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Human microglial models to study host–virus interactions

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Abstract

Microglia, the resident macrophage of the central nervous system, are increasingly recognized as contributing to diverse aspects of human development, health, and disease. In recent years, numerous studies in both mouse and human models have identified microglia as a "double edged sword" in the progression of neurotropic viral infections: protecting against viral replication and cell death in some contexts, while acting as viral reservoirs and promoting excess cellular stress and cytotoxicity in others. It is imperative to understand the diversity of human microglial responses in order to therapeutically modulate them; however, modeling human microglia has been historically challenging due to significant interspecies differences in innate immunity and rapid transformation upon *in vitro* culture. In this review, we discuss the contribution of microglia to the neuropathogenesis of key neurotropic viral infections: human immunodeficiency virus 1 (HIV-1), Zika virus (ZIKV), Japanese encephalitis virus (JEV), West Nile virus (WNV), Herpes simplex virus (HSV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). We pay special attention to recent work with human stem cell-derived microglia and propose strategies to leverage these powerful models to further uncover species- and disease-specific microglial responses and novel therapeutic interventions for neurotropic viral infections.

1. Introduction

1.1. Microglia maintain brain homeostasis in disease and health

Microglia are the primary innate immune cells of the central nervous system (CNS), contributing a significant role in regulation of brain development and maintenance of brain homeostasis during conditions of health and stress. Under homeostatic conditions,

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microglia support the health of neurons and other cells of the CNS by sensing activity of neurons, clearing apoptotic cells and pruning synapses via phagocytosis, and modulating neurogenesis (Borst et al., 2021; Diaz-Aparicio et al., 2020). Microglia exist in a ramified, or "rested" state at homeostasis, but upon activation by stressors such as trauma, ischemia, and pathogens, exhibit diverse responses (Chen et al., 2019). These can include reduced ramifications with a more ameboid morphology (Illes et al., 2020), alterations in phagocytic ability, motility (Fu et al., 2014), and release of proinflammatory cytokines and chemokines (Xu et al., 2022). While a proinflammatory microglial response can be crucial for limiting the pathogenic effects of stressors, in some cases, an inappropriate or prolonged response contributes to disease pathogenesis and exacerbates CNS damage (Westman et al., 2021). In the context of viral infection, the factors contributing to the protective or pathogenic role of microglia are still being discovered. In this review, we will first discuss the human or humanized microglia models that have been developed and their main applications. We will then describe mechanisms by which microglia detect and respond to viral pathogens in the CNS, how microglia have broadly been known to play a role in the clinical pathogenesis of neurotropic viruses, and how these findings have been validated *in vivo*, *ex vivo*, and *in vitro*. Lastly, we will discuss how existing human microglial models are being used to study neurotropic viral pathogenesis. In particular, we will focus on neurotropic viruses that directly infect microglia (HIV-1), infect neurons with some known tropism in microglia (ZIKV, JEV, HSV), and infect neurons with no or little known documentation of microglial infection (SARS-CoV-2, WNV).

2. Model development

Animal models and cultures have assisted in characterizing the role of microglia during neuroinfection, but there are key interspecies differences between animal and human microglia, as well as animal and human disease symptomologies. For instance, humans and mice differ in their immune cell composition (Zschaler et al., 2014), expression of genes related to immune, cell cycle, and age (Gosselin et al., 2017; Hasselmann and Blurton-Jones, 2020), and sensitivity to immune signaling (Zschaler et al., 2014). This may lead to conclusions that are not translatable to human pathologies. Thus, there is a need for clinically relevant, inexpensive, and replicable human microglial models to uncover how human microglia respond to and regulate neuroinfection.

Human microglia are historically challenging to study due to their inaccessibility, heterogeneity, and reliance on the external environment for their tissue-specific cellular identity (Gosselin et al., 2017). Fortunately, several key advances have been made in recent years to enhance modeling of microglia as well as their interactions with other CNS cell types. A graphical summary of these human microglial models and their applications to study neurotropic viral infections is shown in Table 1.

2.1. Primary human microglia

Cultured human fetal and adult microglia isolated from post-mortem brain tissue have contributed to our understanding of basic microglial biology and are one of the most ontogenetically representative models of human microglia (Popova et al., 2021).

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Subsequently developed *in vitro* microglial models are often benchmarked against primary microglia, though the transcriptome of primary microglia changes rapidly upon introduction to the *in vitro* environment (Gosselin et al., 2017). Furthermore, primary human microglia are difficult to access, plagued by highly variable yields, technically difficult to isolate, do not replicate in culture, and are impacted by the postmortem interval and the condition of the donor before isolation (Marsh et al., 2022). Finally, minute deviations in isolation techniques can impact microglial gene expression that may persist if cells are cultured post-isolation (Marsh et al., 2022).

2.2. Immortalized human microglia cell lines

To address the limited yield of primary microglia while maintaining species specificity, numerous immortalized human microglial cell lines have been generated. Many of these lines were created through lentiviral transduction of primary microglia to generate oncogenic gene transformation. Popularly used lines include HMC3 and C13NJ (Garcia-Mesa et al., 2017). However, both of these lines were derived from the CHME- 5 cell line, which has recently been suggested to be contaminated by rat cells (Garcia-Mesa et al., 2017). An alternative are the SV40 microglia lines, which were generated by transducing human embryonic brain- derived macrophages with the large T antigen of the SV40 oncogene and include the hμglia SV40 line (Garcia-Mesa et al., 2017). While human microglial cell lines are accessible, easier to maintain and genetically manipulate, and have a high proliferative capacity, their expression of oncogenes results in unrestrained proliferation that is not representative of primary microglia. The immortalized lines recapitulate some, but not all, of the *in vitro* functionality of primary microglia, an area of ongoing debate (Melief et al., 2016).

2.3. Human stem cell-derived microglia

Techniques to develop stem cell-derived microglia have rapidly advanced the generation of microglial models that are renewable and more representative of not only homeostatic microglia, but also patient- derived disease-associated microglia. The general principle of differentiating induced microglia (iMGs) from human embryonic or induced pluripotent stem cells lines has been to mimic the developmental steps that microglia undergo in normal development or to utilize transcription factor overexpression for directed differentiation.

Contrary to other CNS cell types that are ectodermally-derived, microglia arise from the mesodermal lineage, and more specifically, primitive hematopoiesis from yolk sac macrophages, which develop into erythromyeloid progenitors (EMPs) that infiltrate the brain beginning in the fourth week of conception in humans (Ginhoux et al., 2010; Kierdorf et al., 2013; Menassa et al., 2022). Throughout life, microglia are self- renewing from within their tissue-resident pool, independent of peripherally bone marrow-derived definitive hematopoiesis (Ajami et al., 2007). In mice, the microglial maturation process is dependent on colony stimulating factor receptor 1 (CSF1R) signaling and expression of transcription factors IRF8 and PU.1 (Kierdorf et al., 2013). Mimicking this ontogeny, multiple protocols first differentiate embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) into EMPs mimicking primitive hematopoiesis, before coaxing them into iMGs with a cocktail of growth factors, followed by maintenance with further

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maturation and survival factors. General strategies to generate iMGs include embryoid body (EB)-based differentiations, monoculture-based differentiations, and recently, transcription factor overexpression-based directed differentiation (Table 1). The intricacies and variations between iMG protocols have been reviewed in greater detail in Hasselman and Blurton-Jones and Timmerman et al. (Hasselmann and Blurton-Jones, 2020; Timmerman et al., 2018).

2.3.1. Embryoid body-based differentiation—

One of the first protocols to successfully derive iMGs from ESCs and iPSCs cultured cystic EBs on poly-D-lysine plates with IL-34 for 14 days until they expressed PU.1, a marker for early yolk sac myelogenesis (Muffat et al., 2016). Afterwards, they replated PU.1+ EBs on polystyrene plates to exclude the growth of neuro-ectoderm-derived cells and assessed expression of mature microglial markers at 74 days *in vitro* (DIV). While this step positively selects for iMGs, the initial presence of cells from the neuroectoderm lineage in EBs lowers the resulting iMG yield from each EB. Other EB-based protocols have since been described, generating microglia in as little as 21 DIV (Haenseler et al., 2017; Trudler et al., 2021).

2.3.2. Monoculture-based microglial differentiations—

Another, now foundational, approach utilizes a monoculture instead of EBs to generate myeloid progenitor cells, which results in less heterogeneity and further improves the differentiation efficiency from the starting stem cell population (Douvaras et al., 2017). Additionally, instead of manually selecting EBs, they use fluorescence activated cell sorting or magnetic beads to isolate microglial progenitors, improving the ease of the protocol. Subsequently developed monoculture-based protocols mimic cell-to-cell interactions that are more representative of *in vivo* cellular interactions. These studies drive iPSCs into CD43+ myeloid hematopoietic progenitors, then differentiate these into iMGs using a mixture of microglial differentiation factors, as well as secreted factors from astrocytes and neurons to mimic the brain environment (Abud et al., 2017; McQuade et al., 2018). These protocols assess mature microglial markers from differentiated iPSCs in as little as 38 DIV (Abud et al., 2017; McQuade et al., 2018). They identified macrophage colony- stimulating factor (M-CSF, also called CSF1), IL-34, and transforming growth factor beta (TGFβ−1) as cytokines that are crucial for the formation of homeostatic microglia (McQuade et al., 2018).

2.3.3. Induced microglial differentiation through transcription factor overexpression—

While the aforementioned protocols all require at least 21 days from the starting stem cell population to mature microglia, recently, an eight day induced microglia differentiation protocol has been developed (Drager et al., 2022¨). This approach generated a human iPSC line with inducible expression of six transcription factors that drive microglial differentiation: SPI1 (also called PU.1), MAFB, CEBPα, CEBPβ, IRF5, and IRF8. Induced transcription factor microglia-like cells (iTF-Microglia) recapitulated phagocytic function and immune response and could be applied to patient iPSCs to further uncover host genetic contributors to the neurotropism of infectious diseases. The challenge of this approach is the technical difficulty in generating cell lines, limiting the broad applicability of this approach.

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2.4. Human monocyte/cord blood macrophage-derived microglia

One emerging drawback of ESC-/iPSC-derived cells is that reprogramming patient primary somatic cells to a pluripotent lineage erases epigenetic or age-related changes which can potentially influence the disease process. This has been extensively studied in neurons, with the development of direct differentiation from fibroblasts to neurons (Mertens et al., 2018). Techniques for induced differentiation of primary somatic cells to microglia that overcome the ontological difference between monocytes and microglia has not been reported. However, differentiation of monocytes to monocyte-derived microglia (MDMs), through the addition of microglial-growth factors has been developed (Ohgidani et al., 2014). One of these protocols features culturing human monocytes with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-34 for 14 days and results in ramified microglia-like cells that express CD11b^{high}/CD45^{low} and CX3CR1^{high}/CCR2^{low}, phagocytose and release pro- and anti-inflammatory cytokines (Ohgidani et al., 2014). Subsequent studies have included additional factors, such as M-CSF, nerve growth factor (NGF)-β, and CC chemokine ligand 2 (CCL2), to study HIV (Akiyama et al., 2020; Rawat and Spector, 2017) and JEV infections (Lannes et al., 2017). To investigate maternal-fetal priming of microglia in the context of SARS-CoV-2 infection, one group adapted this protocol to differentiate microglia from umbilical cord blood-derived mononuclear cells (Sheridan et al., 2021). While this approach conserves patient-specific epigenetic factors, it derives microglia-like cells from monocytes, which do not ontologically arise from EMPs. Consequently, when transplanted into immunodeficient mice, they are transcriptionally different compared to transplanted microglia.

2.5. Applications of co-cultures to model CNS cell-cell interactions in vitro

Microglia from any source, studied in isolation *in vitro*, lack the CNS microenvironment and lose a portion of their tissue-resident cell fate. Co-culture of microglia with other CNS cell types not only enhances microglial tissue-resident differentiation (Haenseler et al., 2017; Muffat et al., 2016) but allows for modeling of CNS cellular interactions (Alvarez-Carbonell et al., 2019).

Multiple studies have shown that either 2D or 3D co-culture of microglia with neurons and astrocytes enhances expression of microglial gene signatures (Muffat et al., 2016). For instance, engraftment of iPSC/ESC-derived EMPs into either brain parenchyma or intranasally to access lung tissue resulted in tissue-specific macrophage marker expression, suggesting that stem cell-derived EMPs can take on multiple tissue- resident phenotypes (Takata et al., 2017). Depending on the co- culture setup, it is possible to observe microglial migration kinetics, which could be applied to address important questions about how microglia are involved in the spread and persistence of infectious disease in the CNS. One group generated a 3D culture of neurons and astrocytes in a microfluidic platform, then seeded iMGs in a ring around the culture to observe microglial migration dynamics in the context of Alzheimer's disease (Park et al., 2019). While co-cultures can improve the microglial gene signature and make it possible to observe microglial interaction with other cell types, they can reduce iMG yield and require more downstream processing to separate cell types for analysis.

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Human brain organoids (hBORGs) have emerged as a more representative system to study complex interactions between multiple CNS cell types in a 3D exclusively human environment, while allowing for more faithful recapitulation of the 3D interactions of CNS cells. While hBORGs contain neurons, astrocytes, and oligodendrocyte progenitors and can be generated from one neural progenitor cell (NPC) population, it is more challenging to incorporate microglia, as they originate from a different primary germ cell lineage. Additionally, heterogeneity in microglial and organoid differentiations between protocols, cell lines, and batches present an ongoing challenge (Quadrato et al., 2016). One method that incorporates microglia into hBORGs co-cultures hBORGs with human microglial cell lines, such as the SV40 immortalized microglia (Abreu et al., 2018). While this technique is convenient in that it makes use of a previously generated microglial cell line, it comes with the caveat that immortalized microglia carry oncogenes that confound their application to the study of *in vivo* microglia and may exhibit unrestrained proliferation. To circumvent the use of immortalized microglia, other groups have generated microglia-containing hBORGs from the same stem cell population. One study found that some unpatterned hBORGs already contain a small population of mesodermal cells which can spontaneously differentiate into Iba1+ microglia-like cells, starting at 31 DIV (Ormel et al., 2018). However, this results in a limited amount of microglia in hBORGs (1% of cells), whereas microglia have been reported to make up 5–10% of all CNS cell types, depending on the brain region (Frost and Schafer, 2016). Another study found that co-culturing NPCs with primitive macrophage progenitors resulted in a more standardized and physiologically representative microglia ratio (7%) in hBORGs (Xu et al., 2021). This technique also allows for greater control of the ratio of microglia in the model system. However, deriving different cell types from neuroectoderm and mesoderm lineages incurs even more time and cost.

To address this issue, one group recently developed a method of inducing microglia-like cells, starting at 30 DIV, in cortical hBORGs by co-culturing non-transfected hESCs with hESCs that over express PU.1 in a 9 to 1 ratio (Cakir et al., 2022). While this protocol improves the convenience, consistency, and tunability of previous strategies to generate hBORGs that contain iMGs, still more progress can be made to standardize the distribution, integration and survival of iMGs within the organoid, as well as improve the efficiency of the microglia differentiation steps. The minimum and necessary transcription factors required for microglial identity is an area of debate and ongoing study.

Overall, hBORGs have revolutionized our ability to model complex interactions between multiple cell types in 3D and can even be applied to generate brain region-specific models (Fig. 1A) (Jacob et al., 2020). However, they are more costly and time-intensive to generate than 2D models. Furthermore, their lack of vascularization induces necrosis and immune activation, particularly as the models age, which oftentimes confounds their application to model the aging brain.

2.6. Xenotransplantation models of human microglia

While many of the aforementioned models have built upon one another to improve *in vitro* microglial modeling, the reality is that microglia exhibit substantial transcriptional changes in microglial-specific genes when they are removed from the CNS environment (Gosselin et

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al., 2017). These large-scale transcriptional changes may substantially influence outcomes of *in vitro* studies, including ones that utilize stem cell-derived microglia. Additionally, *in vitro* models lack vasculature, complex myelination, and adaptive immunity. Furthermore, due to the interspecies differences mentioned previously, it is challenging to translate findings from murine studies into humans (Gosselin et al., 2017).

To address the dual issues of *in vitro* microglial modeling and interspecies differences, xenotransplantation of human microglia precursors in immunodeficient mice has been reported. This approach involves peripheral engraftment of human CD34+ cord blood progenitor cells into immunodeficient and/or irradiated mice (Dash et al., 2021). These human cells populate and differentiate in tissue-resident macrophages within their niches, and infiltrate into the CNS to take on a microglia-like cell fate. While some studies have taken advantage of this model system to study microglial HIV-1 infection (Mathews et al., 2019), cord blood contains hematopoietic stem cells and their downstream progenitors, which arise from a different ontology than yolk sac macrophage-derived microglia (Hasselmann and Blurton-Jones, 2020). These ontological differences manifest in transcriptional variations between peripherally transplanted cord blood cells and true microglia, with the former resembling brain infiltrating blood monocytes (Ginhoux et al., 2010).

This model system has been reviewed further in depth in Dash et al. (Dash et al., 2021).

An alternative approach involves transplantation of human stem cell- derived microglial progenitors directly in the murine CNS. These models center around several key discoveries and features of microglial biology and cross-species xenotransplantation. Firstly, xenotransplantation requires eradication of murine B and T cells to prevent rejection of transplanted human cells. Thus, all currently applied models are in a $RAG2^{-/-}$ and $Il2RG^{-/-}$ background. A second fundamental feature is expression of humanized growth factors crucial for microglial survival. It has previously been shown that CSF1 (also called M-CSF) is not only key for microglial survival, but also that the murine CSF1 does not recognize the human receptor (Hasselmann et al., 2019). Therefore, all the applied murine lines have been humanized to express CSF1 ($CSF1^{h/h}$). The contribution of other humanized growth factors may also contribute to engraftment survival and efficacy.

The first murine model utilized is the MITRG mouse line which was developed for humanized hematopoietic engraftment and, in addition to $Rag2^{-/-}$ and $Il2rg^{-/-}$, is humanized for $CSF1^{h/h}$, $IL\text{-}3^{h/h}$, $GM\text{-}CSF^{h/h}$, and $thrombopoietin^{h/h}$ (Rongvaux et al., 2014). The initial study transplanted stem cell-derived hematopoietic progenitors into the lateral ventricles of early postnatal (day 1–4) MITRG mice (Hasselmann et al., 2019). These xenotransplanted microglia (xMGs) express homeostatic microglial markers, such as EGR1, P2RY12, TMEM119, CX3CR1, and SALL1, which were not expressed or are substantially downregulated *in vitro*. Furthermore, they observed higher transcriptional similarities between xMG and *ex vivo* human microglia, compared to iMGs and cultured primary microglia.

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While xenotransplantation studies have revolutionized the study of human microglia *in vivo*, the MITRG model is currently limited by the resident population of murine microglia in the brain parenchyma, which leads to substantial engraftment heterogeneity (Fattorelli et al., 2021). Depletion of all monocyte subsets through deletions of the CSF1R receptor leads to neurodevelopmental and multiorgan dysfunction, making this model unsuitable (Hume et al., 2020). Conversely, deletion of a highly conserved CSF1 receptor super-enhancer, the *fms*-intronic regulatory element (FIRE), is reported to have no developmental deficits and depletion only of microglia and a subset of skin and mammary macrophages (Rojo et al., 2019). A recent study crossed the FIRE murine model with the 5xFAD murine model of Alzheimer's disease in the $CSF1^{h/h}Rag2^{-/-}$ $Il2rg^{-/-}$ background and found greater cerebral amyloid angiopathy, blood brain barrier dysfunction, and brain calcifications, that was rescued by transplantation of wild type microglia (Shabestari et al., 2022). These results indicate the critical role that microglia play in neurodegenerative disease progression and the possibility for microglial transplantation as a potential therapeutic strategy.

Currently, xenotransplantation models have only been applied to the study of neurodegenerative diseases such as Alzheimer's (Mancuso et al., 2019), but this technique has the potential to contribute to our understanding of human microglia in neuroinfectious diseases, such as by transplanting infected microglia to observe their function in the brain parenchyma. However, the use of immunodeficient mice precludes their use in studying interactions between microglia and immune cells, and interactions between human microglia and murine CNS cells may not be completely representative of human microglia-human CNS cell interactions.

To address the issue of a lack of vascularization in brain organoids, groups have pioneered xenotransplantation of human brain organoids into immunodeficient mice (Mansour et al., 2018), neurons from which are capable of projecting into the host brain after transplantation (Revah et al., 2022). With further development and the addition of human microglia, these model systems could be utilized to elucidate complex signaling pathways between many human cell types in the context of neuroinfectious disease.

3. Studying microglia in neuroinfectious diseases

As innate immune cells of the CNS, microglia play an essential role in detecting and responding to viral pathogens. Autopsies of patient brains have exhibited microglial aggregates (Azevedo et al., 2018; Desai et al., 1995; Hackney et al., 2012; Soung et al., 2022; Thakur et al., 2021), microglia in the vicinity of degenerating neurons (Fekete et al., 2018), neuronophagia (Azevedo et al., 2018; Thakur et al., 2021), and infiltration of peripheral immune cells (Azevedo et al., 2018; Soung et al., 2022; Thakur et al., 2021), consistent with the high levels of inflammation and neuronal loss associated with viral infection in the CNS. These findings suggest that microglia become activated and may mediate neuronal loss and other neuropathogenic effects during CNS infection.

Some neurotropic viruses, such as HIV-1, infect microglia, while other viruses are thought to primarily infect neurons or astrocytes, leading to secondary microglia activation. Accordingly, microglia are equipped with receptors that can detect direct viral infection

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and secondary effects of infection by sensing environmental signals from other infected cells. Microglial activation could be neuropathogenic or neuroprotective. In this section, we review how microglia can detect intracellular viruses and environmental signals of infection and how microglia have been implicated in the neuropathogenesis of HIV-1, HSV, SARS-CoV-2, JEV, WNV, and ZIKV using different human microglial models (Fig. 1).

3.1. Microglia directly and indirectly detect viral pathogens

3.1.1. Direct viral sensing by pattern recognition receptors—Pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), and cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) are expressed by microglia (Jeffries and Marriott, 2017; Kigerl et al., 2014) and can detect viral molecules or nucleic acids (Kawai and Akira, 2007; Takeuchi and Akira, 2010). PRRs signal through adapter proteins to activate transcription factors in innate immune signaling pathways, such as nuclear factor-κB (NF-kB) or interferon regulatory factors (IRFs), which, in turn, induce expression of proinflammatory cytokines and type I interferons (IFN) (Kawai and Akira, 2007). Antiviral signaling pathways can prevent viral spread and promote microglial phagocytosis of infected cells. Pathways of microglial pathogen detection via PRRs have been reviewed in detail (Rodríguez-Gomez et al., 2020´).

3.1.2. Indirect viral sensing (Purinergic, glutamatergic, complement, and cytokine signaling)—Microglia express a diverse repertoire of receptors that enable crosstalk between microglia, neurons, and other glia. These receptors include purinergic receptors, glutamatergic receptors (GluR), complement receptor 3 (CR3), and cytokine receptors. Purinergic receptors mediate microglial migration by detecting changes in secreted nucleotides produced by neurons; under stressors like infection, this can act as a chemotactic signal for microglia to detect the affected neuron for phagocytosis (Chen et al., 2019; Liu et al., 2016). Glutamate, a neurotransmitter released by excitatory neurons, can bind to microglial GluR to regulate inflammation. Depending on the specific receptor that is activated, GluRs can modulate the transition into a proinflammatory or anti-inflammatory state (Taylor et al., 2005). When proinflammatory GluRs, such as group II mGlu2 receptors, are engaged, microglia become activated, migratory, and produce reactive oxygen species (ROS) and proinflammatory cytokines (Czapski and Strosznajder, 2021; Taylor et al., 2005). Interestingly, microglia can also produce glutamate when activated (Piani et al., 1991). Piani et al. found that neurotoxicity resulting from lipopolysaccharide (LPS) and IFNγ treatment of primary mouse microglia was mediated by N-methyl-d-aspartate receptor (NDMA), a key glutamate receptor expressed on neurons (Piani et al., 1991). Neuronal loss is also regulated by complement signaling; engagement of CR3 promotes synaptic pruning by microglia during development (Schafer et al., 2012) and elimination of debris, such as protein accumulation, via phagocytosis (Hong et al., 2016). Jiang et al. found an increase in complement signaling and microglial activation in the CNS of mice infected with MERS-CoV; when the pathway is inhibited, viral antigen, microglial activation, and apoptotic signaling in neurons is attenuated, suggesting that complement signaling may mediate neuropathogenesis of viral pathogens (Jiang et al., 2021). During viral infection, cytokines may be released from infected cells, such as neurons and astrocytes, to activate

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microglia, and conversely, microglia may produce cytokines if they detect neuronal stress resulting from infection (Hanisch, 2002). Microglia are one of the major producers of cytokines in the CNS and can produce proinflammatory cytokines in response to stressors including infection, and anti- inflammatory cytokines to promote regeneration (Alvarez-Carbonell et al., 2019; Wang et al., 2022). Primary mouse neuron cultures infected with ZIKV produce increased levels of TNF-α, IL-1β as a marker of inflammasome activation, and glutamate, which induce neuronal apoptosis in uninfected cells (Olmo et al., 2017). Thus, release of proinflammatory cytokines may promote a reactive microglial response as a means to control infection (Hanisch, 2002; Olmo et al., 2017), however, prolonged neuroinflammation can be highly neurotoxic, so the duration and degree of proinflammatory cytokine production is tightly regulated (Rodríguez-Gomez et al., 2020´).

3.2. Microglia as direct or indirect targets in CNS viral pathology

Microglia may contribute to neurotropic infections and disease progression as a site of primary infection or through indirect activation in different viral and clinical contexts. Whether microglia become directly infected or if they respond to environmental signals from other infected CNS cells appears to play a significant role in determining whether microglia are protective or pathogenic during infection in mice. Human microglia are directly infected by HIV-1 in patients and act as a CNS reservoir for this virus. JEV, HSV, and ZIKV antigen has been detected in microglia from post-mortem brains of infected patients; however, it is unclear whether these viruses actively replicate in microglia, or if viral antigen was detected as an artifact of engulfment of infected cells (Desai et al., 1995). Thus far, there is no evidence that SARS-CoV-2 and WNV lead to microglia infection in human patients (Cheeran et al., 2005; Thakur et al., 2021). However, even in the absence of direct infection, microglia may still play an important role in disease pathogenesis (Soung et al., 2022; Thakur et al., 2021). Interestingly, *in vivo* mouse studies and *in vitro* data from primary human microglial cultures and cell lines suggest that HSV, JEV, and ZIKV can infect microglia (Diop et al., 2018; Jeffries et al., 2020; Kumar et al., 2020; Lannes et al., 2019, 2017; Lokensgard et al., 2001; Lum et al., 2017; Martinez Viedma and Pickett, 2018; Muffat et al., 2018; Retallack et al., 2016). This emphasizes the discrepancy between primary human brain studies which, by necessity, involve late-stage disease and may be affected by prolonged postmortem intervals, as opposed to *in vitro* or nonhuman studies, which carry the caveat of environmental and species differences.

3.2.1. Human immunodeficiency virus 1—HIV-1 causes AIDS and is associated with neurologic diseases, including HIV associated neurocognitive disorder (HAND) and HIV associated dementia (HAD), also termed HIV-1 encephalitis (Clifford and Ances, 2013). Patients with HAND exhibit memory loss and decreased cognitive ability (Clifford and Ances, 2013). Cortical neuronal damage and synapse loss are key characteristics of fatal cases of HAND (Ru and Tang, 2017), and microglia are thought to contribute to this loss. Autopsy studies of brain tissue from individuals with HAND have identified infected microglia as an important cellular reservoir for HIV-1 as well as microglial aggregates, suggestive of microglia activation (Bagasra et al., 1996; Cosenza et al., 2002).

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Microglia are the main CNS cellular target for HIV-1 infection. Neurons are not infected, while astrocytes have shown variable permissiveness to infection (Cevallos et al., 2022; Kaul et al., 2001). Animal models and *in vitro* studies support the contribution of microglia to HIV-associated neuropathogenesis. Microglia depletion from primary mouse cortical cultures and murine spinal cords leads to a reduction in synapse loss following exposure to the neurotoxic HIV-1 gp120 protein (Ru et al., 2019). HIV-1 gp120 treatment of primary rat mixed glial/ neuron co-cultures also induces glial production of ROS that stimulates IL-1β-mediated neuronal death (Viviani et al., 2001). These studies suggest that microglial activation by HIV-1 gp120 could contribute to neurotoxicity. Treatment of a human neuronal cell line, primary human fetal cortical cultures, and primary rat neurons with HIV-1 Tat, a secreted protein in HIV-1 infection, has also been shown to induce high levels of neuronal death (New et al., 1998). Tat-induced neuronal death was found to be driven by TNF-α induction and glutamate receptor activation (New et al., 1998). Given that Tat is a secreted viral protein and that neurons were not co-cultured with microglia in this study, this finding implicates mechanisms of HIV-1-mediated neurotoxicity that are independent of microglia. However, Tat is also known to induce expression of TNF-α and other proinflammatory factors in a mouse microglia cell line (BV-2) that could similarly contribute to neuronal death (Silveira et al., 2022). It should be noted that these studies treated cells with purified HIV-1 proteins instead of infectious virus; it is possible that infection, which includes co-expression of many HIV-1 proteins, may have different effects.

3.2.1.1. HIV-1: microglial models.: Primary human microglia models have been utilized to further the understanding of HIV-1 regulation of neuroinflammation. As discussed above, neuronal loss is a hallmark of HAND, and proinflammatory factors produced by microglia are known to drive neuronal death during HIV-1 infection in murine cell cultures (Ru et al., 2019; Silveira et al., 2022; Viviani et al., 2001). HIV-1- infected primary human fetal microglia produce glutamate, which contributes to neuronal death of primary rat cortical cultures treated with conditioned media from the infected microglia (Fig. 1B) (Huang et al., 2011). Additionally, Zhao and colleagues found that HIV-1- infected microglia act as the principal producers of the proinflammatory cytokine, IL-16 (Fig. 1B) (Zhao et al., 2004). IL-16 production in the CNS may contribute to HIV-1 progression by recruiting CD4+ T cells that subsequently become infected (Hridi et al., 2021). Furthermore, infiltration of immune cells in the CNS could lead to additional neurologic injury and thus, progression of HAND.

Beyond their role in studying HIV-1 associated inflammatory activation, microglia have also been used to study HIV tropism and mechanisms of latency. Using primary human microglia and blood-derived macrophages, Strizki et al. found that neurotropism of HIV-1 is isolate- dependent, and is likely determined by differences in V3 loop sequences (Strizki et al., 1996). After HIV-1 enters the CNS, the virus establishes latency in microglia; Castellano et al. found that acute HIV-1 infection of primary microglia results in high rates of microglial death and that HIV- 1 establishes latency in surviving cells, possibly in a BCL-2-interacting mediator of cell death (Bim)- dependent manner (Fig. 1B) (Castellano et al., 2017). This suggests that apoptosis may be a host defense mechanism during HIV-1

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infection. Additional studies are needed to determine how microglial death following HIV-1 entry into the CNS affects latency and progression of HAND.

HIV-1 latency in microglia has also been investigated utilizing stem cell-derived (iMGs) and human blood monocyte-derived microglia (MDMs). Alvarez-Carbonell et al. probed the role of neurons in regulating HIV latency in microglia (Fig. 1B) (Alvarez-Carbonell et al., 2019); HIV-1 latently infects iMGs and primary human microglia in the presence of healthy neurons. However, in the presence of damaged neurons, HIV replication resumes (Fig. 1B) (Alvarez-Carbonell et al., 2019). Furthermore, basal replication of HIV-1 in microglia results in neurotoxicity later in infection. The exact factors driving neurotoxicity during basal HIV-1 replication in microglia have not been identified, and it is unclear if there is a difference between neurotoxicity during primary CNS invasion of HIV-1 compared to basal replication following HIV-1 latency.

Different studies utilizing MDMs have demonstrated differential susceptibility to HIV-1 infection. Thus, the applicability of applying these microglial models to the study of HIV is a topic of debate. Rawat et al. demonstrated that HIV-1 viral production and release of HIV-1 in infected MDMs is comparable to that of primary human microglia (Rawat and Spector, 2017). HIV-infected MDMs take on an activated phenotype, consistent with that of activated primary human microglia (Rawat and Spector, 2017). However, Akiyama et al. found that infection of human MDMs and iMGs with HIV-1 results in infection of iMGs but not MDMs (Akiyama et al., 2020). This is contradictory to other studies that have documented HIV-1 infection of human MDMs and could potentially be explained by differences in differentiation protocols or cellular ontogeny.

Because crosstalk between infected microglia and other CNS cell types is a major feature of HAD/HAND, hBORG models have been applied to probe these interactions. The first study to incorporate HIV-1- infected microglia in a hBORG system found that increased IL-1β expression correlates with viral production (Fig. 1B) (dos Reis et al., 2020). There was also decreased neuronal viability in the setting of HIV- 1 infection without evidence of astrocyte infection. Given the role of IL- 1β inflammasome activation in mediating neuronal death in primary rat glial/neuron cultures following HIV-1 gp120 treatment, it is possible that microglia similarly induce IL-1β in a ROS-dependent manner, and that the gp120 protein is driving this phenomenon (Viviani et al., 2001). Ryan et al. modeled some of the cell-cell interactions that drive progression of HAND in a tri-culture model of iPS neurons, astrocytes, and microglia (Fig. 1B) (Ryan et al., 2020). The authors found that in HIV- infected tri-cultures, neurons and astrocytes exhibit moderate changes in inflammatory response, and microglia become proinflammatory, producing IL-8 and IL-1β (Fig. 1B) (Ryan et al., 2020). The continued development of hBORG models to study HAD/HAND may enable the discovery of new biomarkers and therapeutics that rescue detrimental effects of HIV-infected microglia.

Lastly, Mathews et al. established chimeric models of HIV-1 infection with humanized bone marrow mice, engrafted with human hematopoietic stem and progenitor cells (HSPCs) that infiltrate the brain and differentiate into microglia-like bone marrow-derived cells (Mathews et al., 2019). They found that these mice become productively infected with

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HIV-1 and exhibit appropriate antiviral and inflammatory gene induction (Mathews et al., 2019). The development of complex iPS tri- culture and humanized mouse models to study microglial-mediated neuropathology in HIV-1 provide opportunities to investigate how microglia respond to other neurotropic viruses.

3.2.2. Herpes simplex virus—HSV-1 is the most frequent cause of sporadic fatal encephalitis worldwide. Following primary infection, HSV-1 and HSV-2 become latent in sensory ganglia and can reactivate under stress or immunosuppression (Cohen, 2020). Symptoms of HSV infection range from mild to severe, from herpetic skin lesions to meningitis and encephalitis (Zhu and Viejo-Borbolla, 2021). In most cases, cell death occurs in the temporal lobe (Whitley, 2006). Fatal cases of neonatal herpes simplex encephalitis (HSE) exhibit infiltration of macrophages and leukocytes in the parenchyma, parenchymal calcifications, and neuronophagia (Baumann et al., 1985).

The precise mechanisms of HSE neuropathogenesis are not known, however both direct virus-mediated and indirect immune-mediated mechanisms likely contribute to neuronal and glial cell death (DeBiasi et al., 2002; Lokensgard et al., 2001). Activated microglia are a well- documented phenotype in severe HSV infections. Microglial nodules have been found in severe cases of HSV-1 CNS infection (Hackney et al., 2012), and microglia can be found surrounding sites of HSV-1-infected neurons (Fekete et al., 2018). *In vivo* mouse studies support a protective role of microglia in regulating the CNS response to HSV-1 infection, as microglia depletion increased HSV-1 titers and progression of CNS disease (Katzilieris-Petras et al., 2022). Katzilieris-Petras et al. and Uyar et al. demonstrated that an early response to HSV-1 infection is critical to prevent mortality and disease progression, and Katzilieris-Petras et al. uncovered that type I interferon (IFN I) produced by microglia may mediate these protective effects (Katzilieris-Petras et al., 2022; Uyar et al., 2020). Thus, mounting a protective microglial response during CNS viral infection may be dependent upon timing.

3.2.2.1. HSV: microglial models.: Few studies have delineated the role of human microglia in HSV infection, however human microglia cell lines, iMGs, and primary human microglia have been applied to varying degrees. Jeffries et al. documented that the human microglia cell line, hμglia, express the DNA sensors cGAS and IFI16, but the ability of HSV- infected microglia to produce and secrete HSV viral particles is not attenuated by the presence of these PRRs (Jeffries et al., 2020).

Primary microglia have been utilized to understand HSV-1 replication dynamics and characterize the proinflammatory factors that are produced in infection. Primary human microglia were non-productively infected by HSV-1 but produced high levels of proinflammatory cytokines, TNF-α, IL-1β, IP-10 (also known as CXCL10), and RANTES (Fig. 1C) (Lokensgard et al., 2001). Thus, microglia may produce proinflammatory signals during HSV-1 infection that promote neuronal loss without amplifying virus (Marques et al., 2004).

Only one study has employed iMGs to model HSV-1 infection; Bodda et al. infected iMGs with HSV-1 in order to understand the mechanisms by which HSV-1 inhibits IFN

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induction (Bodda et al., 2020). The authors found that iMGs infected with HSV-1 lacking the deubiquitinase activity of the VP1–2 protein induced high levels of IFN in human and mouse microglial models and exhibited reduced viral replication in the brain (Bodda et al., 2020). This study showed that VP1–2 deubiquitination of STING is a mechanism of immune evasion and highlighted the potential for human microglial-HSV-1 studies to add to our understanding of disease pathogenesis.

3.2.3. SARS-CoV-2—Infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) may manifest in neurological symptoms, such as mild cognitive impairment, commonly referred to as “brain fog”, even long after the initial infection (Monje and Iwasaki, 2022). Retrospective clinical studies have defined new-onset and exacerbated neuropsychiatric disorders in SARS-CoV-2 disease patients. One study found that out of 214 patients, 53 [24.8%] had CNS symptoms such as headaches and dizziness, and 19 [8.9%] had PNS neurological symptoms such as loss of taste and smell (Mao et al., 2020). A systematic review showed that 26 [65%] of 40 patients with acute SARS-CoV-2 experienced delirium, a sudden severe confusion state (Rogers et al., 2020). To improve treatment of the neurologic sequelae of SARS-CoV-2, it is important to determine if and how microglia contribute to disease.

SARS-CoV-2 infects cells primarily through the ACE2 receptor (Letko et al., 2020). Single cell sequencing of the middle temporal gyrus revealed that the ACE2 receptor is expressed in neurons, astrocytes, oligodendrocytes, as well as endothelial cells lining the choroid plexus, but not microglia (Fernandez-Castáneda et al., 2022˜). Although SARS- CoV-2 may be incapable of infecting microglia, it nevertheless may exert secondary effects, either through the infection of other CNS cell types or through its effects on the peripheral immune system. For instance, postmortem SARS-CoV-2 patients exhibit higher microglial activation levels and increased IL-1β and IL-6 expression, especially in the hippocampus and medulla oblongata (Soung et al., 2022). Infection of other CNS cell types may lead to secondary upregulation of proinflammatory cytokines from microglia, and in severe cases, may result in a cytokine storm which can be detrimental to surrounding CNS cells (Vargas et al., 2020). Altogether these intersecting pathways lead to activation of astrocytes and greater recruitment of peripheral immune cells into the brain parenchyma, further driving neuronal dysfunction and death (Vargas et al., 2020).

3.2.3.1. SARS-CoV-2: microglial models.: Other studies with iPSC- derived CNS cells aimed to discover how SARS-CoV-2 gains access to the CNS. One study using iPSC-derived monolayer and organoid cultures found that SARS-CoV-2 productively infected choroid plexus organoids, but not cortical neurons, astrocytes, or microglia (Fig. 1A) (Jacob et al., 2020). However, another study reported SARS-CoV-2 infection of neurons in human brain organoids and validated these findings by observing SARS-CoV-2 in cortical neurons of deceased patients (Song et al., 2021). Notably, infection was not accompanied by immune cell infiltration. These conflicting results could be explained by discrepancies in the studied neuronal subtypes, the neuronal maturation stage, or possibly the susceptibility of individual donor lines to infection. Future studies exploring the conditions that allow for SARS-CoV-2 to infect neurons and neuronal subtypes are needed to identify the exact mechanisms of

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microglial-neural crosstalk in SARS-CoV-2. Although not directly infected, microglia may be an important instigator of the detrimental effect that SARS-CoV-2 has on brain function. The use of iMGs, in conjunction with co-cultures with other CNS cell types and genetic screens, could be integral in elucidating the role of human microglia in this disease.

3.2.4. West Nile virus—While the majority of infections with neurotropic flaviviruses are asymptomatic, infection can result in serious neurologic disease in a subset of patients (Chauhan et al., 2022). Individuals with neuroinvasive WNV infections can present with meningitis, encephalitis, acute flaccid paralysis, and movement disorders (Sejvar et al., 2003). In addition to acute neurological manifestations, patients with WNV may also experience prolonged neurologic symptoms, such as fatigue and confusion and only 37% of patients with symptomatic WNV infection reported an absence of prolonged symptoms one year after illness (Klee et al., 2004).

Neurons are the main CNS cellular hosts for WNV infection in patients, and neuronal loss is a hallmark of neurologic WNV infection (Peng and Wang, 2019). In severe neurologic manifestations of WNV, neurons in the anterior and ventral horns and brainstem are the main cellular targets and may undergo cell death (Guarner et al., 2004). WNV has also been found to infect human astrocytes *in vitro* (Fig. 1A) (Cheeran et al., 2005). While there is a lack of clinical data supporting direct infection of microglia by WNV, microglia are still thought to contribute to WNV infection. Several case studies of fatal WNV have reported neuronophagia, microglial nodules surrounding dying neurons, and immune infiltration of CD8+ T cells and perivascular B cells (Guarner et al., 2004). Garber et al. propose that loss of presynaptic termini in WNV-infected mice is driven by CD8+ T cell-produced IFNγ and subsequent microglial-mediated elimination of neurons (Garber et al., 2019), and it is possible that these mechanisms are conserved across species. Complement factors, which mediate synapse pruning, may also contribute to the neuronal loss observed in patients with severe WNV. Vasek et al. found that microglia-mediated synapse loss and neuronophagia in mice infected with a mutant neuroinvasive strain of WNV requires C3 and the C3 receptor (Vasek et al., 2016). While microglia likely play a role in mediating neuronal loss in severe WNV, they may also play a neuroprotective role. Microglia depletion in mice infected with WNV resulted in increased viral titer (Seitz et al., 2018). Consistent with these findings, Stonedahl et al. ablated microglia in an *ex vivo* mouse brain slice and observed increased WNV replication and cell death in the absence of microglia (Stonedahl et al., 2022). It is possible that an early microglial response may limit viral spread and neuronal death, but once the virus spreads to multiple target cells, microglial elimination of neurons may become detrimental.

3.2.4.1. West Nile virus: microglial models.: To our knowledge, Cheeran et al. is the only study that utilizes human microglia to model WNV pathogenesis (Cheeran et al., 2005). Primary human brain cultures were infected with WNV; while WNV productively infected astrocytes and neurons, microglia were not infected (Fig. 1A) (Cheeran et al., 2005). However, in response to changes in the extracellular environment, microglia produced high levels of proinflammatory cytokines and chemokines: IL-6, TNF-α, CXCL10, and CCL2 (Fig. 1A) (Cheeran et al., 2005). Additionally, the MAPK pathway was activated

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in microglia and found to be at least partially responsible for driving proinflammatory chemokine production (Cheeran et al., 2005). This is consistent with *in vivo* studies, and production of these factors may drive immune cell infiltration and contribute to neuronal loss. Although clinical and *in vitro* evidence suggests that WNV does not infect human microglia, the contribution of this cell type to modulating neuroinflammation during infection is not negligible and future studies should consider co-culture models that incorporate microglia.

3.2.5. Japanese encephalitis virus—Similar to WNV, most cases of JEV are asymptomatic, and symptomatic cases present with neurologic symptoms such as meningitis, encephalitis, myelitis as well as flaccid paralysis and seizures (Grewe et al., 2022). JEV was found to be the primary pathogen resulting in tropical arbovirus encephalitis in children, and prolonged recovery has been documented in children and adults (Chow and Dehority, 2021; Griffiths et al., 2014). In patients with severe JEV, brain lesions have been observed in the thalamus, basal ganglia, midbrain, hippocampus, and cerebral cortex (Sunwoo et al., 2017).

In the CNS of patients, JEV mainly targets neurons, particularly in the thalamus and brainstem (Desai et al., 1995). JEV has also been demonstrated to infect human astrocyte cell lines (Mishra et al., 2008). Microglial nodules have been observed in post-mortem tissue from patients infected with JEV (Desai et al., 1995), sometimes in the vicinity of degenerating neurons in the temporal cortex (Desai et al., 1995). This data suggests that microglia become activated upon neurologic infection with these viruses and may play a role in neuronal death (Das et al., 2008; Ghoshal et al., 2007). Additionally, one study that examined the brains of patients who succumbed to JEV infection found JEV antigen localized to microglia (Desai et al., 1995). Although this could suggest JEV infects microglia, it is possible that the antigen was derived from phagocytosed JEV-infected neurons.

Neuronal loss and microglial activation are a hallmark of JEV infection in patients, *in vivo* and *in vitro* (Das et al., 2008; Desai et al., 1995; Ghoshal et al., 2007). *In vitro* studies have found that secreted factors from microglia during JEV infection lead to neuronal death (Das et al., 2008; Ghoshal et al., 2007). Das et al. found that activated microglia and astrocytes produce IL-18 and IL-1β in JEV-infected mice, and that these cytokines amplify proinflammatory cytokine release and subsequent neuronal death (Das et al., 2008); this provides a potential explanation of the factors that may lead to neuronal loss in JEV patients. While microglia appear to mediate several neuropathologic effects during JEV infection, microglial depletion in mice has been associated with an increase in viral load, suggesting a protective role of microglia in JEV infection (Seitz et al., 2018). Similar to WNV, this could be explained by a temporal-dependent response of microglia during viral infection.

3.2.5.1. Japanese encephalitis virus: microglial models.: Immortalized microglial cell lines have been leveraged to probe innate signaling mechanisms and efficacy of antiviral compounds during JEV in (Kumar et al., 2020). Kumar et al. found that the antiviral compound, belladonna, restricts viral replication of JEV in the CHME3 microglial cell line (Fig. 1C) (Kumar et al., 2020); belladonna reduces JEV replication and expression

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of proinflammatory and pro-apoptotic factors, such as BAD, BAX, caspase-3, caspase-8, IL-1β, and CXCL10. Given the known role of these factors in driving neuroinflammation, neuronal death, and trafficking of infiltrating immune cells, belladonna could potentially ameliorate the neurologic manifestations of JEV in patients, although further *in vivo* examination is needed.

Primary human microglia and MDMs have also been used to model the neuroimmunology of JEV infection. Lannes et al. utilized both human primary microglia and MDMs to probe the role of microglia in JEV pathogenesis (Fig. 1C) (Lannes et al., 2017). The authors showed that MDMs produce high levels of proinflammatory chemokines, CCL2, CXCL9, and CXCL10 following JEV infection and that both models upregulate fractalkine receptor, CX3CR1, which has been implicated in cell-to-cell communication between neurons and microglia. This study is the first and only application of primary human microglia for modeling JEV infection. In a subsequent paper, Lannes et al. aimed to understand how microglia mediate infection of other CNS cell types using MDMs (Fig. 1C) (Lannes et al., 2019). Ultimately, the authors reported that cell- to-cell contact is crucial for viral transmission, and the CXCL1-CXCR1 axis plays a role in this method of viral transmission.

3.2.6. Zika virus—In the last fifteen years, ZIKV has reemerged as a threat in the Pacific Islands, South America, Central America, Africa, and Asia (Giraldo et al., 2022; Sekaran et al., 2022). Similar to other flaviviruses, ZIKV may present as undifferentiated febrile illness (Cerbino-Neto et al., 2016; Khan et al., 2016), but exhibits distinct neuropathology. In severe cases, mothers infected with ZIKV during pregnancy may give birth to infants with microcephaly (Acosta-Reyes et al., 2017; Lazear and Diamond, 2016). In adults and adolescents, ZIKV infection may uncommonly manifest in meningoencephalitis (Chauhan et al., 2022), myelitis (Chauhan et al., 2022), and Guillain-Barre syndrome (Cao-Lormeau et al., 2016; Lazear and Diamond, 2016). Case reports of Congenital Zika Syndrome have revealed loss of brain tissue, reduced neuronal migration, attenuated CNS maturation, loss of NPCs, calcifications, necrosis, neuronophagia, infiltrating lymphocytes and macrophages, and activated glial cells (Acosta-Reyes et al., 2017; Azevedo et al., 2018). Interestingly, one case report that documented high levels of neuronal damage and death in the cortex and expression of proinflammatory cytokines, also observed an upregulation of caspase 3 in the parenchyma (Azevedo et al., 2018), suggesting that caspase 3-mediated apoptosis may contribute to the neuronal loss observed in patients.

Similar to clinical findings, *in vitro* susceptibility of human NPCs to ZIKV infection and NPC death and cell cycle arrest have been well documented (Tang et al., 2016; Wells et al., 2016). Devhare et al. uncovered a lineage-dependent induction of caspase 3-mediated apoptosis of ZIKV-infected NPCs, finding that MR766, an African ZIKV lineage, induced cleavage of poly (ADP-ribose) polymerase (PARP) and caspase- 3, but PRVABC59, an Asian lineage, did not (Devhare et al., 2017). PRVABC59-infected NPCs exhibited arrest of cell cycle, as measured by elevated p53, p21, and PUMA levels (Devhare et al., 2017). Despite this lineage-dependent difference in attenuating NPC development, infection of NPCs with both lineages resulted in cell death (Devhare et al., 2017). Costa et al. found that inhibition of NMDA signaling in ZIKV- infected primary mouse neurons rescues neuronal death without attenuating viral replication (Costa et al., 2017). *In vivo*, NMDA receptor

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inhibition in ZIKV-infected mice was also associated with a reduction in activated microglia and neurodegeneration (Costa et al., 2017). As a follow-up to this finding, Olmo et al. uncovered that neuronal death during ZIKV infection occurs in uninfected cells (Olmo et al., 2017); these authors propose that infected cells produce proinflammatory factors, such as TNF-α and IL-1β, which signal through NMDA receptors to increase the Ca2+ intake and thus the excitotoxicity of uninfected cells, resulting in cell death (Olmo et al., 2017). Whether microglia participate in this circuit remains unclear, but given their susceptibility and activation in ZIKV-infected patients (Azevedo et al., 2018; Martines et al., 2016) and their role in mediating cell death in JEV and WNV, it is likely that microglia contribute to neurotoxicity during ZIKV infection.

In vivo, microglia have been found to be both neuroprotective and neuropathogenic during ZIKV infection. Enlow et al. found that depletion of microglia in young adult mice infected with ZIKV resulted in elevated viral antigen levels in neurons and astrocytes, suggesting a neuroprotective role of microglia (Enlow et al., 2021). However, Xu et al. demonstrate that depletion of mouse yolk sac-derived microglia in fetuses of ZIKV-infected pregnant mice resulted in decreased ZIKV titer, suggesting that microglia may be pathogenic in vertical transmission of ZIKV (Xu et al., 2020). Additional studies are needed to determine if developmental stage affects the role of microglia in the pathogenesis of ZIKV and other neurotropic viruses. Both studies depleted microglia by inhibiting CSF1R; Enlow et al. used a pharmacological inhibitor, PLX5622, while Xu et al. utilized an anti-CSF1R antibody. It is important to note that both methods of CSF1R inhibition affect other macrophage populations and can impair recruitment of inflammatory monocytes, which may confound these results (Spiteri et al., 2022).

3.2.6.1. Zika virus: microglial models.: Interactions between microglia and ZIKV have been examined using iMGs, primary human microglia cultures, human organoids co-cultured with microglia, and human immortalized microglia cell lines. Retallack et al. utilized primary cultures of human cortical tissue to demonstrate that microglia, NPCs, astrocytes, and oligodendrocyte precursors are infected by ZIKV, whereas neurons were infected at a lower incidence (Fig. 1C) (Retallack et al., 2016). Meertens et al. provide insight as to the mechanism of viral entry in microglia and astrocytes, specifically finding that AXL, the key attachment factor for ZIKV, is expressed by both primary human astrocytes and microglia (Meertens et al., 2017), supporting evidence that ZIKV directly infects microglia. Lum et al. infected primary human brain cultures with ZIKV, finding that microglia are highly susceptible to infection, and that IL-6, TNF-α, IL-1β, and MCP-1 are produced following infection (Fig. 1C) (Lum et al., 2017). It is possible that production of these cytokines are released from activated microglia and contribute to the sensitization of NDMA-mediated Ca2+ flux, as in Olmo et al., but future studies are needed to determine if this is true in human tissue as well (Olmo et al., 2017).

In the microglial CHME-5 line, metabolomic analysis of ZIKV infection revealed that proinflammatory factors IL-6, TNF-α, IL-1β, inducible nitric oxide synthase, and nitric oxide synthase are produced, and the chemokine receptor CX3CR1 is upregulated (Fig. 1C) (Diop et al., 2018). Martinez Viedma et al. found that the TLR7/8 signaling pathway is antagonized during ZIKV infection, but the NF-κB pathway, which can be activated

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downstream of TLR7/8, is induced (Martinez Viedma and Pickett, 2018). Both of these studies support a proinflammatory role for microglia during ZIKV infection.

Using iPS-derived NPCs, microglia, and astrocytes, Muffat et al. found that ZIKV can infect all three cell types, but only induces cell death in NPCs (Muffat et al., 2018). These authors uncovered that ZIKV- infected iMGs serve as viral reservoirs for extended periods of time, and can transmit infectious viral particles to neurons in a co-culture/ neuronal spheroid model (Fig. 1C) (Muffat et al., 2018). This suggests a neuropathogenic contribution for microglia in ZIKV infection (Muffat et al., 2018). Mesci et al. found that ZIKV-infected iMGs upregulate mRNAs of proinflammatory cytokines, such as IL-1β and IL-6 and produce high levels of GM-CSF and IL-1β in a strain-dependent manner (Fig. 1C) (Mesci et al., 2018). Co-culture of iMGs with infected NPCs revealed attenuation of neuronal cell death (Mesci et al., 2018). This was not found in Muffat et al., although this inconsistency could be due to neuron development stage, cell line and differentiation protocol heterogeneity (Mesci et al., 2018; Muffat et al., 2018). Xu et al. reveal that ZIKV-infected microglia become activated during infection of human brain organoids that incorporate microglia (Fig. 1C) (Xu et al., 2021); proinflammatory cytokines such as IL-6, TNF-α, and IL-1β are upregulated, and complement factors CR3 and C3 production increases as well (Fig. 1C) (Xu et al., 2021). Corroborating these findings, Abreu et al., found that ZIKV infection of neural progenitor assembloids co-cultured with microglia results in upregulation of IL-6, IL-1β, TNF-α, and CCL2 compared to infected assembloids that lack microglia, suggesting that microglia drive inflammation in response to ZIKV infection (Fig. 1C) (Abreu et al., 2018). Several of these studies suggest that ZIKV infection elicits proinflammatory factors that drive immune infiltration into the brain and neuronal death. Future studies are needed to determine if these factors are necessary and sufficient to mediate loss of NPCs in the context of ZIKV infection.

4. Discussion

In this review, we summarize the importance of microglia in responding to CNS viral infection with a specific emphasis on findings generated from human microglial models (Fig. 1). We review the existing human microglial models, their application to neuroinfection, and the benefits and limitations of each (Table 1). We also summarize current literature regarding how microglia contribute to the pathogenesis of neurotropic viruses and review important findings from different human microglial models.

A major challenge of modeling neurotropic viral infections is one of benchmarking, given the diversity of lines and models each with their own strengths and weaknesses. C20 and HMC3, immortalized human microglial cell lines, express many of the typical myeloid markers (Rai et al., 2020). However, clustering analysis reveals that these models are more transcriptionally similar to each other than to human MDMs, iMGs, and primary microglia (Rai et al., 2020). Additional clustering analysis determined that, based on expression of HIV-relevant genes such as viral receptors and attachment factors, iMGs and MDMs again are more transcriptionally similar to primary human microglia, and human microglial cell lines are more similar to each other but different from the other models (Rai et al., 2020). Furthermore, high rates of productive HIV-1 infection are exhibited by primary human

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microglia and MDMs, but not human brain organoids that incorporate microglia or human immortalized cell lines, SV40 and HMC3 (Gumbs et al., 2022). This is an important caveat to studies that model neurotropic viral infection using microglial cell lines alone or in co-culture with hBORGs, but supports the relevance of primary human microglia, iMG, and MDM models.

Xenotransplantation models address the main constraints of microglial hBORG co-cultures: vascularization and mature myelination. While humanized mice peripherally engrafted with CNS-infiltrating iHPCs have been applied towards the study of HIV infection, brain-engrafted models have not yet been leveraged to study other neurotropic viral infection. As mentioned above, these models enable the study of neurotropic infection in the context of a whole organism and could be particularly useful to understand crosstalk between different CNS cell types during infection (Fig. 2C). However, a prominent limitation of this model is the absence of a functional adaptive immune system, which plays an important role in mediating neuroinflammation during neurotropic viral infection via immune infiltration (Table 1).

Primary human microglia have been the most commonly utilized microglial model to probe microglial-virus interactions; however, in recent years, the development of MDMs and iMGs has expanded their application to modeling neurotropic viral infection as well. These primary human microglia are ontogenetically appropriate; however, these cells can be difficult to access, vary in replicability depending on isolation protocol and donor genetics, and may lose expression of key microglia transcription factors in the absence of the CNS microenvironment (Table 1). Conversely, MDMs are highly accessible, have been utilized to study HIV-1 and JEV, and, depending on the protocol, differentiate more rapidly than iMGs (Table 1; Fig. 2B). While some studies have demonstrated that MDMs and primary human microglia are similarly susceptible to infection and exhibit similar phenotypes following infection (Rawat and Spector, 2017), others found that MDMs were less susceptible to HIV-1 infection compared to primary human microglia (Akiyama et al., 2020). Furthermore, these cells are differentiated from monocytes, which are derived from the bone marrow, while microglia originate from the yolk sac. The advent of stem cell- derived iMGs has gained momentum in recent years as an advantageous model to study virus-microglial interactions. As such iMGs are renewable, patient-derived, transcriptionally similar to primary human microglia (Table 1) (Gumbs et al., 2022), and, in contrast with MDMs, are reproducibly susceptible to HIV-1 infection. Furthermore, co-culture of iMGs with other iPS-derived CNS cells can be used to probe interactions between CNS cells during infection (Fig. 2C). An important caveat to iMGs is the known significant effect of sex on microglial behavior, compounded by variable and unpredictable loss of X chromosome inactivation (also called X chromosome erosion) in female lines (Brenes et al., 2021).

The development of varying human microglia models offers clinically relevant means to dissect the contribution of microglia to the neuropathogenesis of viral infection. Of the neurotropic viruses reviewed here, ZIKV and HIV-1 -microglial interactions are the best characterized. In contrast to the other reviewed viruses which primarily target neurons with some evidence in favor of microglial infection for HSV, JEV, and ZIKV, microglia are recognized as the main CNS target for HIV-1. Thus, understanding the contribution

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of infected microglia to the pathogenesis of HAND versus the contribution of activated but uninfected microglia may be an interesting topic of study. Additionally, given that microglia are not infected by SARS-CoV-2 and WNV, future studies that aim to uncover the interactions between SARS-CoV-2 and the CNS should consider co-culture of iPS microglia, neurons and astrocytes, or primary human brain cultures (Fig. 2C). These approaches could help uncover how microglia contribute to neurotoxicity in the absence of direct infection. These co-culture methods as well as primary human microglial models, could be applied to JEV, HSV, ZIKV, and HIV- 1 to study replication dynamics and host cell response during direct microglia infection.

As mentioned above, neuronal death and microglial activation have been observed in clinical cases of all reviewed viruses and have been documented *in vitro* and *in vivo* as well. While the contribution of PRRs, glutamatergic signaling, and proinflammatory cytokines to human microglial mediated neuronal death has been consistently reported, the role of complement factors and purinergic signaling in mediating neuronal death during viral infection require additional study. Additionally, microglia-produced IL-1β and TNF-α were broadly implicated in driving neuronal death across multiple neurotropic viruses; Olmo et al. proposes a model by which microglial-produced ROS drives IL-1β production, and this contributes to the sensitization of NMDA-mediated Ca2+ flux in neurons, leading to cell death (Olmo et al., 2017). It is possible these mechanisms are conserved across neurotropic viruses that cause significant neuronal death, but additional research is required.

In addition to communicating with cells in the CNS, microglia recruit adaptive immune cells through secretion of cytokines and signaling molecules and present antigens using MHC I and MHC II (Giunta et al., 2008; Goddery et al., 2021; Lind et al., 2021; Moseman et al., 2020; Salemi et al., 2011; Tsai et al., 2016; Wheeler et al., 2018). Microglial recruitment of CD8+ T cells may contribute to the resolution of some neurotropic infections. However, some neurotropic viruses evade detection by interfering with the ability of microglia to communicate with the adaptive immune system. For instance, HSV-1 downregulates CNS expression of CXCL9, a CD8+ T cell chemoattractant, resulting in increased encephalitis and mortality in mice (Koyanagi et al., 2017). Since microglia secrete CXCL9 upon IFN-γ stimulation in a cell-restricted manner, HSV may suppress microglial CXCL9 production, thereby decreasing T cell infiltration and viral clearance (Ellis et al., 2010; Koyanagi et al., 2017). Similarly, ZIKV downregulates genes involved in adaptive immune activation in a human microglia cell line, suggesting that ZIKV also interferes with microglia-T-cell signaling and antigen presentation (Tiwari et al., 2017). T cells signaling to microglia can also mitigate or exacerbate post-infection recovery (Garber et al., 2019; Herz et al., 2015). After ZIKV or WNV infection, microglia may be chronically activated by CD8+ T cell-derived IFNγ, leading to increased neuronal elimination (Garber et al., 2019). Neuronal loss was associated with prolonged recovery, including spatial learning deficits, suggesting that infiltrating CD8+ T cells may negatively impact neurologic recovery following infection with these viruses (Garber et al., 2019). Additionally, in post-mortem brain tissue from patients with COVID-19, T-cell- microglial interactions were associated with increased microglia activation and neuroinflammation (Schwabenland et al., 2021). These studies provide insight into the importance of microglia-T cell communication during viral infection, but many questions remain, including whether microglia-T cell communication

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exacerbates disease or enhances viral clearance and whether interactions are mediated by soluble signaling molecules or direct antigen presentation by microglia. Additionally, the majority of studies investigating microglia-T cell interactions have been conducted in mice. Therefore, complex human models are needed to clarify the contribution of adaptive and innate immune crosstalk during CNS infection in humans.

Overall, these novel modeling approaches have expanded our understanding of how microglia can initiate and drive progression and damage in varying types of neurotropic viral infections. However, significant gaps in knowledge both about pathogenesis and the application to therapeutic discovery remain. For instance, whether mechanisms other than apoptosis contribute to cell death, and the extent to which microglia drive neuronal death during infection with these viruses remains to be determined. A second such area is how microglial heterogeneity and disease-associated microglial phenotypes contribute to neuroprotective or pathological roles of microglia in neurotropic infections. Future areas of innovation include modeling of microglial heterogeneity using xenotransplantation, regional-specific organoids, or mixed microglial and peripheral hematopoietic transplantation paired with viral infection (Fig. 2C). Lastly, the application of these *in vitro* and *in vivo* models to screen candidate therapeutics or small molecule libraries and lead to meaningful preclinical data for targeted drug development for the treatment of neurotropic viruses remains to be shown.

Declaration of Competing Interest

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Data availability

No data was used for the research described in the article.

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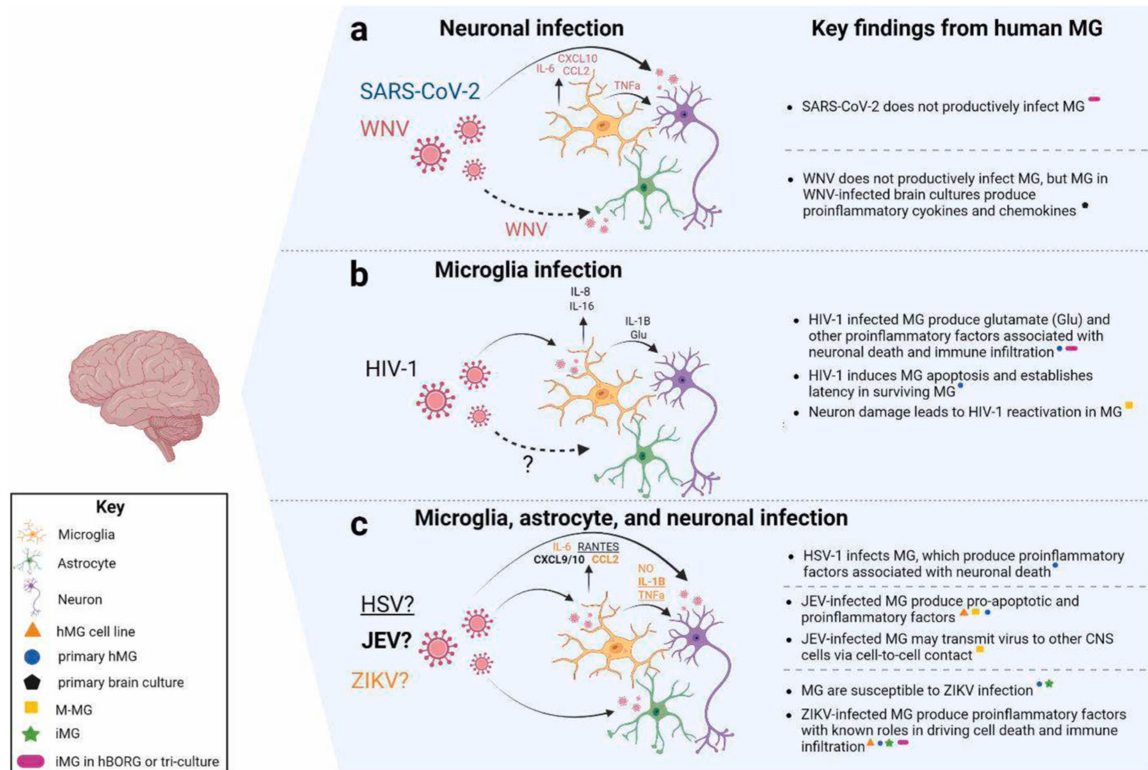

Fig. 1.

Human microglial modeling of neurotropic viral infection. MG = microglia. (a) SARS-CoV-2 and WNV infect neurons but not microglia (Jacob et al., 2020; Cheeran et al., 2005), and WNV -infected hMG produce proinflammatory factors (Cheeran et al., 2005). (b) HIV-1 infects microglia in the CNS, which produce glutamate and other proinflammatory factors that contribute to neuronal death and recruitment of immune cells (Huang et al., 2011; Zhao et al., 2004; dos Reis et al., 2020; Ryan et al., 2020), and HIV-1 establishes latency in MG that survive cell death following HIV-1 CNS entry (Castellano et al., 2017) and can be reactivated by neuronal damage (Alvarez-Carbonell et al., 2019). (c) HSV-1, JEV, and ZIKV infect MG (Lokensgard et al., 2001; Kumar et al., 2020; Lannes et al., 2017; Retallack et al., 2016; Lum et al., 2017), which produce proinflammatory factors that may drive neuronal loss and immune infiltration (Lokensgard et al., 2001; Kumar et al., 2020; Lannes et al., 2017; Lum et al., 2017; Diop et al., 2018; Muffat et al., 2018; Mesci et al., 2018; Xu et al., 2021; Abreu et al., 2018), and JEV-infected MG may infect neighboring cells by cell-to-cell contact (Lannes et al., 2019). Created with BioRender.com.

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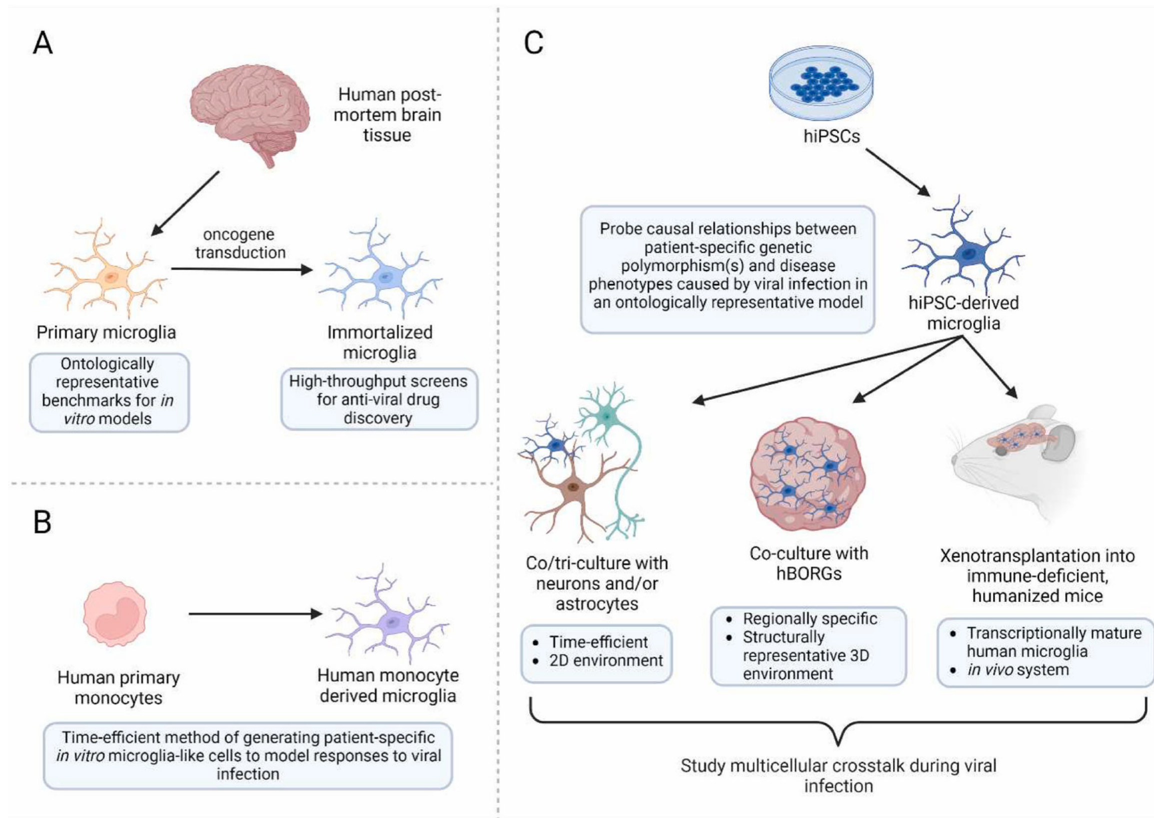

Fig. 2.

Strategies to investigate neurotropic viral infections using human microglial models. (a) Primary microglia isolated from human post-mortem brain tissue can be immortalized through oncogenic transduction. (b) Microglia-like cells can be derived from primary monocytes, though they are derived from different progenitors. (c) hiPSC-derived microglia can be cultured in isolation or applied to a wide variety of multicellular and *in vivo* systems. Created with BioRender.com.

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Table 1:

Human microglia models and their applications to neuroinfectious or neurological diseases

Model		Description	Benefits of Model	Drawbacks of Model	Diseases Applications
Human Primary Microglia		Fetal and adult microglia isolated from post-mortem brain tissue	Ontogenically representative models, serves as benchmark for other *in vitro* microglial models	Transcriptomic alterations occur rapidly following *in vitro* culture, difficult to access, highly variable yield, confounded by post-mortem interval, condition of the donor, and deviations in isolation protocol	**JEV** (Lannes et al., 2017), **WNV** (Cheeran et al., 2005), **ZIKV** (Meertens et al., 2017; Retallack et al., 2016), **HIV** (Castellano et al., 2017; dos Reis et al., 2020; Huang et al., 2011; Strizki et al., 1996; Zhao et al., 2004), **HSV** (Lokensgard et al., 2001; Marques et al., 2004)
Immortalized human microglia cell lines	HMC3	Embryonic microglia that have undergone SV40-dependent immortalization	Accessible, easier to maintain and genetically manipulate, and have a high proliferative capacity	Express oncogenes, does not recapitulate all functionality of in-vitro microglia, transcriptionally distinct from human monocyte-derived microglia, iMGs, and human primary microglia (Rai et al 2020)	**ZIKV** (Martinez Viedma and Pickett, 2018), **JEV** (Rastogi and Singh, 2019), **HIV** (dos Reis et al., 2020)
	CHME3				**JEV** (Kumar et al., 2020; Mishra et al., 2022; Rastogi and Singh, 2020)
	CHME-5				**ZIKV** (Diop et al., 2018)
	iμhu SV40 line	Embryonic brain-derived macrophages transduced with the large T antigen of the SV40 oncogene			**Dengue** (Abreu et al., 2018), **ZIKV** (Abreu et al., 2018), **HSV** (Jeffries et al., 2020)
Human monocyte-derived microglia (MDMs)		Direct differentiation of iMGs from peripheral blood cells	Conserves patient-specific age-related/ epigenetic changes	Monocytes do not arise from the same EMPs as microglia, resulting in transcriptional differences when monocyte-derived microglia and iMGs are transplanted into immune-deficient, humanized mice	**HIV** (Akiyama et al., 2020; Leone et al., 2006; Rawat and Spector, 2017), **JEV** (Lannes et al., 2019, 2017), **SARS-CoV-2** (Sheridan et al., 2021)
Human stem cell derived microglia (iMGs)	EB differentiation	Positive selection for mesoderm-lineage cells from PU.1+ EBs	Renewable, representative of homeostatic and disease-associated microglia derived from patients, applied to model crosstalk between other CNS cell types	Reprogramming may erase pertinent age-related/ epigenetic changes, in vitro culture results in more transcriptional similarities with fetal than adult microglia, sex differences can be an added confound	**ZIKV** (Muffat et al., 2018), **HSV** (Bodda et al., 2020), **Zika** (Xu et al., 2021)
	Monolayer differentiation	Uses a monoculture to generate myeloid progenitor cells			**HIV** (Ryan et al., 2020), **ZIKV** (Mesci et al., 2018)
	Commercially obtained				**SARS-CoV-2** (Jacob et al., 2020 sourced from

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Model		Description	Benefits of Model	Drawbacks of Model	Diseases Applications
					BrainXell, Inc. BX-0900)
	Transcription factor overexpression	Generates iPSC line with inducible expression of six transcription factors that drive microglial differentiation			**N/A** (Dräger et al., 2022)
	2D Co/Tri-culture	Co-culture iMGs with other CNS cell types to further drive differentiation			**HIV** (Alvarez-Carbonell et al., 2019; Ryan et al., 2020), **ZIKV** (Mesci et al., 2018; Muffat et al., 2018)
Human brain organoids	Unguided organoids	Some unguided hBORGs contain a small population of mesodermal cells which spontaneously differentiate into microglia	Model cross-talk between different CNS cell types in a 3D environment, recapitulates brain structure and organogenesis more faithfully than 2D culture, can be regionally specific	Costly and time consuming, lack of vascularization in non-grafted organoids results in necrosis and immune activation as models age	**N/A** (Ormel et al., 2018)
	Assembloids	hBORGs co-cultured with microglia			**Dengue** (Abreu et al., 2018), **ZIKV** (Abreu et al., 2018), HIV (dos Reis et al., 2020), Zika (Xu et al., 2021)
	Grafted organoids	hBORGs transplanted into immune-deficient mice			**N/A** (Mansour et al., 2018; Revah et al., 2022)
Xenotransplantation	Peripherally engrafted	CD34+ cord blood cells peripherally transplanted in humanized immune deficient mice, which infiltrate the CNS	Peripheral monocytes infiltrate the brain to become microglia-like MDMs	Models interactions between human microglia and the murine CNS, rather than other human CNS cell types, disease manifestations in immune-deficient mice may not be representative of infection in immunocompetent patients, such as immune cell infiltration	**HIV** (Mathews et al., 2019)
	Brain engrafted	Stem cell derived hematopoietic progenitors transplanted into lateral ventricles of CSF1 humanized, immune deficient mice	Transplanted microglia are more transcriptionally similar to ex-vivo microglia compared to primary microglia and iMGs		**Alzheimer's disease** (Mancuso et al., 2019; Rojo et al., 2019)