment of bacterial and viral pathogens. Key steps in the process of autophagy include the nucleation step to form a phagophore, elongation of the phagophore membrane and vesicle completion to produce an autophagosome, and fusion of the autophagosome with lysosomes that results in vesicle breakdown and degradation of the inside cargo (Bhattacharya and Eissa 2015). As depicted in Figure 1, during infection, the process of autophagy results in engulfment of intracellular pathogen into autophagosomes followed by fusion with lysosomes to form the autolysosome, which carries hydrolases that help degrade the pathogen.

Progression of autophagy through its multiple steps is mediated by specific protein complexes that contain autophagy-related (ATG) proteins. For instance, the UNC-51-like-

kinase complex (ULK), which consists of ATG101, ATG13, FIP200 and ULK1/2, and the vacuolar protein sorting mutant 34 (Vps34) type I complex, which contains BECLIN1, VPS34, p150 and ATG14, participate in the process of phagophore formation and elongation (Chen et al. 2014; Ktistakis and Tooze 2016). As part of this, ULK is recruited through binding of ATG16L1 protein within the ATG12–ATG5-ATG16L1 complex to reinforce the activity of the Vps34 complex (Ktistakis and Tooze 2016). Another example is the ATG7–ATG3 complex, which similar to the ATG5– ATG12 complex is a ubiquitin-like conjugation system and allows the covalent linkage of ATG8 (referred to as LC3B in mammals) to phosphatidylethanolamine (PE) on the growing autophagosomal membrane (Bhattacharya and Eissa 2015; Ichimura et al. 2000; Mizushima et al. 1998). The last stage, which is fusion of autophagosome with the lysosome, is mediated by SNARE and Rab7 proteins. This fusion result in the formation of autolysosomes (Huotari and Helenius 2011; Hyttinen et al. 2013). ATG14, which is part of the Vps34 complex, binds the SNARE core domain of STX17 to stabilize the STX17-SNAP29 complex on the surface of the autophagosomes and priming it to interact with VAMP8 in order to promote autophagosome-lysosome fusion (Ktistakis and Tooze 2016).

In order to understand autophagy regulation at a molecular level, an in-depth comprehension of the signaling network mechanisms is essential. Multiple host signaling pathways have been identified that regulate autophagy. The main pathways and their connectivity, including the proteins that affect autophagy in microglia and are involved in response to infection, are shown in Figure 2. For instance, AKT activation leads to activation of mammalian target of rapamycin complex 1 mTORC1 through phosphorylation of ATG13 and ULK1/2 (Bestebroer et al. 2013), resulting in negative regulation of autophagy (Kim and Guan 2015). AKT itself can get activated by focal adhesion kinase (FAK), which can also activate STAT-3 to cause autophagy inhibition (Figure 2). Work from our group has demonstrated that at 8 hours post infection with *Yersinia pestis* (Yp) bacteria, a coordinated response to downregulate autophagy occurs that involves the functions of several proteins depicted in Figure 2 (Alem et al. 2015), including AKT, p53 (Alem et al. 2015), FAK, STAT3, and ERK1/2 (Alem *et al.*, manuscript in preparation). The FAK-Src-STAT3 signaling network has also been shown to regulate autophagy in response to some other infections, including *Toxoplasma gondii* (Portillo et al. 2017) and HIV-1 (Van Grol et al. 2010). Interestingly, some pathogens rely exclusively on activation of autophagy in the host cell for survival. For instance, recent studies show that JEV activates autophagy in order to grow and replicate (Ke 2018). Recent work also shows that methamphetamine along with HIV-Tat induce autophagy in the presence of ATG5 and ATG7 as indicated by increased protein levels of Beclin-1 and LC3II, increasing the risk of exposure to HIV-1 and contributing to neuronal dysfunction in HIV-associated neurocognitive disorders (Li et al. 2018). Given that some of the signaling proteins shown in Figure 2 such as mTORC1 or Beclin 1 are known to regulate autophagy in microglia and also play a role in exosome-autophagy interactions, analysis of their contributions to exosome-autophagy crosstalk during microglial response to infection are important to perform in future studies.

Autophagy-exosome interactions

Recent discoveries have highlighted the presence of cross regulatory mechanisms between exosome and autophagy pathways (Figure 1). Considering this interplay and the fact that autophagy is a major mechanism for regulating activation of microglia during infection (Su et al. 2016), such as during response to neuroAIDS (El-Hage et al. 2015), it is of great interest to understand how exosome-autophagy crosstalk influences microglial immune function. Several recent findings have demonstrated the importance of this crosstalk in microglial response. In one study, it was shown that neuronal cell line SH-SY5Y transfected with α-synuclein released exosomes that display overexpression of miR-19a-3p. Upon release, these exosomes stimulate recipient microglia to express miR-19a-3p. The increased expression of miR-19a-3p in exosomes also suppresses autophagy in recipient microglia, as confirmed by increased expression of P62 and decreased expression of LC3-II (Zhou et al. 2019). The modulation of autophagy by the miRNA is by targeting PTEN/AKT/mTOR signaling pathway in microglia (Zhou et al. 2019). Another recent study has shown that increased levels of miR-124–3P in microglial exosomes provides neuroprotection against traumatic brain injury through inhibition of neuronal autophagy (Li et al. 2019), an effect that may also play a role during neuronal infection. Influence on autophagy by exosome-associated miRNA has also been shown during response to infection by macrophages, the lineage to which microglia belong. Macrophages infected with M.tb secrete exosomes that are rich in miR-18a, which promotes intracellular survival of M.tb through inhibition of autophagy (Yuan et al. 2019). It has been shown that miR-18a prevents autophagy through inhibition of Ataxia-Talangiectasia Mutated (ATM) kinase, which is known to activate autophagy by suppressing mTORC1 (Tripathi et al. 2013). Furthermore, another very recent report has shown that autophagy has a role in the exosome-associated inflammatory responses by microglia (Xia et al. 2019). In this *in vivo* study, it was shown that exosomes derived from PD patients preferentially target microglial BV2 cells in mouse brain. Transfer of the exosomes led to inhibition of autophagy in the BV2 cells, as indicated by decreased Beclin1 and LC3-II protein levels, and accumulation of α-synuclein. It was shown that the inhibition of autophagy is achieved through activation of the Akt-mTOR signaling pathway in BV2 cells (Xia et al. 2019). Following the uptake of PD-derived exosomes, BV2 cells showed increased proliferation and increased secretion of proinflammatory cytokines and nitric oxide.

Both the autophagy pathway and the exosome biogenesis pathway are well conserved across different cell types. Exosomal biogenesis processes use highly conserved complexes that have been given different names depending on their cellular origin (Li et al. 2019). Similarly, the complexes and mechanisms involved during the autophagy process are highly conserved across all eukaryotes (Parzych et al. 2014). Consequently, the autophagy-exosome interaction mechanisms in other cell types are also directly relevant to what may occur in microglia and their consideration is quite informative. Emerging evidence shows that several ATG proteins are involved in regulation of exosome release or biogenesis in multiple cell types, including mouse embryonic fibroblast cells (MEFs), MDA-MB-231 breast cancer cells, and HCT116 colorectal cancer cells (Guo et al. 2017; Murrow et al. 2015). Of special note is the contributions of ATG16L1 and ATG5 to exosome production. ATG5 mediates the detachment of a regulatory component of vacuolar proton pumps from the MVBs,

preventing the creation of an acidic environment inside the lumen of MVBs and thereby allowing exosome release (Guo et al. 2017). This was demonstrated in part by the observation that knocking out ATG5 and ATG16L1 genes leads to a significant reduction in exosome release and alteration of exosomal content. Furthermore, when treated with V-ATPase inhibitor, ATG5 knockout cells regained the ability for efficient exosome release. This affirms the importance of luminal pH influence on the ultimate fate of the MVBs and whether they fuse with plasma membrane to release their content or fuse with lysosomes for degradation. It has been shown that treatment with alkaline agents that inhibit lysosomes increases EV secretion, and inhibition of lysosomes by Bafilomycin A treatment of neuronal cell line SH-SY5Y overexpressing α-synuclein increases the levels of α-synuclein released in EVs (Alvarez-Erviti et al. 2011). Bafilomycin A treatment of human bladder epithelial cells in which autophagy is already activated by either infection with *E. coli* K-12 or by rapamycin treatment also increases EV secretion (Miao et al. 2015). These and other similar findings (Eitan et al. 2016) suggest that vesicle accumulation due to inhibition of fusion with lysosomes forces the cell to extrude EVs such as exosomes in order to maintain vesicular homeostasis and dispose of unwanted materials. Indeed, findings suggest that the ability of HIV-1 to inhibit autophagosome degradation shifts the virus removal process during autophagy towards exosome export in cells within the CNS (Ojha et al. 2017). The function of ATG7 protein for potential effects on the exosome pathway has also been analyzed. ATG7 knockdown does not affect exosome release, suggesting that autophagosome formation is not involved (Jing et al. 2018). However, knockdown of ATG7 or Beclin1 in human hepatocytes infected with HCV significantly decreases the release of exosomes that carry the virus, suggesting that both of these core autophagy proteins are important for HCV incorporation into vesicles (Shrivastava et al. 2016). These findings suggest that while ATG7 does not affect EV release it may nevertheless have a role in packaging of EVs. On the other hand, the ATG9 protein may be involved in regulating EV release as ATG9 has been implicated in the formation of intraluminal vesicles (ILVs) in Drosophila (Bader et al. 2015). While several of the ATG proteins function to induce EV release, modulation of autophagy can also lead to its inhibition. Conditions that induce autophagy such as rapamycin treatment and starvation cause the fusion of autophagosomes with MVBs to form amphisomes, thereby preventing release of exosomes (Fader et al. 2008). Furthermore, recent findings show that induction of autophagy reduces exosome release of the pathological isoform of prion (PrP^{Sc}) in both infected central and peripheral neuronal cells ScCAD5 and ScN2a (Abdulrahman et al. 2018). Upon activation of autophagy in the infected cells by treatment with rapamycin, a significant decrease in exosome release is observed, along with an alteration in exosomal content (Abdulrahman et al. 2018). This reduction in exosome release is so significant that it nearly parallels the level of reduction observed in cells that are treated with GW4869, an established and widely used inhibitor of exosome release (Abdulrahman et al. 2018). On the other hand, downregulation of autophagy through inhibition of class III PI3K leads only to a slight increase in exosome release that is not statistically significant although the exosomal content of PrP^{Sc} and infectivity of ScCAD5 are significantly increased under these conditions (Abdulrahman et al. 2018).

Although the importance of LC3 for exosome pathway is not fully understood, recent investigations demonstrate that exosomes are preferentially enriched in lipid-modified LC3-

II (Guo et al. 2017). Also, the ATG12-ATG3 complex, which is involved in catalysis of LC3-II conjugation, interacts with Alix (ALG-2 interacting protein X) to regulate exosome biogenesis in mouse embryonic fibroblasts (MEF) (Murrow et al. 2015). Knockdown of Alix reduces autophagy flux in nutrient-rich conditions, indicating regulatory relationships between exosome biogenesis and autophagy (Murrow et al. 2015).

In the exosome-autophagy interplay, components associated with the exosome pathway have also been found to regulate autophagy. The tetraspanin CD63, a well-known exosomal marker, has been shown to play a key role in regulation of autophagy. CD63 downregulates Epstein-Barr virus LMP1-mediated mTORC1 activation, resulting in autophagy induction (Hurwitz et al. 2018). Furthermore, mTORC1, a known inhibitor of autophagy, also inhibits exosome release both in vivo (hepatocytes) and in vitro (MEFs and HeLa cells) (Zou et al., 2019). mTORC1 inhibition rescues exosome release without altering exosomal cargo, suggesting that while mTORC1 modulates exosome release it does not influence exosome formation.

Considering the above findings, the implications of autophagy-exosome interplay for microglial response to infection within the CNS merit strong attention in future studies. Aspects discovered in other cell types and discussed above, such as involvement of specific ATGs in exosome packaging during infection, or the influence of exosome-associated tetraspanins on autophagy response during infection, are likely to apply to microglia as well and need to be addressed in future investigations. As described earlier, the influence of exosome-autophagy crosstalk in microglia has been shown for PD, a neurodegenerative disease that is thought to be triggered by infectious agents. Microglial function is also important for neuroAIDS, another major neurological disease of concern that is caused by infection and for which the relevance of exosome functions and autophagy pathway has been amply demonstrated. A deep understanding of how different ATG proteins may be influencing microglia-derived exosomal release and content, and how in reverse the contents of microglia-derived exosomes may regulate autophagy response in infected host, will provide key insights into the mechanisms of CNS pathologies such as PD and neuroAIDS that are impacted by microglia.

FINAL REMARKS AND FUTURE DIRECTIONS

For over a decade, the study of the communicative roles of exosomes and other EVs have enjoyed tremendous attention and growth. While much excellent work has been done in the EV field, many unanswered questions and significant gaps in knowledge still remain given the complexity of these vesicles and also the complexity of their interactions with their surrounding milieu. In particular, their role in regulation of innate immune response is still in relative infancy. One aspect of this is understanding EV/EX functions related to microglia as part of their innate immune roles in the CNS. For instance, a great deal remains to be elucidated as to how EV-based communication between microglia and other cell types of the CNS such OLs, neurons, and astrocytes coordinate responses to pathogenic infections. *In vitro* co-culture experiments in the context of infection and *in vivo* approaches that target specific CNS cell types or molecular pathways in infected animal models will provide important insights. Mouse models such as CX3CR1^{CreER} or CD11b-HSVTK^{mt-30}, which

allow microglial ablation in adult mice CNS, or mice deficient in specific components of the autophagy machinery (e.g., conditional brain-specific *Atg* knockout mice), provide unique opportunities for *in vivo* mechanistic investigations. In addition, proteomic approaches that allow quantitative analysis of the signaling events that form the foundation of EV effects during pathogenic infections need to be applied extensively in order to form a more in-depth picture of the underlying complexity. Platforms such as reverse-phase protein microarrays (RPMA), which we have successfully applied for high-throughput quantitative profiling of signaling changes in response to infection or exosome treatments (Alem et al. 2015; Fleming et al. 2018; Fleming et al. Under review) can be applied to provide a wealth of knowledge about the molecular mechanisms involved. The work by our laboratory and others have identified specific innate immune responses that are induced by exosomes derived from infected monocytes or macrophages (Figure 3). In this regard, it will be of interest to study whether some of the same responses occur in the CNS cells that receive exosomes released by infected microglia (Figure 3). Understandably, in general, there also exists a continuing need to develop new assays that allow better differentiation of exosomes from other EV subtypes. Comprehensive proteomic analysis of the EV subtypes to identify set of marker proteins specific to each will be worthwhile to perform. This information would also allow development of antibody-based multiplexed platforms for improved separation of different EV subtypes.

One emerging and fascinating facet of exosomal regulation of innate immune response to infections is the exosome-autophagy cross regulatory mechanisms. The full nature of signals that determine the fate of the MVBs, dictating whether they fuse with the plasma membrane to release exosomes or are used to make amphisomes, and how this influences infectious diseases, need to become determined. As it was discussed in this review, several of the proteins that are involved in autophagy also affect exosome biogenesis and release. How such interplay influences infectious disease progression remains to be fully investigated. This includes an understanding of how autophagy-exosome interaction impacts microglial function as resident macrophages of the CNS.

Exosomes present with attractive features for development of novel therapeutic and vaccine platforms, and the knowledge from the current and future studies of how they regulate immune response will certainly aid such endeavors. In addition to having the advantage of being naturally produced, their excellent target interaction properties and ability to shield their cargo from immune recognition makes them attractive vehicles for drug and nucleic acid delivery. This is particularly attractive for CNS applications as exosomes derived from brain cells display brain-specific surface proteins, a feature that together with the small size of exosomes allows them to cross the blood-brain barrier (BBB) for targeted drug delivery to the brain. The next decade promises to be an extremely exciting time for the EV field and many breakthrough advances are anticipated.

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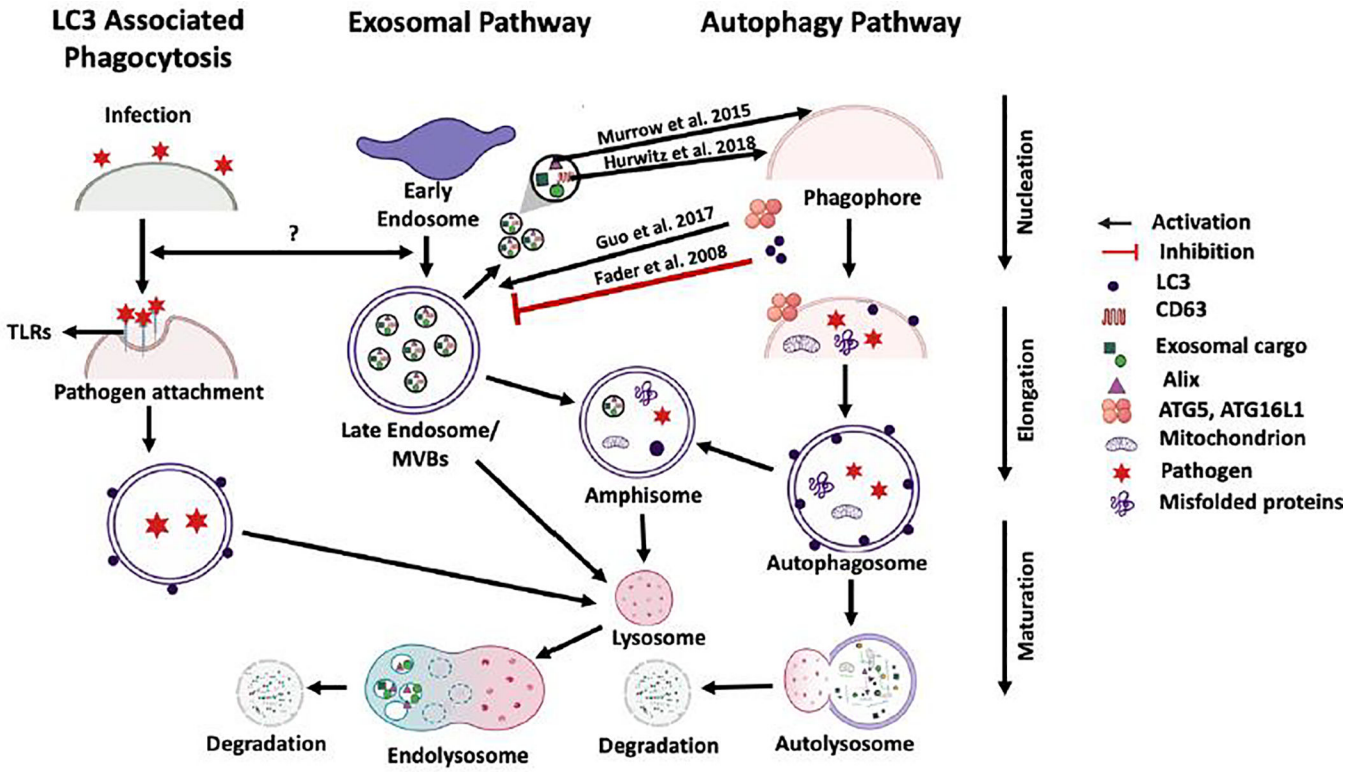

Figure 1: Exosome-Autophagy Interplay.

The pathways for LC3-associated phagocytosis (LAP autophagy), exosome biogenesis and release, and macroautophagy are indicated. The last stage of progression for all three pathways can be fusion with lysosomes for degradation of vesicular components. For the exosome pathway, the MVB can either be shuttled to the plasma membrane to release exosomes or fuse with autophagosome from the autophagy pathway to form amphisomes that subsequently fuse with lysosomes. Arrows between the pathways indicate the nature of the crosstalk between them, with the red line indicating inhibition and black arrows indicating activation or interaction. LC3 protein has been shown to negatively regulate exosome release while ATG5 and ATG16L1 proteins show positive regulation. CD63 and Alix proteins associated with released exosomes induce autophagy. It is not clear at this time whether regulatory cross talk exists between the LAP process and exosome biogenesis and release pathways.

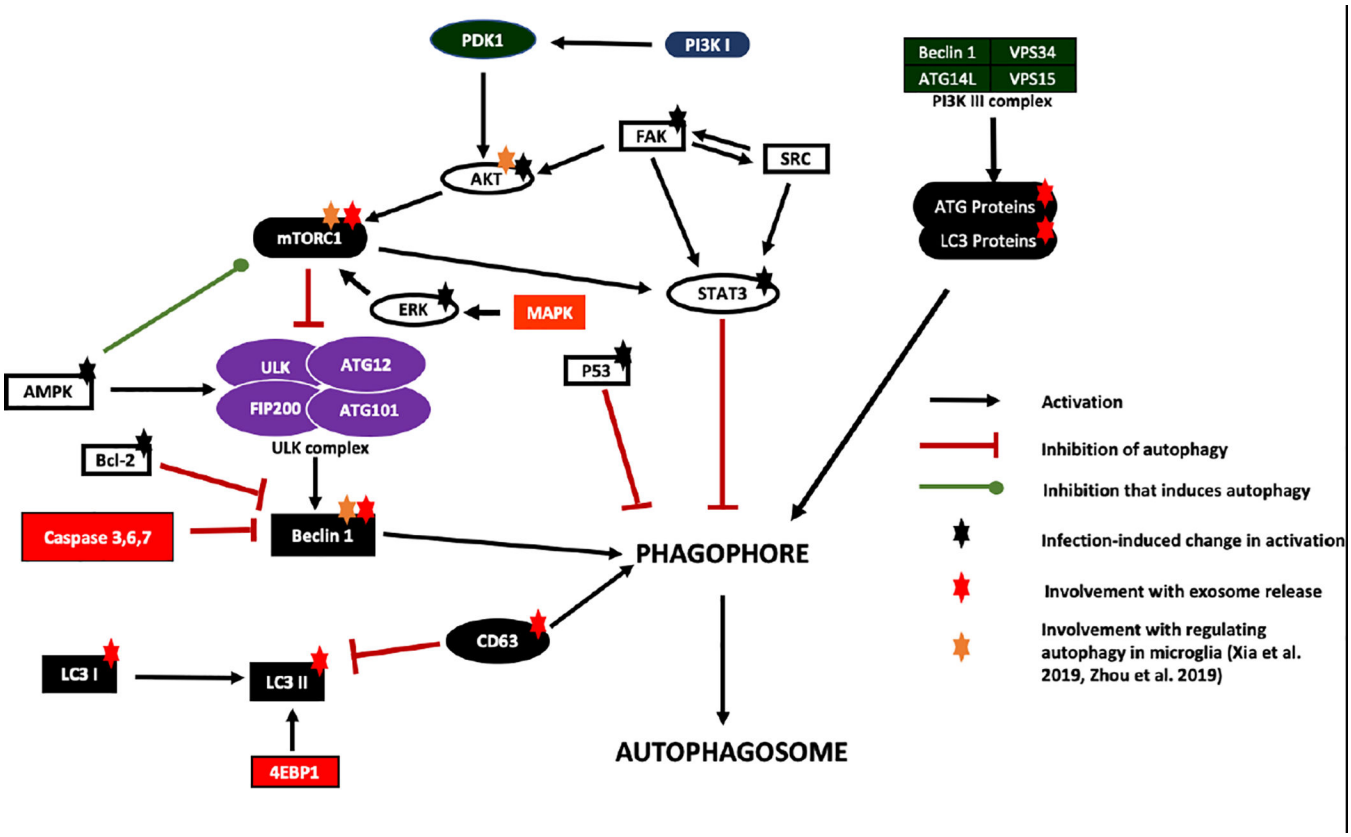

Figure 2: Protein signaling networks regulating autophagy.

Main signaling proteins and pathway networks that carry the task of regulating autophagy through effects on phagophore formation or autophagosome formation are shown. Proteins that undergo activity changes during infection, resulting in modulation of the autophagy response, are designated with a black star. Proteins involved in the exosome pathway that also regulate autophagy are designated with a red star. The regulatory proteins that have been shown to influence autophagy response in microglia are designated with a gold star. Red lines indicate inhibition. Green line designates inhibition that ultimately results in induction of autophagy. Black arrows indicate activation.

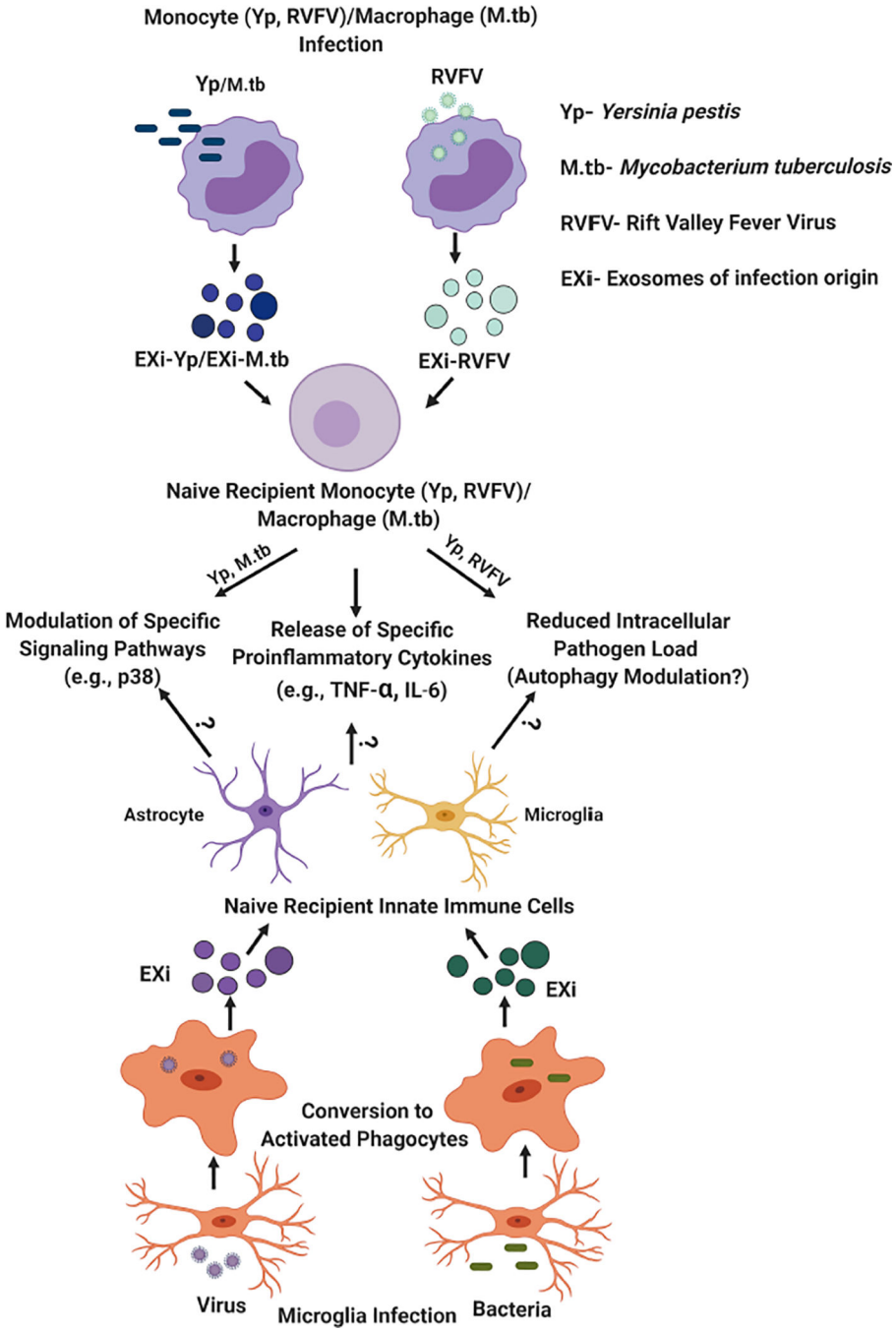

Figure 3: Regulation of innate immune response by exosomes derived from infected monocytes/ macrophages (EXi) and potential implications for functions of exosomes produced by infected microglia.

As summarized by the upper half of this figure, the work from our laboratory and published studies from other groups, such as findings for *M. tuberculosis* infection (Bhatnagar et al. 2007; Singh et al., 2012), have demonstrated that exosomes derived from human monocytes/ macrophages infected with bacteria such as *Y. pestis* or *M. tuberculosis,* or with the RVFV virus, regulate innate immune responses to infection in specific ways. These include inducing naïve recipient monocytes/macrophages to release proinflammatory cytokines such

as TNF-α or IL-6, and inducing significant changes in the activation states of specific signaling proteins in these cells such as the p38 MAP kinase. Our work with both Yp and RVFV has also demonstrated that these exosomes make the naïve recipient cells refractory to subsequent infection, an effect that is likely to involve autophagy since significant changes in autophagy occur as part of host response to both Yp and RVFV (Alem et al. 2015; Moy et al. 2014). The bottom half of this figure summarizes our speculation/ hypothesis about microglia, given their role as resident macrophages of the CNS, the findings summarized in the top half of the figure, and the published evidence discussed in the manuscript on effects of exosomes released from infected microglia. We propose that exosomes produced by infected microglia may also have very similar effects on recipient innate immune cells within the CNS (uninfected microglia and astrocytes) as part of mounting an effective response. These may include induction of proinflammatory cytokine release, induction of specific signaling changes, and induction of increased ability for pathogen clearance through modulation of the autophagy pathway.