



Human microglial models to study HIV infection and neuropathogenesis: a literature overview and comparative analyses

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Abstract

HIV persistence in the CNS despite antiretroviral therapy may cause neurological disorders and poses a critical challenge for HIV cure. Understanding the pathobiology of HIV-infected microglia, the main viral CNS reservoir, is imperative. Here, we provide a comprehensive comparison of human microglial culture models: cultured primary microglia (pMG), microglial cell lines, monocyte-derived microglia (MDMi), stem cell-derived microglia (iPSC-MG), and microglia grown in 3D cerebral organoids (oMG) as potential model systems to advance HIV research on microglia. Functional characterization revealed phagocytic capabilities and responsiveness to LPS across all models. Microglial transcriptome profiles of uncultured pMG showed the highest similarity to cultured pMG and oMG, followed by iPSC-MG and then MDMi. Direct comparison of HIV infection showed a striking difference, with high levels of viral replication in cultured pMG and MDMi and relatively low levels in oMG resembling HIV infection observed in post-mortem biopsies, while the SV40 and HMC3 cell lines did not support HIV infection. Altogether, based on transcriptional similarities to uncultured pMG and susceptibility to HIV infection, MDMi may serve as a first screening tool, whereas oMG, cultured pMG, and iPSC-MG provide more representative microglial culture models for HIV research. The use of current human microglial cell lines (SV40, HMC3) is not recommended.

Keywords Microglia · HIV · HIV-associated neurocognitive disorder · Neuropathogenesis · Central nervous system · Organoid

Introduction

Currently, 38 million people are estimated to be living with HIV (www.who.int). Implementation of antiretroviral therapy (ART) resulted in effective suppression of viral replication and substantially reduced AIDS-related morbidity and mortality (Global HIV & AIDS Statistics 2020). However, ART neither eliminates HIV that persists in a latent state nor suppresses HIV expression and production from cellular reservoirs in the body (Sengupta and Siliciano 2018). Hence, despite long-term ART, HIV-1 persists in the central nervous system (CNS), which upon ART cessation contributes to the rekindling of viral infection and replication. Persistence of HIV in the CNS indirectly and directly results in a wide range of CNS manifestations in up to 50% of ART-treated HIV-infected individuals, collectively termed HIV-associated neurocognitive disorders (HAND) (Caruana et al. 2017; Heaton

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et al. 2010, 2011; Wang et al. 2020; Winston and Spudich 2020). In addition to viral factors, the onset or progression of HAND is exacerbated by systemic inflammation, myeloid activation, and a variety of common comorbid conditions including cardiovascular disease, chronic lung disease, diabetes, anemia, obesity, and substance abuse (Ances et al. 2021; Ances and Letendre 2019; Heaton et al. 2010, 2015). Besides optimizing the antiretroviral drug combination for better CNS penetration and encouraging the patients' adherence to treatment, no other clear recommendation can be formulated for the treatment of HAND (Calcagno et al. 2017; Nosik et al. 2021; Ulfhammer et al. 2018; Winston and Spudich 2020). To develop novel therapeutic strategies that target this CNS reservoir and diminish HIV-associated pathogenesis in the CNS, it is therefore of primary interest to understand how HIV reservoirs in the brain are established and maintained.

HIV-1 enters the CNS within 2 to 4 weeks after initial infection. Viral RNA has been detected in the cerebrospinal fluid (CSF) as well as in brain tissues of both asymptomatic and symptomatic individuals (Davis et al. 1992; Enting et al. 2001; Pilcher et al. 2001; Tambussi et al. 2000). Later on, throughout the course of infection, compartmentalized HIV-1 populations, genetically distinct from viral populations replicating in the periphery, can be detected in the CSF or brain tissue of several HIV-infected individuals (Bednar et al. 2015; Harrington et al. 2009). Viral analyses showed that the CNS cells are predominantly infected by macrophage-tropic HIV-1 variants that utilize the CCR5 co-receptor and require a low density of the CD4 receptor for efficient cell entry and infection (R5 M-tropic) (Arrildt et al. 2015; Joseph et al. 2015; Joseph and Swanstrom 2018). Based on these cellular characteristics, microglia are thought to constitute the main viral reservoir in the CNS and support productive HIV infection allowing for viral compartmentalization, evolution and escape (Wallet et al. 2019).

Investigation of the pathobiology of HIV-infected microglia and how this potential viral reservoir is established and maintained is urgently needed but is restricted due to the difficulty of studying primary microglia. A great variety of microglial culture models have been developed in the past decade (Timmerman et al. 2018). The present study aimed to investigate the validity and similarity of these models to primary microglia in regard to HIV research. In the present study, we provide a comprehensive overview of five different human microglial culture models: cultured primary microglia (pMG), microglial cell lines (SV40, HMC3, C20), monocyte-derived microglia (MDMi), stem cell-derived microglia (iPSC-MG), and microglia grown in 3D cerebral organoids (oMG). For each model, we describe how it compares to human primary microglia in situ and in vitro across the characteristics morphology, gene expression, immune function, and HIV infection, as well as practical strengths and limitations. By using publicly available RNA-seq data,

we evaluated the transcriptomic similarity of the models to uncultured primary microglia in the context of a microglia-specific core signature and HIV-relevant genes. Together, this study aims to provide researchers working on HIV with a guide to choosing a suitable microglial culture model for studying HIV infection and neuropathogenesis.

Comprehensive overview: model description and comparison

Description of the models

Isolation and culture of fetal and adult pMG from human brain tissue for in vitro studies has been performed already for a long time (Grenier et al. 1989; Hassan et al. 1991; Hayes et al. 1988; Lee et al. 1992). Microglia can be isolated by generating a cell suspension of the brain tissue followed by subsequent further microglia-specific enrichment techniques (Mizee et al. 2017; Olah et al. 2012; Rustenhoven et al. 2016; Zhang et al. 2016). Post isolation, cells can be cultured in vitro for weeks to months. Loss of phenotypic characteristics of primary microglia during isolation procedures is, however, well documented and known to aggravate once the cells are cultured (Gosselin et al. 2017; Marsh et al. 2020). Adding factors such as GM-CSF and IL-34 partly but not fully prevents this loss of phenotype (Gosselin et al. 2017). The limited availability of human brain tissue and the subsequent limited number of viable microglial cells present further difficulties in performing experiments with human pMG.

To combat these restrictions, pMG have been immortalized through viral transduction with different oncogenes to generate microglial human cell lines. Examples of these immortalized cell lines are HMC3, SV40, and C20 (Garcia-Mesa et al. 2017; Janabi et al. 1995). For over a decade, SV40 and HMC3 have been the only human microglial cell lines available. A comprehensive review of the HMC3 cell line was recently published by Dello Russo and colleagues reporting that the HMC3 cell line has been circulated and used in various laboratories with different denominations, i.e., C13NJ, CHME-3, and CHME-5 cells (Dello Russo et al. 2018). Notably, a recent study has reported that some of the circulating HMC3-based cell lines are impure, as the cells are of rat origin (Garcia-Mesa et al. 2017).

The natural plasticity of monocytes has been exploited in vitro to differentiate these cells towards dendritic cells and macrophages (Davies and Lloyd 1989; Zhou and Tedder 1996). These differentiated cells have been extensively used to study HIV biology (Ancuta et al. 2006; Ciborowski et al. 2007; Fairman and Angel 2012; Suzuki et al. 2000; Taya et al. 2014; Tsunetsugu-Yokota et al. 1995; Tuttle et al. 1998). Several protocols have been developed to direct monocytes

towards a microglia-like phenotype, within 10 to 14 days, referred to as monocyte-derived microglia (MDMi). The generation of MDMi is based on the culture of monocytes in either astrocyte-conditioned medium (Bertin et al. 2012; Leone et al. 2006; Ormel et al. 2020), extracellular matrix (Sellgren et al. 2017), or serum-free medium (Etemad et al. 2012; Ohgidani et al. 2014; Rawat and Spector 2017; Ryan et al. 2017) in the presence of human recombinant cytokines (such as GM-CSF, M-CSF, NGF- β , CCL2, TGF- β , IFN- γ , and IL-34) that have been identified to drive microglia development and survival (Butovsky et al. 2014; Kierdorf et al. 2013).

In addition to MDMi, recent technological advancements with induced pluripotent stem cells (iPSCs) have led to the generation of several protocols to differentiate iPSCs into representative microglial cells (iPSC-MG) (Haenseler and Rajendran 2019; Hasselmann and Blurton-Jones 2020; Speicher et al. 2019). First, iPSCs are induced to a hematopoietic lineage and into erythro-myeloid or hematopoietic progenitors via stimulation with simple growth factor cocktails (minimally BMP4, VEGF, SCF, followed by IL-3, M-CSF). Subsequently, microglial differentiation is achieved either by co-culture with neural cells (Haenseler et al. 2017; Muffat et al. 2016; Pandya et al. 2017; Takata et al. 2017), by application of neural precursor conditioned media (Banerjee et al. 2020), or by addition of cytokines secreted from neurons and astrocytes to the culture media to mimic their presence (Abud et al. 2017; Douvaras et al. 2017; McQuade et al. 2018; Muffat et al. 2016).

As it is becoming evident that the phenotype of microglia is dependent on the CNS environment, co-culture with astrocytes and/or neurons and 3D culture systems have been shown to further induce the maturation of microglia. Cerebral organoids recapitulate many structural, developmental, and functional features of the human brain, including cytoarchitecture, cell diversity, and transcriptional profile (Chan et al. 2021; Chiaradia and Lancaster 2020; Qian et al. 2019; Sidhaye and Knoblich 2021). The generation of cerebral organoids can be divided in two categories: non-patterned or patterned. Non-patterned organoids are spontaneously differentiated from iPSCs via endogenous patterning cues and self-organize into various brain regions, ranging from the retina to hindbrain. Alternatively, patterned organoids are differentiated into one specific brain region through the addition of exogenous signaling molecules and growth factors to induce iPSC differentiation towards the desired lineage (Qian et al. 2019; Sidhaye and Knoblich 2021; Kim et al. 2021). A potential limitation of patterned cerebral organoids is the inhibition of mesoderm and endoderm formation including the cells derived from these germ layers in particular microglia. Recently, Ormel and colleagues reported the innate development of microglia, referred to as organoid-derived microglia (oMG, within unpatterned cerebral organoids (Ormel et al.

2018). Other CNS-microglia co- and tri-culture approaches include co-culturing iPSC-derived microglia with cerebral organoids (Abreu et al. 2018; Abud et al. 2017; Brownjohn et al. 2018; dos Reis et al. 2020; Lin et al. 2018; Muffat et al. 2016), or adding iPSC-derived astrocytes and neurons to iPSC-derived microglia (Haenseler et al. 2017; Park et al. 2018; Ryan et al. 2020; Takata et al. 2017).

Comparison of microglia morphology

In the human adult brain, a variety of morphological forms is seen, with differences between gray and white matter (Geirsdottir et al. 2019; Salamanca et al. 2019; Torres-Platas et al. 2014). A so-called ramified morphology is classically assigned to be specific to microglia. This ramified morphology is one of the main characteristics used as a read-out parameter to optimize microglia in vitro culture models. A ramified morphology refers to cells with a small soma and very long and fine arborized processes, with primary, secondary, tertiary, and even quaternary branches (Walker et al. 2014) (Fig. 1a). These processes are highly dynamic and used for the active surveillance of the surroundings of the cells (Davalos et al. 2005; Nimmerjahn et al. 2005). Pathological events in the CNS, such as an infection, bleeding, hypoxia, cell death, and neurodegeneration, lead to activation of microglia, which is accompanied by a rapid change in microglia morphology (Wolf et al. 2017). In the first stage, the processes of the microglia are shortened and widened, a morphology referred to as reactive microglia. In the next stage, the soma of the microglia is enlarged with no or very few processes. This stage is referred to as an amoeboid morphology (Davalos et al. 2005; Nimmerjahn et al. 2005; Stence et al. 2001).

Following brain isolation, fetal and adult microglia show an amoeboid morphology with no or few processes. After a few days in culture, the cells will develop processes with some ramifications but these processes are never as arborized and complex as the microglia in situ (Levtova et al. 2017; Lue et al. 2019; Mizee et al. 2017; Tewari et al. 2021) (Fig. 1b). This disparity in morphology is evident across every in vitro culture model (Table 1). Commercially available human microglial cells lines SV40 and HMC3 have a varied morphology of globular and spindle-shaped cells with short processes and a few primary branches that fuse together when confluence is reached (Akhter et al. 2021; Chiavari et al. 2019; Dello Russo et al. 2018) (Fig. 1c). Similar morphology was observed in the novel microglial cell line h μ glia clone 20 (C20) (Garcia-Mesa et al. 2017). Initial monocyte differentiation protocols generated MDMi with an elongated or small cellular body and a few unbranched processes; however, further optimization of the differentiation medium, e.g., the addition of IL-34 and GM-CSF, generated MDMi with a round or ovoid cell body with several primary processes with

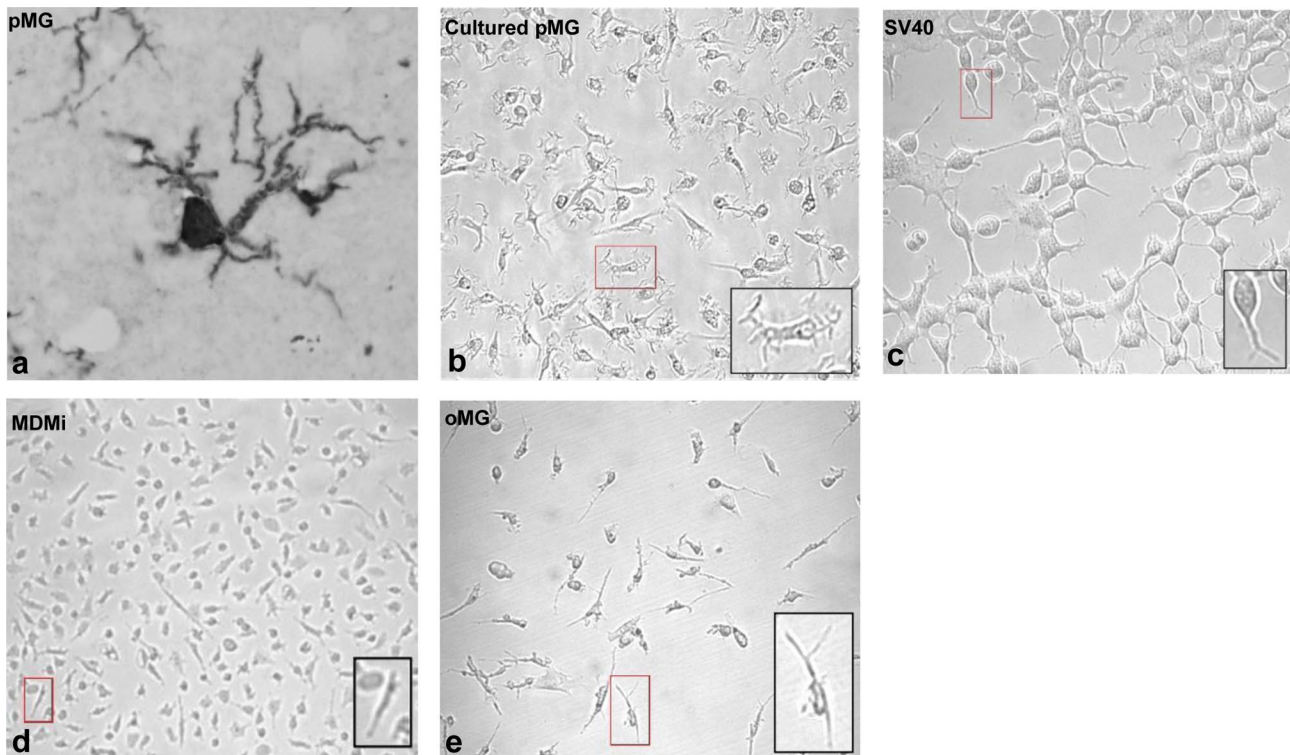


Fig. 1 Morphology of human primary microglia and four different microglial in vitro culture models. **a** IBA-1 stained microglia in DAB-stained human brain sections of GFM at 40× magnification. **b–e** Phase-contrast images of microglial culture models: adult primary

microglia at 7 days post isolation **b**, SV40 microglial cell line **c**, monocyte-derived microglia **d**, and organoid-derived microglia **e**. Magnification= 10× **d** and 20× **b, c, e**

primary and a few secondary branches (Bertin et al. 2012; Leone et al. 2006; Ohgidani et al. 2014; Ryan et al. 2017; Sellgren et al. 2017) (Fig. 1d).

Out of all the models, iPSC-MG and oMG have the closest resemblance to the morphology of microglia in situ. iPSC-MG cultures consist mainly of amoeboid-shaped cell bodies from which several primary processes emerge with secondary and a few tertiary branches (Abud et al. 2017; Banerjee et al. 2020; Brownjohn et al. 2018; Douvaras et al. 2017; Muffat et al. 2016; Takata et al. 2017). oMG exhibits an elongated or amoeboid-shaped cell body with a few secondary branched processes (Ormel et al. 2018). Upon isolation and culture, oMG has a spindle shape with a few processes from which some 2nd-degree fine spines emerge (Ormel et al. 2018) (Fig. 1e).

Comparison of microglia immune function

Under normal physiological conditions, microglia control neuronal viability, phagocytose degenerating neurons, remove excessive synaptic elements, and guide angiogenesis to support the establishment of functional neural circuits (Kierdorf and Prinz 2017; Wolf et al. 2017). Under pathological conditions, microglia secrete a broad spectrum of

cytokines, chemokines, reactive oxygen species, and neurotrophic factors to promote and/or control inflammation and phagocytose apoptotic cells and cellular and myelin debris (Colonna and Butovsky 2017; Galloway et al. 2019; Rodríguez-Gómez et al. 2020). However, these immune functions are shared with other myeloid cells in the CNS including non-parenchymal macrophages and infiltrating macrophages from the periphery (Abe et al. 2020; Li and Barres 2018). Several studies reported that microglia and macrophages have different functions during or following injury (Evans et al. 2014; Greenhalgh and David 2014; Plemel et al. 2020; Ritzel et al. 2015; Yamasaki et al. 2014). However, as microglia lose their ramified morphology in vitro and transform into an amoeboid phenotype in response to injury in vivo, it becomes increasingly difficult to discriminate between them. Consequently, it has been difficult to assign how microglia are different from other myeloid cells in terms of function.

Though not exclusive to microglia, we assessed two of the primary functions of microglia in each microglial model, namely the ability to phagocytose and the ability to induce an inflammatory response (Table 1). Each culture model reported to have phagocytic capabilities ranging from engulfment of zymosan- or iC3b-coated microbeads to

Table 1 Overview of morphology, phagocytosis, and inflammatory responsiveness of microglial in vitro culture models

Culture model	Difficulty	Culture days to microglia	Coating	Morphology	Function
Adult primary microglia	Intermediate	0	Poly-L-lysine, none	Spindle shape with a few processes and some 1st-degree branching	Phagocytosis: pHrodo-labeled myelin, fluorescent beads Inflammation response to: IL-1 β , LPS, IFN γ , dexamethasone, IL-4
Human microglial cell lines	Easy	0	None	Globular and spindle-shaped cells with short processes	Phagocytosis: pHrodo-labeled synaptosomes, live neural progenitor cells, dead neuronal cells, A β 42 Inflammation response to TNF- α , IL-1 β , IFN γ , LPS
Monocyte-derived microglia	Easy	10–16	Poly-L-lysine, Geltrex, none	Round or ovoid cell body with several processes with 1st-degree branching	Phagocytosis: iC3b-coated beads, pHrodo-labeled synaptosomes, live neural progenitor cells, fluorescent-labeled <i>S. aureus</i> , latex beads, zymosan particles Inflammation response to LPS, IL-6, dexamethasone
iPSC-derived microglia	Intermediate	25–74	CellBIND, Primaria, Matrigel, poly-L-ornithine, fibronectin, gelatin, none	Amoeboid shaped cell body with several 1st- and 2nd-degree branched processes	Phagocytosis: zymosan-coated microbeads, <i>Escherichia coli</i> / <i>S. aureus</i> particles, fibrillar beta-amyloid, tau oligomers, and synaptosomes Inflammation response to IL-1 β , IFN γ , LPS
Organoid-derived microglia	High	> 31	Matrigel embedment	Spindle-shaped cells with several processes with 1st- and 2nd-degree fine spines	Phagocytosis: iC3b-coated beads Inflammation response to LPS, dexamethasone

more CNS-relevant substrates such as synaptosomes, neural progenitor cells, apoptotic neurons, and myelin (Abud et al. 2017; Amos et al. 2017; Banerjee et al. 2020; Douvaras et al. 2017; Garcia-Mesa et al. 2017; Hendrickx et al. 2014; McQuade et al. 2018; Mizze et al. 2017; Ormel et al. 2018; Pandya et al. 2017; Rawat and Spector 2017; Sellgren et al. 2017; Takata et al. 2017). Direct comparison to cultured pMG revealed a similar phagocytic ability with MDMi and oMG, whereas SV40 had limited phagocytosis (Ormel et al. 2018; Rawat and Spector 2017; Sellgren et al. 2017).

Like phagocytosis, the inflammatory responses of microglial culture models are validated using a variety of pro- and anti-inflammatory stimuli and read-out parameters (Table 1). Stimulation of cultured pMG with the pro-inflammatory stimulus LPS (lipopolysaccharide) classically leads to activation of microglia with increased secretion of IL-6, IL-1 β , and TNF- α (Lee et al. 2002; Melief et al. 2012, 2016; Nagai et al. 2001; Rustenhoven et al. 2016). To compare the models with each other, secretion of IL-6, IL-1 β , and TNF- α was assessed and compared to cultured pMG post stimulation with LPS. LPS stimulation of SV40, iPSC-MG, and oMG led to high induction of TNF- α release, whereas the secretion of IL-6 and IL-1 β varied from modest and moderate in the cell lines MDMi and iPSC-MG to significant in oMG and cerebral organoids (Abreu et al. 2018; Abud et al. 2017; Banerjee et al. 2020; Ormel et al. 2018, 2020; Pandya et al. 2017; Patel et al. 2016). Direct comparison to cultured pMG revealed a significantly higher inflammatory response (IL-6 and IL-1 β) in oMG (Ormel et al. 2018).

As a whole, each microglial in vitro culture model is capable of phagocytosis and showed varying degrees of LPS responsiveness. However, as previously mentioned, these functions are not exclusive to microglia but shared with other myeloid cells such as macrophages. Therefore, to further investigate the microglial phenotype of the culture models, we compared the transcriptome of the cultured pMG, the MDMi, the iPSC-MG, and the oMG to the transcriptome of uncultured adult pMG.

Gene expression

Markers that have classically been used to identify microglia in brain tissue, such as HLA-DR and CD68, are also expressed by other myeloid cells and can therefore not be used to determine whether a cell model reflects microglia or another myeloid cell type. Recent transcriptomic studies have compared human microglia with other myeloid cells (Böttcher et al. 2019; Gosselin et al. 2017; Kracht et al. 2020; Ormel et al. 2018) and identified a specific gene signature for adult and fetal microglia. This includes markers such as *AIF1*, *TMEM119*, *P2RY12*, *CX3CR1*, *CSF1R*, and *TREM2* (Bennett et al. 2016; Butovsky et al. 2014;

DePaula-Silva et al. 2019; Galatro et al. 2017; Gautiar et al. 2012; Gosselin et al. 2017; Haage et al. 2019; Hickman et al. 2013; Konishi et al. 2017; Mildner et al. 2017; Ormel et al. 2018) (Table 2).

Following isolation, cultured adult pMG lose part of the microglia-specific gene signature, with a downregulation of *P2RY12*, *TREM2*, and *TMEM119*, as well as an upregulation of inflammatory- and stress response-associated genes (Bohlen et al. 2017; Gosselin et al. 2017). This mature microglia signature, therefore, seems to be much dependent on the CNS environment (Gosselin et al. 2017). Accordingly, lower expression levels of *CX3CR1*, *P2RY12*, and *TMEM119* were generally reported in the microglial cell lines (Melief et al. 2016; Rai et al. 2020), MDMi (Melief et al. 2016; Ormel et al. 2020; Rai et al. 2020; Rawat and Spector 2017), and oMG (Ormel et al. 2018) compared to those in adult pMG.

To further characterize the transcriptomic similarity of microglial culture models to adult pMG, we compared previously published RNA-seq data of cultured adult pMG (Gosselin et al. 2017; Lopes et al. 2020), fetal pMG (Douvaras et al. 2017), iPSC-MG (Brownjohn et al. 2018; Douvaras et al. 2017), MDMi (Ormel et al. 2020), monocytes (Gosselin et al. 2017; Ormel et al. 2020), and oMG (Ormel et al. 2018) to that of uncultured adult pMG (Gosselin et al. 2017; Ormel et al. 2018, 2020) leveraging (i) Pearson correlation, (ii) principal component analysis (PCA), and (iii) unsupervised hierarchical clustering.

Comparison of the full transcriptome

To examine the general relationship between the gene expression of the various culture models, we correlated the counts of a collapsed version of the three uncultured adult pMG datasets with the counts of the cultured microglial model datasets. The regression coefficients of the full transcriptome datasets of uncultured adult pMG and the different culture models were all high, ranging from 0.91 for the cultured pMG, to 0.71 for the oMG (Fig. S1). The results of our principal component, correlation, and unsupervised clustering analyses of cluster-defining and microglia genes show a high similarity of same cell-type samples despite their origin from different studies, supporting the validity of our comparative analyses (Figs. 2 and 3).

To thoroughly understand the similarities and dissimilarities of the transcriptomic architecture of the culture models to uncultured adult pMG, we then decided to shift the perspective towards a more specific examination of our RNA-seq data by performing our analyses on two selected gene sets: (i) the 500 most variable genes (i.e., the genes that are most distinguishing across samples in terms of expression) and (ii) a microglia-specific core signature recently defined by Patir et al. (2019).

Table 2 Overview of microglia-enriched markers

Marker (gene) Name	Cell type	Function	Reference
TMEM119	Microglia	Uncertain	Bennett et al. (2016), Satoh et al. (2016)
P2RY12	Microglia	Purinergic receptor required for microglia chemotaxis in response to CNS injury	Bennett et al. (2016), Butovsky et al. (2014)
CSF1R	Microglia and other myeloid lineage cells	Cell surface receptor that directly controls the development, survival, and maintenance of microglia and plays a pivotal role in neuroinflammation	Chitu et al. (2016), Erbllich et al. (2011), Nandi et al. (2012)
CX3CR1	Microglia and other myeloid lineage cells	Chemokine receptor critical in controlling microglia numbers, synaptic pruning, and functional brain connectivity	Jones et al. (2010)
TREM2	Microglia and other myeloid lineage cells	Regulatory protein involved in microglia activation and phagocytosis of apoptotic neurons	Colonna (2003), Neumann and Takahashi (2007)
AIF1	Microglia and other myeloid lineage cells	Cytoplasmic protein involved in microglia motility, membrane reorganization and phagocytosis	Imai et al. (1996), Sasaki et al. (2001)

Most variable genes

Leveraging the most variable genes only (i.e., those most defining of inter-sample difference), we found that datasets of the same model system were moderately ($r > 0.6$; iPSC-MG, cultured adult pMG) to strongly correlated ($r > 0.7$; uncultured adult pMG, monocytes), presenting evidence for the validity of our comparisons (Fig. 2a).

Only the cultured adult pMG by Gosselin et al. (2017) were moderately correlated with uncultured adult pMG ($r > 0.4$). These results were mirrored in the inter-sample distances we observed on our PCA based on the 500 most variable genes: samples of monocytes or uncultured adult pMG across different datasets clustered together, respectively (suggesting high intra-cell-type similarity), while the cultured model systems, MDMi disregarded, formed an interspersed cluster apart from uncultured adult pMG and monocytes (Fig. 2b). To further extract information about transcriptomic cell-type similarity, we interrogated the data on hierarchical clustering (Fig. 2c). Again, we found small intra-cell-type sample distances where samples of the same cell type but different datasets corresponded to the same clusters. In line with our correlation results, the cultured adult pMG samples also clustered more proximal to the uncultured adult pMG samples than would our PCA suggest, further emphasizing the similarity of these model systems. These results corresponded to those of previously conducted comparative analyses (Ormel et al. 2018).

Expression of microglia core genes

Next, we aimed to examine the transcriptomic similarity of cell types by repeating our analyses in the context of a microglia-specific core signature of 249 genes defined by Patir et al. (2019). Again, we found moderate ($r > 0.6$; iPSC-MG, cultured pMG) to strong ($r > 0.8$: uncultured adult pMG, monocytes) intra-cell-type sample correlations across datasets (Fig. 3a). Opposed to earlier results, cultured pMG now were correlated only moderately with uncultured adult pMG ($r > 0.6$), followed by oMG and MDMi ($r > 0.5$). Similarly, the PCA displayed that inter-sample distances for the uncultured adult pMG and monocyte datasets were diminishingly small within cell types but distinguishably large between cell types (Fig. 3b). MDMi samples now showed a smaller distance to samples of microglial culture models while these remained an interspersed cluster on their own. The hierarchical clustering analysis as well underlined the internal consistency regarding intra-cell-type sample distances (Fig. 3c). Contrary to the inter-sample distances on our PCA, MDMi clustered closer to monocytes which together formed a cluster separate from the microglial culture model samples. Interestingly, oMG samples represented, together with the

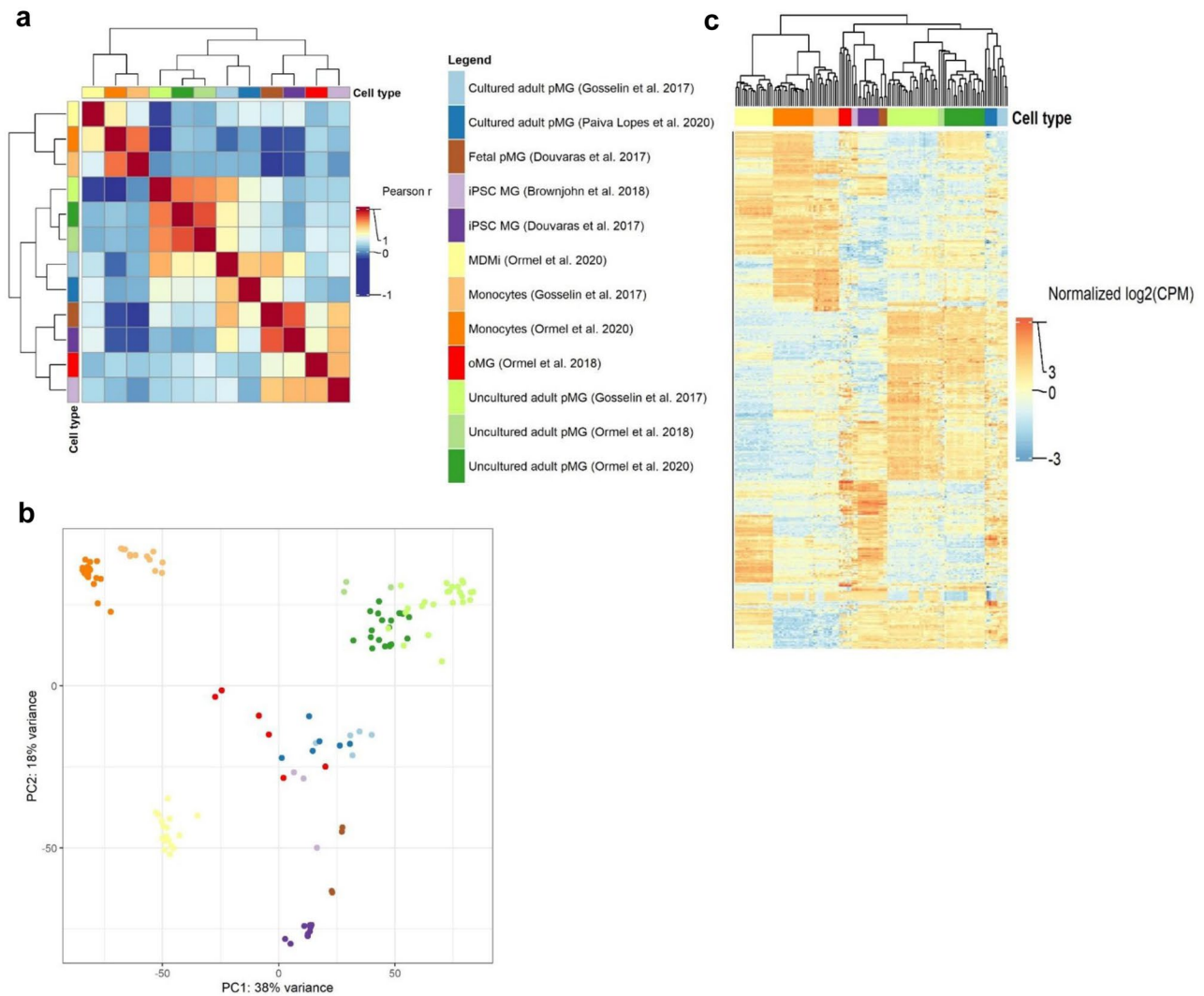


Fig. 2 Gene expression analysis of microglial culture models on the 500 most variable genes. Legend shows color coding for cell type. **a** Heatmap depicting the Pearson r correlation effect sizes between cell types based on the 500 most variable genes. Clustering dendrogram is based on Euclidean distances. **b** PC plot depicting cell-type dis-

tances based on expression variance in the 500 most variable genes. Clustering dendrogram is based on Euclidean distances. **c** Heatmap of $\log_2(\text{CPM})$ expression values for the 500 most variable genes depicted for each cell type

iPSC-MG by Brownjohn et al. (2018), a cluster distinct from all other cell types. These observations, however, aligned with the results of our correlation analysis. This suggests that the examined model systems break down in reflecting the microglia phenotype on a transcriptomic level when focusing on microglia-specific expression programs. In line with previous transcriptomic studies, microglia-specific genes including *TMEM119*, *P2RY12*, *CX3CR1*, and *CSF1R* were low in the in vitro culture models compared to uncultured adult pMG. Interestingly, adult pMG kept in culture for 7 and 10 days had a higher expression of *CSF1R* and *TMEM119* compared to uncultured adult pMG. Whether this is due to prolonged culture or a response to cell death and/or cell debris remains to be

determined in future studies (Fig. S2). MDMi and iPSC-MG also had a higher expression of *IBA1* and *TREM2* compared to cultured adult pMG.

HIV-relevant gene expression

To identify microglial culture models that are suitable for HIV research, we evaluated the similarity of the culture models with uncultured adult pMG at the level of HIV-relevant genes (Fig. 4). Undoubtedly, a good HIV microglia model must express the CD4 receptor and CCR5 co-receptor ideally at levels similar to microglia in situ. *CD4* gene expression in uncultured adult pMG was similar to oMG, monocytes, cultured pMG from Gosselin et al. (2017) and iPSC-MG from

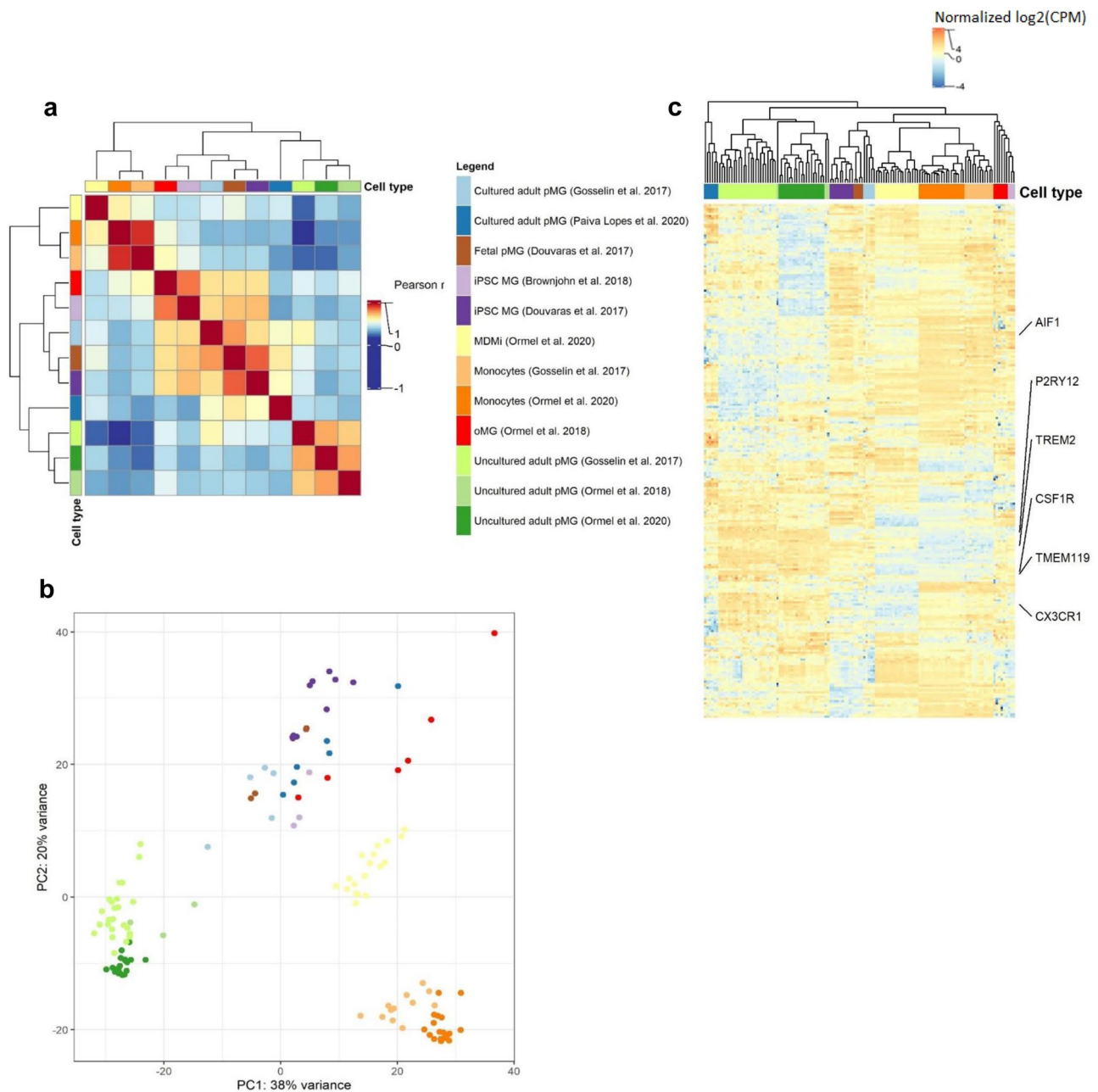


Fig. 3 Gene expression analysis of microglial culture models on a microglia-specific core signature. **a** Heatmap depicting Pearson r correlation effect sizes between the cell types based on microglia core gene expression. Clustering dendrogram is based on Euclidean dis-

tances. Clustering dendrogram depicts Euclidean distances. **b** PC plot depicting cell-type similarities based on expression variance within microglia core genes. **c** Heatmap of $\log_2(\text{CPM})$ values for microglia core genes extracted from Patir et al. (2019)

Brownjohn et al. (2018), but considerably higher than MDMi, cultured pMG from Lopes et al. (2020), and iPSC-MG from Douvaras et al. (2017) (Fig. 4b). Opposite to *CD4*, expression of *CCR5* in the culture models was generally similar to that in uncultured pMG; however, the uncultured adult pMG from Ormel et al. (2018) had considerably lower expression compared to the other uncultured adult pMG datasets. Although the viruses found in the CNS predominantly use *CCR5*, we

also analyzed the expression of the *CXCR4* co-receptor. In general, *CXCR4* expression in the models was similar to that in uncultured adult pMG, except for the lower expression seen in iPSC-MG. Besides the major HIV receptors, we also assessed the expression levels of several restriction factors that were found to restrict HIV infection, replication, and/or spread. *TRIM5* and *APOBEC3G* expression in the culture models was similar to that in uncultured adult pMG, although

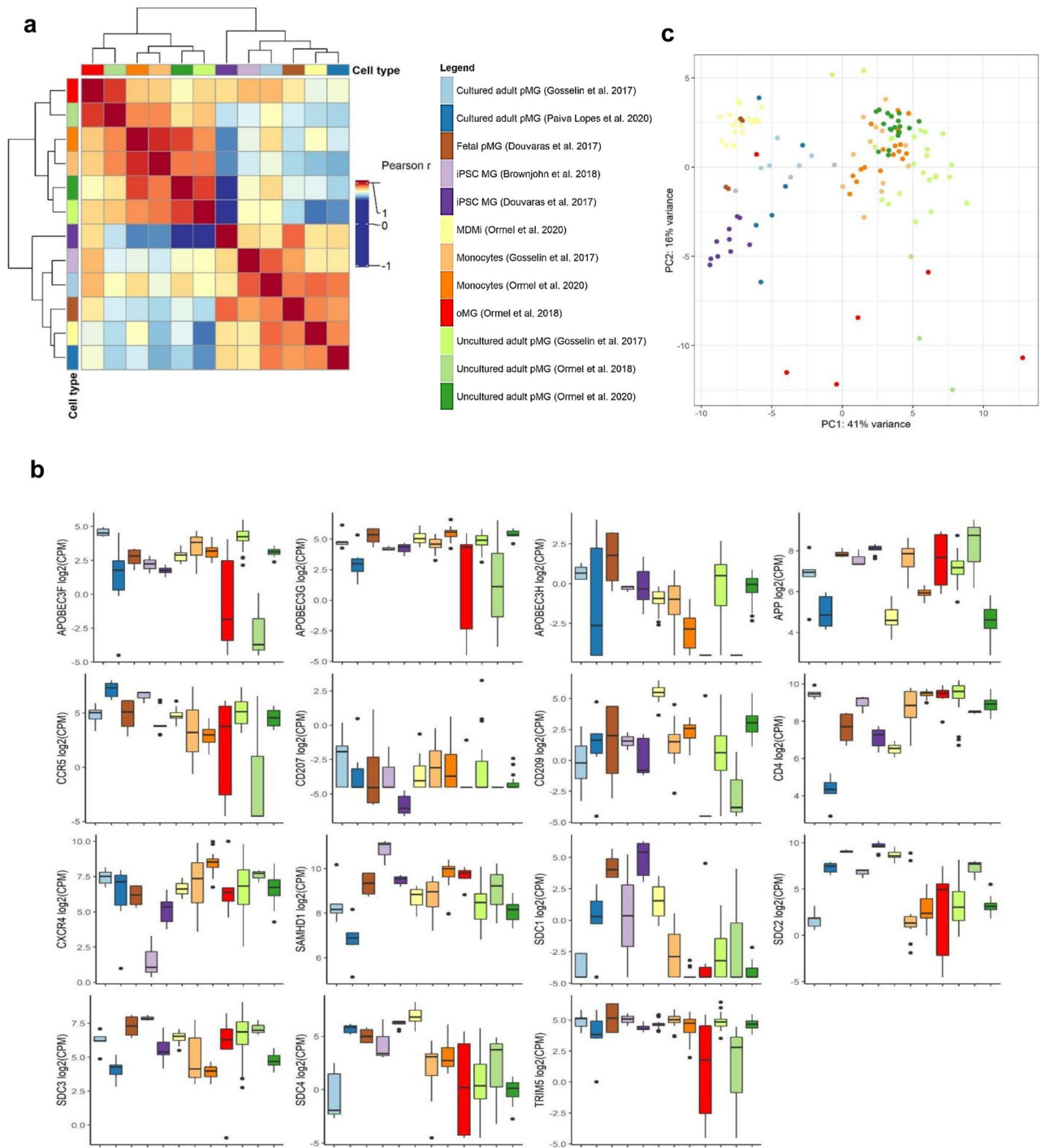


Fig. 4 Gene expression analysis of microglia culture models on HIV-relevant genes. **a** Heatmap of Pearson r for between each cell type (cluster distances are Euclidean). **b** Boxplot of log₂(CPM) for

selected HIV genes. **c** PC plot depicting cell-type distances based on expression variance within selected HIV genes

oMG had a lower *TRIM5* expression compared to the other models. Expression of *SAMHD1* was lower in cultured adult pMG (Lopes et al. 2020) and higher in iPSC-MG (Brownjohn et al. 2018) compared to that in uncultured adult pMG.

Looking at overall model similarity, strikingly, Pearson correlation coefficients were very high ($r > 0.8$) among MDMi and cultured pMG, as well as among monocytes, uncultured adult pMG, and oMG, respectively (Fig. 4b). Unlike before,

cell-type-specific clusters of samples started to diffuse and became less apparent. That is, on our PCA the distances between monocytes and uncultured adult pMG diminished, MDMi clustered with the highly interspersed cultured adult pMG, and oMG now scattered across the first two PCs (Fig. 4c). This suggests that the HIV-relevant gene expression profile is not specific to one model. Hierarchical clustering of samples supported the strong notion of diffusion but provided a higher conservation of the same-cell-type sample cluster identity. Notably, in all three analyses, monocytes displayed the highest degree of similarity to uncultured adult pMG in terms of the examined HIV gene expression, suggesting high conservation of HIV gene expression compared to uncultured adult pMG. CCR5 expression, however, was low.

Comprehensive overview: HIV research on microglia models

Overview of HIV DNA and RNA in microglia of human brain tissues

That microglia are a site of HIV infection in the CNS is supported by the detection of (integrated) HIV DNA in microglia of HIV-positive individuals by laser capture microdissection coupled with polymerase chain reaction (PCR) (Churchill et al. 2006; Thompson et al. 2011; Trillo-Pazos et al. 2003). Churchill and colleagues detected integrated HIV DNA in the isolated CD68 + microglial/macrophage cells in all 3 examined HIV-positive individuals that died with HIV-associated dementia (Churchill et al. 2006). Trillo-Pazos and colleagues also consistently detected HIV DNA in microdissected brain tissue from all four HIV + individuals (2 pediatric and 2 adult patients) with HIV encephalitis (HIVE) (Trillo-Pazos et al. 2003). HIV DNA levels were quantified in duplicate pools of 100 CD68 + microglia/macrophage cells, and extrapolation to a standard curve revealed that about 1–10% of the investigated cells are likely to harbor HIV DNA. HIV *gag* DNA was more prominent in cases with notable microgliosis (Trillo-Pazos et al. 2003). Another study by Thompson and colleagues detected HIV DNA in both HIV-positive encephalitic patients, with evident microglia activation and/or microglial nodules, and in 4 of the 5 HIV-positive presymptomatic individuals who died before pathological evidence of HIVE (Thompson et al. 2011). HIV DNA levels were quantified in pools of 200 CD68 + parenchymal microglial cells (distinguished by shape and location from the perivascular macrophages), and 10% of the analyzed replicates were positive by triple-nested PCR in both symptomatic patients and the majority of the presymptomatic patients (Thompson et al. 2011).

However, these post-mortem studies do not reflect modern-day combined antiretroviral therapy (cART) patients with

effective suppressive therapy. Lamers and colleagues measured HIV DNA in tissues of 20 virally suppressed HIV + individuals by real-time PCR and droplet digital PCR (ddPCR) and found HIV DNA in 48 of 87 brain tissues (Lamers et al. 2016). A more in-depth cellular analysis was done by Tso and colleagues, who detected HIV RNA and/or DNA within CD68 + cells in 3 out of 4 virally suppressed individuals infected with HIV subtype C using ddPCR and RNA/DNA scope ISH (Tso et al. 2018). The distribution of the HIV viral genome was proposed to most likely be the result of a random event as no obvious distribution pattern was observed among the various brain compartments (frontal lobe, cerebellum, hippocampus, basal ganglia, temporal lobe, parietal lobe, and occipital lobe) (Tso et al. 2018). This being said, viral strain compartmentalization has been found between different brain regions (frontal lobe, occipital lobe, and parietal lobe) (Brese et al. 2018).

A recent study by Ko and colleagues confirmed the persistence of HIV DNA in virally suppressed patients with ($n = 8$) and without ($n = 8$) HAND using a highly specific DNAscope in situ hybridization technique (Ko et al. 2019). In all 16 cases, HIV DNA was found exclusively in CD68 + microglia/perivascular macrophages. Small clusters of isolated HIV RNA signals, which were infrequent and very focal, were observed in a small group ($n = 6$) of virally suppressed patients with ($n = 3$) and without HAND ($n = 3$) (Ko et al. 2019). The evidence of low copies of HIV RNA in some cases suggests either spontaneous viral reactivation or ongoing low level replication despite suppressive cART (Ko et al. 2019; Tso et al. 2018).

Overview of HIV infection in cultured microglia models (Table 3)

As mentioned earlier, viruses detected in the CNS are predominantly R5 M-tropic (Arrildt et al. 2015; Joseph et al. 2015; Joseph and Swanstrom 2018). Accordingly, primary microglia isolated from fetal and adult brain tissue in culture were shown to be primarily susceptible to HIV infection with R5 M-tropic HIV strains (HIV_{ada}, HIV_{Bal}, HIV_{YU-2}, HIV_{JRFL}, HIV_{SF162}) (Albright et al. 1999, 2000; Asahchop et al. 2017; Castellano et al. 2017; Cenker et al. 2017; Garcia-Mesa et al. 2017; Ghorpade et al. 1998; Huang et al. 2011; Lee et al. 1993; Mamik and Ghorpade 2014; Schuenke and Gelman 2003; Strizki et al. 1996; Tatro et al. 2014; Zenón et al. 2015). It is noteworthy to mention that although every study observed HIV infection with an R5 M-tropic virus, different methods were used to isolate microglia that were subsequently grown in different culture media for 1 day up to 3 weeks before infection. As previously mentioned, this leads to significant changes in the microglial phenotype, which in turn could affect the

Table 3 Overview of the characteristics of all the microglial in vitro culture models

Microglia model	Name	Co-culture	Microglia markers	HIV research citations (n) ^a	Reference
Primary microglia	Primary microglia		CD11b, CD45, TMEM119, CD68, HLA-DR, P2RY12, CX3CR1, PU.1	(13) Albright et al. (1999), Asahchop et al. (2017), Castellano et al. (2017), Cenker et al. (2017), Garcia-Mesa et al. (2017), Ghorpade et al. (1998), Huang et al. (2011), Lee et al. (1993), Mamik and Ghorpade (2014), Schutenke and Gelman (2003), Strizki et al. (1996), Tatro et al. (2014), Zenón et al. (2015)	Mizee et al. (2017), Olah et al. (2012), Rustenhoven et al. (2016), Zhang et al. (2016)
Human microglia cell lines	SV40 huglia		IBA1, TREM2, CD11b, CD68 CD68, P2RY12, CD11b	(4) Alvarez-Carbonell et al. (2017), Garcia-Mesa et al. (2017), Ingram et al. (2020), Rai et al. (2020)	Chiavari et al. (2019) Garcia-Mesa et al. (2017)
	HMC3, CHME3/5, C13NJ		CD68, CD11b, CD45, IBA1, CX3CR1	(18) Ambrosius et al. (2017), Campbell et al. (2017, 2019), Chai et al. (2017), Chugh et al. (2007), Delaney et al. (2017), dos Reis et al. (2020), Francis et al. (2020), Ingram et al. (2020), Jadhav et al. (2014), Lisi et al. (2016), Malikov and Naghavi (2017), Mishra et al. (2012), Rai et al. (2020), Samikkannu et al. (2016), Tomitaka et al. (2018), Wires et al. (2012), Zenón et al. (2015)	Dello Russo et al. (2018), Janabi et al. (1995)
Monocyte-derived microglia	MDMi		HLA-DR, IBA1	(2) Cherrier et al. (2011), Leone et al. (2006)	Leone et al. (2006)
	iMG		CX3CR1, HLA-DR, CD45		Ohgidani et al. (2014)
	iMG		TMEM119, P2RY12, PU.1		Selgren et al. (2017, 2019)
	MMG		IBA1, CD11b, CD45, HLADR _{Low} , P2RY12, CD68	(2) Rawat et al. (2019), Rawat and Spector (2017)	Rawat and Spector (2017)
	ML	Astrocytes	CD11b, TREM2, IBA1	(1) Bertin et al. (2012)	Noto et al. (2014)
	MDMi		IBA1	(1) Rai et al. (2020)	Bertin et al. (2012)
	MDMi		P2RY12, CSF1R, TREM2		Ryan et al. (2017)
	iMG		TREM2, HLADR		Ormel et al. (2020)
	M-MG		CX3CR1		Etemad et al. (2012)
iPSC-derived microglia	pMGLs		CD45, IBA1, P2RY12, TMEM119		Muffat et al. (2016)
	iPSC-MG		CD11b, CX3CR1, IBA1, P2RY12, TMEM119		Douvaras et al. (2017)

Table 3 (continued)

Microglia model	Name	Co-culture	Microglia markers	HIV research citations (n) ^a	Reference
	iMGLs		CD45, CX3CR1, P2RY12, TREM2, PU.1, CSF1R, CD11b		Abud et al. (2017)
	iMGLs		IBA1, TMEM119		Xu et al. (2019)
	iPSC-derived microglia		IBA1, CD45, TREM2		Brownjohn et al. (2018)
	Co-pMG	iPSC-derived neurons	CD11b, CD45, IBA1		Haenseler et al. (2017)
	iMicros	iPSC-derived neurons	IBA1		Takata et al. (2017)
	iPS-MG	Astrocytes	CD11b, CD45, CX3CR1, HLA-DR, IBA1, TREM2		Pandya et al. (2017)
	ScMglia		CX3CR1, P2RY12, TREM2, CSF1R, IBA1, CD11b		Amos et al. (2017)
	hiPSC-MG		CSF1R, P2RY12, TMEM119, TREM2, CX3CR1		Banerjee et al. (2020)
	iMg		CX3CR1, TMEM119, IBA1, P2RY12	(1) Ryan et al. (2020)	Ryan et al. (2020)
Cerebral organoids	Brain spheres + SV40 cell line	Neurons, astrocytes, oligodendrocytes	TMEM119, IBA1		Abreu et al. (2018)

Table 3 (continued)

Microglia model	Name	Co-culture	Microglia markers	HIV research citations (n) ^a	Reference
	3D cortical organoids + iPSC-derived microglia	Neurons, astrocytes	ND		Brownjohn et al. (2018)
	3D BORG + iPSC-derived microglia	Neurons, astrocytes, oligodendrocytes	ND		Abud et al. (2017)
	hBORG + HMC3 cell line	Neurons, astrocytes	ND	(1) dos Reis et al. (2020)	dos Reis et al. (2020)
	Cerebral organoids	Neurons, astrocytes, oligodendrocytes	IBA1, CD68, CD11b, TREM2, CX3CR1, HLA-DR, CD45		Ormel et al. (2018)

ND not determined

^aResearch article of HIV infection studies with human lab strains

susceptibility to HIV infection. This being said, cultured fetal and adult pMG seem to have a higher susceptibility to HIV infection compared to in vivo cells, with an infection rate of 40–50% at 72 h (Alvarez-Carbonell et al. 2019; Cenker et al. 2017), 75% at day 5 (Garcia-Mesa et al. 2017), and about 90% at day 6 post infection (Cenker et al. 2017). Moreover, HIV-infected microglia formed giant multinucleated syncytia that accumulated in the cultures over time and correlated with peaks in HIV capsid (p24) levels (Castellano et al. 2017; Cenker et al. 2017; Lee et al. 1993). This cytopathic effect is reminiscent of microglial nodular lesions observed in the brains of HIV-positive individuals with HAND and AIDS (Budka et al. 1987; Nebuloni et al. 2000), among which are the HIV-positive individuals with HIVE in the previously mentioned studies by Trillo-Pazos et al. (2003) and Thompson et al. (2011). Furthermore, addition of CCR5 inhibitor maraviroc on day 1 post infection blocked the increase in infection, indicating spread of HIV infection in these cultures (Cenker et al., 2017). Most laboratory-adapted T-cell tropic HIV strains require high surface density of the CD4 and CXCR4 receptor and hence were found to replicate inefficiently in human primary microglia (HIV_{HXB2}, HIV_{NL4-3}, HIV_{LAI}) (Ghorpade et al. 1998; Strizki et al. 1996).

In line with cultured pMG, the HMC3 cell line, MDMi and iPSC-MG were reported to be susceptible to infection by several R5 M-tropic HIV strains (HIV_{bal}, HIV_{ada}, HIV_{YU-2}, HIV_{jago}) (Bertin et al. 2012; Chugh et al. 2007; Leone et al. 2006; Rai et al. 2020; Rawat and Spector 2017; Ryan et al. 2020; Samikkannu et al. 2016; Zenón et al. 2015). Zenón and colleagues directly compared HIV infection of cultured fetal pMG with infection of the microglial cell line HMC3 and showed tenfold higher levels of p24 production in the pMG cells as compared to HMC3 (Zenón et al. 2015). In contrast, Rai and colleagues could not detect infection of the C20 and HMC3 cells, which is in line with the fact that they were also unable to detect expression of the primary CD4 receptor in these cell lines (Rai et al. 2020). Rawat and Spector compared cultured fetal primary microglia with MDMi and showed similar HIV infection kinetics and indicated that after 20 days of infection the majority of cells were p24 positive (Rawat and Spector 2017). Productive HIV infection of MDMi is also shown in other in vitro studies (Bertin et al. 2012; Leone et al. 2006; Rai et al. 2020). Rai and colleagues directly compared iPSC-MG to MDMi and the C20 and HMC3 cell lines and showed productive infection in the iPSC-MG as well as in the MDMi (Rai et al. 2020). iPSC-MG infection resulted in virus levels that peaked at day 8 post infection and then declined. MDMi, in contrast, continued to produce virus over the 2-week experiment, albeit at about tenfold lower levels as seen in the iPSC-MG. Ryan and colleagues showed that iPSC-MG are highly susceptible

to HIV infection with 95% of cells p24 positive after 15 days with extensive multinucleation exclusively associated with infection (Ryan et al. 2020). As observed for the pMG also, the HMC3 cell line (Chugh et al. 2007) and MDMi (Bertin et al. 2012) were found to be refractory to infection with the T-cell tropic HIV strain HIV_{NL4-3}. To the best of our knowledge, there are no published studies of HIV research with organoid-derived microglia or 3D cerebral organoids in which microglia develop innately.

Head-to-head comparison of HIV infection in microglial culture models

As previously mentioned, numerous studies have been published on the susceptibility of each culture model to HIV infection. However, there is a gap in literature in how the different microglial culture models compare with each other and most importantly to cultured pMG. Therefore, we performed a head-to-head comparison of the different microglial culture models by infecting each model, except for iPSC-MG, with the R5 M-tropic HIV strain HIV_{bal}, equipped with a luciferase tag, under the same experimental conditions (Fig. 5). Extracellular virus production was

measured indirectly by quantifying the release of luminescence over time.

As expected, cultured adult pMG were highly susceptible to HIV_{bal} infection and showed continuous virus production with a peak infection on day 15 (Fig. 5a). A similar infectivity and pattern was observed in MDMi that peaked on day 13 (Fig. 5b). To investigate whether oMG are susceptible to HIV infection, we isolated oMG innately developed within human cerebral organoids before HIV_{bal} infection. Organoid-derived microglia were susceptible to HIV_{bal} infection; however, contrary to cultured adult pMG and MDMi, peak infection was reached on day 9 and luminescence was also substantially lower (> 150-fold) in oMG compared to cultured adult pMG (Fig. 5c, d). Pre-incubation of microglia with the CCR5 inhibitor maraviroc successfully prevented viral infection in all culture models, indicating that infection occurred primarily through the CCR5 receptor. Microglial cell lines SV40 and HMC3 did not support the infection of HIV_{bal} (Fig. 5e).

Next, we also infected cultured pMG and MDMi with HIV_{bal} equipped with a GFP tag to evaluate the fraction of HIV-infected cells and the effect of HIV infection on cell morphology. GFP+ microglia could be detected as early as

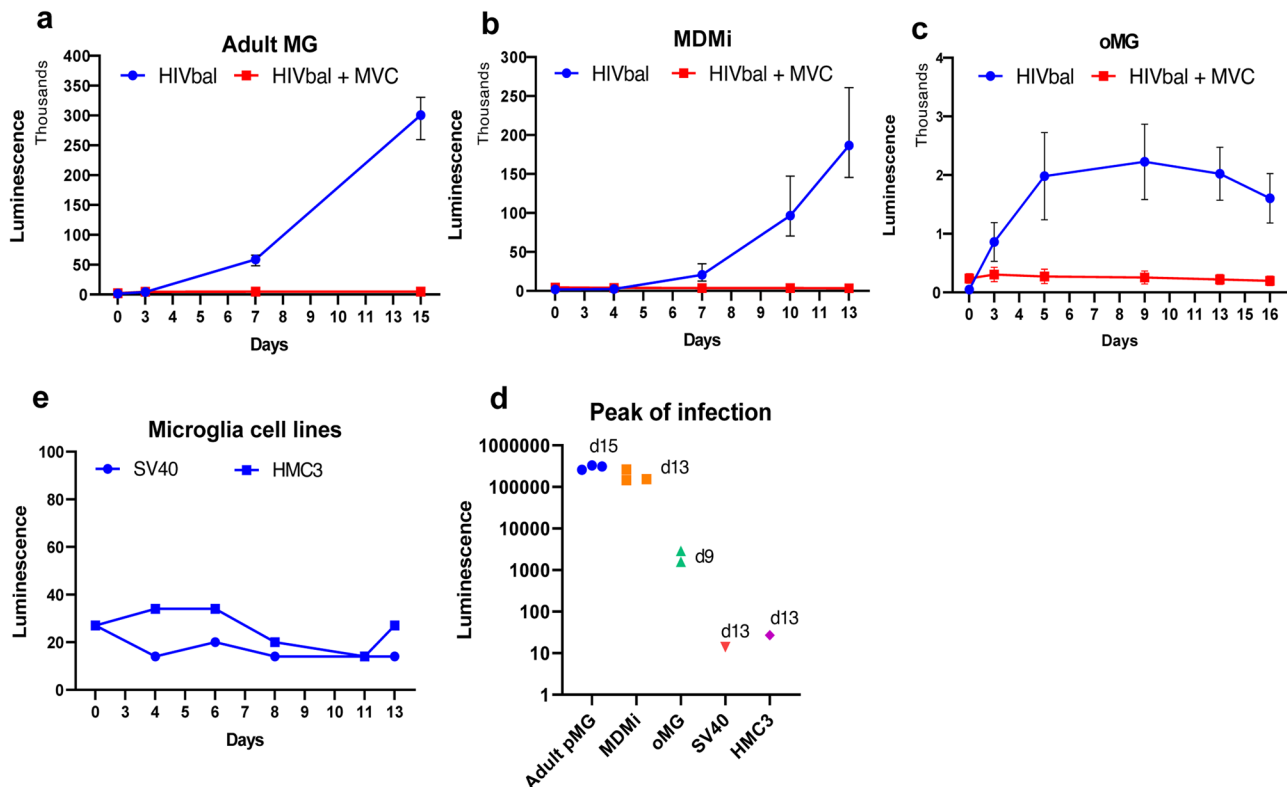


Fig. 5 HIV infection and virus production in microglial culture models. Adult primary microglia **a**, MDMi **b**, oMG **c**, and microglial cell lines SV40 and HMC3 **e** were infected with 10 ng (p24 Gag) HIV_{bal} with a luciferase tag and cultured for the indicated days. Supernatant

was collected post infection on the indicated days, and virus production was measured with luminescence. **d** Peak infection day of each culture model

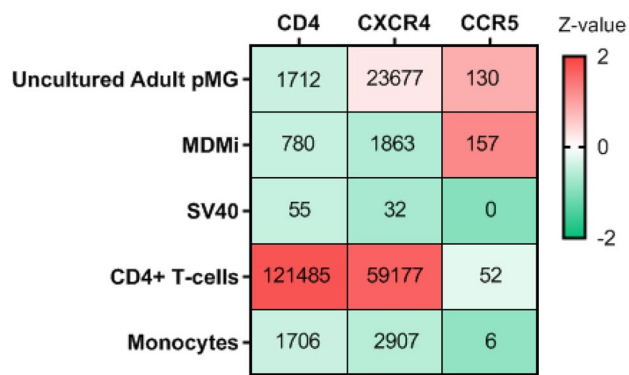


Fig. 6 Gene expression of major HIV receptors in in vitro culture models. Median (IQR) gene expression of *CD4*, *CXCR4*, and *CCR5* in primary microglia (pMG), monocyte-derived microglia (MDMi), SV40 microglial cell line, CD4+ T-cells and monocytes assessed by RT-PCR and normalized to the reference gene *GAPDH*. All cells are color-coded according to their Z-value (color bar on the right-hand side)

4 days post infection in both culture models, which increased to ~90–95% on day 15. GFP expression was also exclusively found in giant multinucleated cells (data not shown).

Hereafter, we performed real-time PCR for the main HIV receptor genes, *CD4*, *CXCR4*, and *CCR5*, in three microglial culture models (Fig. 6). *CD4* and *CXCR4* expression were the highest in uncultured adult pMG, followed by MDMi and then SV40 with very low expression. The expression of *CCR5*

was slightly higher in MDMi compared to that in uncultured adult pMG and was not expressed in SV40. Interestingly, our real-time PCR data revealed that on a transcriptomic level, uncultured adult pMG express *CXCR4* at considerably higher levels than *CCR5*. This being said, uncultured adult pMG have a substantially lower expression of *CD4* and *CXCR4* and a higher expression of *CCR5* compared to CD4+ T-cells. This further corroborates the theory that R5 M-tropic HIV strains, unlike R5 T-tropic strains, can infect cells expressing relatively low levels of *CD4* including pMG.

Discussion

Microglia are thought to constitute the main viral reservoir for HIV in the CNS (Wallet et al. 2019), but insight into the dynamics of HIV-microglial interactions has been limited due to the difficulty of obtaining human brain tissues and the limited number of viable microglial cells after isolation. This has led to a boost in the development of novel human microglial culture models, each having its own set of advantages and limitations (Table 4).

The suitability of these models for HIV research, however, remains to be established. We, therefore, aimed to provide researchers in the field of HIV with an encompassing guide to selecting a suitable human microglial in vitro culture model for studying the interplay between HIV and

Table 4 Benefits and limitation of microglial in vitro culture models

Culture model	Benefits	Limitations
Cultured primary microglia	Moderately easy to culture Susceptible to HIV infection	Difficult to obtain fresh human brain tissue Limited number of viable cells Limited life span Transcriptomic deficiencies induced by in vitro culture
Microglial cell lines	Commercially available Easy to culture Mass production Long-term culture Genetic modifications: HIV latency	Transcriptomic profile does not cluster with adult or fetal primary microglia Not susceptible to HIV infection
Monocyte-derived microglia	Easy to obtain and culture Mass production Susceptible to HIV infection	Limited life span Transcriptomic profile does not cluster with adult or fetal primary microglia Expensive
iPSC-derived microglia	Mass production Long-term culture Genetic modifications Susceptible to HIV infection	Transcriptomic profile clusters more closely with fetal microglia Technically complex and time consuming Very expensive
3D organoids	Recapitulate in vivo CNS structure Cell–cell interaction with other CNS cell types Microglia developed in a 3D micro-environment Transcriptomic profile cluster with adult primary microglia Long-term culture	High inter- and intra-variability between organoids Variability in differentiation protocols; patterned and non-patterned Technically complex and time consuming Lack vasculature Ethical concerns Very expensive

microglia. A good microglial model should strongly resemble uncultured *ex vivo* adult microglia based on morphology, immune functions, and gene expression profile. Furthermore, the ideal microglial model for HIV research should express HIV receptors and restriction factors at similar levels as uncultured *ex vivo* adult microglia and support productive HIV infection representative of HIV infection in the CNS. To evaluate the various microglial culture models, we (i) performed a literature review on the morphology, immune functions, expression of microglia-enriched genes, and the susceptibility of each model to HIV infection and (ii) leveraged real-time PCR and RNA-seq data to further characterize the similarity of the various culture models to cultured and uncultured pMG on a transcriptomic level. Our approach, therefore, was novel in so far as to interrogate the transcriptomic data of seven cell types stemming from twelve distinct datasets on a variety of gene signatures to give viability to our interpretation.

The morphology of microglial *in vitro* culture models is commonly described as having the typical “ramified” morphology of microglia. Although iPSC-MG and oMG showed a closer resemblance to microglia *in situ* with primary and secondary branches, this is only a minimal representation of the complexity in the arborizations observed in *in situ* microglia in human brain tissue. Functional characterization of the models based on phagocytosis and LPS responsiveness revealed that every model has the capacity to phagocytose and the ability to induce a pro-inflammatory cytokine response (IL-1 β , TNF- α , IL-6). However, most studies did not perform comparative analysis with cultured pMG, rendering it difficult to accurately determine whether these responses are representative. Furthermore, as previously highlighted, these immune functions are not exclusive to microglia. Therefore, we strongly advise to follow up with gene expression profiling, as we did, to more accurately validate the microglial phenotype of a (novel) culture model.

Transcriptome analysis of the microglial models, except for the immortalized cell lines, showed that overall cultured pMG and oMG have the highest level of similarity to the microglial transcriptome gene profile of uncultured adult pMG, followed by iPSC-MG and then MDMi. Focusing on the microglia-specific core gene signature, we noted that none of the models including cultured adult and fetal pMG clustered with uncultured adult pMG. This highlights that unfortunately none of the current microglial models is capable of fully recapitulating the microglial transcriptome of *ex vivo* pMG analyzed immediately after isolation. Notably, considering the degree of phenotypic changes observed in pMG after culture (Gosselin et al. 2017; Gosselin et al. 2017), it is highly probable that the phenotype of freshly isolated *ex vivo* pMG does not fully recapitulate the phenotype of *in vivo* microglia. The most probable cause for this transcriptomic discrepancy is the absence of certain chemical or

physical factors from the complex CNS microenvironment and lack of intercellular communication with other CNS cell types (Bohlen et al. 2017; Butovsky et al. 2014). As many of these homeostatic signature genes are involved in the communication between microglia, neurons, and astrocytes, conclusions on the impact of HIV-infected microglia on neurons and neuronal tissue, including the levels of neurotoxicity, should be interpreted with care.

Recent studies revealed that the morphological and transcriptomic deficits can be corrected in part via co-culture with neurons and/or astrocytes (Haenseler et al. 2017; Park et al. 2018; Ryan et al. 2020; Takata et al. 2017), incorporation into 3D cerebral organoids (Abreu et al. 2018; Abud et al. 2017; Brownjohn et al. 2018; dos Reis et al. 2020; Lin et al. 2018; Muffat et al. 2016), or transplantation of iPSC-derived hematopoietic progenitors into humanized mice (Abud et al. 2017; Hasselmann et al. 2019; Mancuso et al. 2019; McQuade et al. 2018; Svoboda et al. 2019). Transplanted microglia acquired a highly ramified morphology, reminiscent of the complex arborization patterns seen *in situ*, and a gene expression profile that more closely resembled uncultured pMG rather than cultured pMG, including significantly higher expression of key microglia-specific genes such as *P2RY12*, *CX3CR1*, and *CSF1R* (Hasselmann et al. 2019; Mancuso et al. 2019; Svoboda et al. 2019). This indicates that the regulation of key microglial specific genes is dynamically dependent on the environment and can be rescued by mimicking the CNS microenvironment. In this regard, another attempt to drive these cells closer to a microglia fate has been by optimizing the culture media composition with the addition of cytokines (TGF- β , CX3CL1, and CD200) critical for maintaining microglial homeostasis (Abud et al. 2017; Butovsky et al. 2014; Cardona et al. 2006; Hoek et al. 2000; Kierdorf and Prinz 2013) or the use of neural progenitor cell conditioned medium (Banerjee et al. 2020).

Despite the difficulty in maintaining the homeostatic microglial transcriptome in culture, this progressive field of microglial culture models holds great promise for the advancement of HIV research on microglia and the CNS. Thus, to identify which microglial models are suitable for HIV research, we thoroughly investigated the transcriptome similarity of the microglial models, except for cell lines, on the expression of HIV genes that are relevant for HIV infection and replication. HIV receptor expression levels in uncultured adult pMG shared the most similarities with oMG followed by cultured adult pMG. We noted dissimilarities in *CD4* gene expression between cultured and uncultured pMG and a surprisingly low expression of *CD4* and *CXCR4* in iPSC-MG compared to uncultured pMG. A recent study by Rai and colleagues also reported a low expression of both co-receptors in iPSC-MG compared to cultured pMG (Rai et al. 2020). This being said, the expression levels in

cultured adult pMG and iPSC-MG differed between datasets corroborating the significance of the culture environment on the microglial transcriptome. The expression of the HIV restriction factors *TRIM5*, *APOBEC3G*, and *SAMHD1* was generally similar to that in uncultured adult pMG and are mostly conserved between the microglial culture models.

Based on the transcriptomic similarity to uncultured adult pMG across all gene signatures we evaluated, oMG and cultured adult pMG are the most representative culture models when considering HIV research in microglia. However, we acknowledge that cerebral organoids have high inter-organoid variability and that a definitive conclusion should only be drawn upon a transcriptomic evaluation of oMG encompassing a sample size that accounts for such high variability. Furthermore, the statistical approach can be strengthened by leveraging count statistics other than CPM (counts per million) to make the analyses less biased to technical factors evoking artificial variance in the data unassociated with true biological differences between the model systems. Correction approaches accounting for known and unknown technical factors could then be applied without overwriting the interesting biological information within the data.

Infection of the microglial culture models, except for iPSC-MG, with HIV_{bal} exposed distinct differences in virus production. Cultured adult pMG and MDMi both continuously produced virus up to the last day in culture (days 15 and 13). This pattern is consistent with previous studies that showed continuous virus production in cultured pMG and MDMi up to the third week after infection with HIV_{bal} (Albright et al. 2000; Ghorpade et al. 1998; Leone et al. 2006; Rai et al. 2020; Rawat and Spector 2017; Watkins et al. 1990). Interestingly, HIV_{bal} infection of oMG was significantly low compared to that of cultured adult pMG and, contrary to pMG and MDMi, peaked at day 9 and decayed over the following week. On a transcriptomic level, oMG has the closest resemblance to microglia in situ, suggesting that HIV infection in oMG is also the most representative of HIV infection in the CNS. HIV DNA is found irrespective of ART treatment and/or HAND, in a focally distributed small population of CD68 + microglia/macrophage cells (1 to 10%) (Ko et al. 2019; Thompson et al. 2011; Trillo-Pazos et al. 2003; Tso et al. 2018). Besides the sporadic detection of HIV RNA, this indicates that HIV infection, replication, and spread within the microglia population in the CNS is limited. In this regard, the low initial HIV_{bal} infection in oMG is reflective of the small HIV-infected microglial population observed in situ. Interestingly, this decline in viral production after the 1st week of infection was also recently reported by Rai et al. after infection of iPSC-MG with HIV_{bal} (Rai et al. 2020). However, despite being widely used in the HIV research field as an R5 M-tropic virus, HIV_{bal} is a laboratory-adapted strain with replication kinetics and biological properties that might not be representative of R5 M-tropic

HIV strains circulating in the human CNS. It will be interesting in future studies to investigate whether this decline in virus production in oMG and iPSC-MG is also observed with viral strains from the CNS of HIV-infected individuals and whether this is due to reversion to a latent state as is proposed for the HIV-infected microglial population in the CNS (Wallet et al. 2019). In summary, different conclusions may be drawn on the level and kinetics of microglia infection, the underlying mechanisms, and potential therapies, based on the model that is chosen. We therefore, propose to be mindful of these potential model-specific effects, and to cross-validate important findings with different models.

Altogether, based on the transcriptome and infection analysis, we find oMG, cultured adult pMG, and iPSC-MG to be suitable microglia in vitro culture models to further research on the interplay between HIV and microglia. However, we acknowledge that all three models, particularly oMG and iPSC-MG, have laborious, costly, and lengthy protocols limiting their widespread use. We do not recommend using the human microglial cell line SV40 or the HMC3-based cell lines for microglia or HIV research as they have large transcriptomic and phenotypic discrepancies with primary microglia and do not support HIV infection. A more affordable, fast, and straightforward model would be MDMi, which is superior to microglial cell lines on a morphological, transcriptional, and functional level. Researchers interested in a cost-effective model with low inter-assay variability for the initial assessment of large cohort studies would benefit from the use of MDMi as a first-line screening tool. Alternatively, we acknowledge the novel hµglia/HIV latent microglial cell line as an exception and a promising model that can be used for the initial assessment of HIV latency reversal on microglia or other HIV latency-related research (Garcia-Mesa et al. 2017). This being said, oMG, cultured pMG, iPSC-MG, and MDMi provide an assessment of HIV-microglia interactions outside of the context of the CNS microenvironment and other CNS cell types. A recent study by Alvarez-Carbonell et al. showed HIV expression in primary microglia was silenced following co-culture with primary neurons (Alvarez-Carbonell et al. 2019). Another study by Ryan et al. showed a reduction in viral production after culturing HIV-infected iPSC-MG with IPSC-derived neurons and astrocytes (Ryan et al. 2020). Thus, it is important to validate experiments done in monoculture in more complex and representative models such as pMG and iPSC-derived co- and tri-culture models or cerebral organoids.

Ultimately, the best model has to be chosen on a case-by-case basis based on the research question and genes of interest and should take into account the capability and resources of the laboratory. We will with no doubt continue to see great technological advancements in this field leading to great improvements in these protocols.

Methods

Literature search strategy

For this article, we evaluated five human *in vitro* microglial culture models and their application in HIV research. We searched the PubMed database for articles describing the generation and characterization of each microglial culture model, including the morphology, inflammatory response, and phagocytic ability of the model. Next, we searched for articles that examined the presence of the HIV genome in microglia in human brain tissue. Finally, we searched for HIV studies performed with human HIV lab strains on any of these culture models.

Human microglia isolation and culture

Fresh post-mortem brain tissue from the subventricular zone ($n = 5$) was provided by the Netherlands Brain Bank (www.hersensbank.nl). Informed consent was obtained from all donors. Human microglia were isolated and cultured according to the protocol described before (Sneeboer et al. 2019). In short, a mechanical and enzymatic dissociation with DNase 1 (200 $\mu\text{g}/\text{ml}$; Roche Diagnostics GmbH) and trypsin was done to obtain a single cell suspension, followed by a Percoll gradient to remove myelin and red blood cells. Microglia enrichment was achieved by positive selection for CD11b expression using CD11b + MACS (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Microglial cells were cultured in 200 μl microglia medium ((RPMI 1640; Gibco Life Technologies, USA) supplemented with 10% FCS, 1% penicillin–streptomycin (Gibco Life Technologies, USA) and 100 ng/ml IL-34 (Miltenyi Biotec, Germany)) for 1 day before infection.

Isolation and culture of other microglial cell models

The SV40 human immortalized microglial cell line, originally derived from microglia isolated from the embryonic spinal cord and cortex immortalized with SV40 virus, was obtained from Applied Biological Materials Inc. (Janabi et al. 1995). SV40 were maintained in RPMI 1640 (Gibco Life Technologies, USA) supplemented with 10% FCS and 1% penicillin–streptomycin (Gibco Life Technologies, USA).

Monocytes were isolated from PBMCs by CD14 + MACS (Miltenyi Biotec, Germany) according to the manufacturer's protocol. Monocytes were then differentiated to monocyte-derived microglia according to the protocol of (Ormel et al. 2020). In short, monocytes were cultured in monocyte culture medium (RPMI 1640 (Gibco Life Technologies, USA), 2 mM L-glutamine, 1% penicillin–streptomycin (Gibco Life

Technologies, USA)) + 25% astrocyte-conditioned medium (ACM) (SCC1811, ScienCell, USA). On the fourth and eighth day in culture, the medium was replaced with monocyte-derived microglia (MDMi) medium (RPMI 1640, 2 mM L-glutamine, 1% penicillin–streptomycin, 25% ACM, 10 ng/ml M-CSF, 10 ng/ml GM-CSF, 20 ng/ml TGF β , 12.5 ng/ml IFN γ , and 100 ng/ml IL34 (all cytokines from Miltenyi Biotec, Germany)). Infection and qPCR analyses were done on day 10 post differentiation.

Three-dimensional cerebral organoids were generated as we have published before (Ormel et al. 2018). Three-dimensional organoids were dissociated with enzymatic dissociation using papain (18.6 U/ml, Worthington, LK003176) and DNase 1 (337 U/ml Worthington, LK003170), followed by microglia isolation using CD11b + MACS (Miltenyi Biotec, Germany) according to the protocol published before (Ormel et al. 2018). Organoid microglia (oMG) were cultured in poly-L-lysine hydrobromide (PLL)-coated 96-well plates in microglia medium. Infection experiments were performed on day 1 post isolation.

Construction of HIV-1 reporter virus

An HXB2 molecular clone (pHXB2PS) was used to construct a molecular gp160 deletion vector with a luciferase reporter gene (HxB2 Δ ENVluc). pHXB2PS is derived from pHXB2WT (Van Maarseveen et al. 2006), which expresses the full-length HIV-1 sequence HXB2 (9719 bp, GenBank accession number [K03455.1](https://www.ncbi.nlm.nih.gov/nuccore/K03455.1)), with all bacterial sequences non-essential for bacterial expression and replication removed. To create the HxB2 env deletion vector, a unique BtgZI site was introduced at position 6112 in pHXB2PS by site-directed mutagenesis PCR. The envelope coding region was removed through digestion with BtgZI and BsmBI (6112–8850) and replaced with a linker sequence. Hereafter, we cloned the NanoLuc luciferase gene (pNL1.3) (Promega) into the nef gene using the unique restriction site Bpu1102I as described before (Lebbink et al. 2017). Undesired NgoMIV and BtgZI restriction sites in the NanoLuc luciferase gene were removed by silent mutation to facilitate envelope cloning.

To generate the HxB2bal luciferase reporter virus (HxB2Balgp160Luc), we first amplified the envelope coding region of the R5 laboratory-adapted HIV-1 strain Bal (obtained through the NIH HIV Reagent Programs (<https://www.hivreagentprogram.org/>)), using the SuperScript III one-step RT-PCR system with Platinum *Taq* High Fidelity DNA Polymerase (Thermo Fisher Scientific, USA), according to the manufacturer's protocol. The real-time RT-PCR reaction was done with the primers OeVif-1forw 5'-GGTCAGGGAGTCTCCATAGAATGGAGG-3' and HIV-R-end-rev1 5'-GCACACAACGCGTGAAGCACTCAA GGCAAGCTTTATTGAGGC-3', followed by a nested PCR

with the primers gp160-fw 5'-TAGTAGTAGCASYAATCA TCGCAATAGTTGTGTGG-3' and gp160-rv 5'-CTCGTCTCA TTCTTTCCCTTACMKCAGGCCATCC-3'. The PCR product and the HxB2ΔENVluc vector were digested with BtgZI (6112) and BsmBI (8850) and subsequently ligated with T4 ligase.

To generate the NL4-3bal GFP reporter virus (NL4-3Bal-gp160GFP), we cloned the envelope coding region of HIV-1 BaL into an NL4-3 GFP reporter molecular clone (NL4-3GFPwt) (a kind gift from Theo Geijtenbeek (AMC, the Netherlands)) using the unique restriction sites SalI (5785) and NotI (8797). In brief, two gBlock gene fragments (Integrated DNA Technologies (IDT)) encoding for the HIV-1 BaL envelope gene and a 25-nt overlap with the NL4-3GFPwt vector at both the 5' and 3' ends were cloned into the NL4-3GFPwt molecular clone using the NEBuilder® HiFi DNA Assembly Master Mix kit following the manufacturer's instructions (New England BioLabs). We also introduced two silent mutations in the vpu gene to create a new, unique AfeI restriction site at position 6091/6092 to facilitate envelope cloning. Single colonies were picked and expanded, and plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Thermo Scientific). All HIV constructs were verified by nucleotide sequencing.

Replication competent viral stocks were generated by transfecting HEK-293 T cells with the chimeric infectious plasmids (HxB2Balgp160Luc and NL4-3Balgp160GFP) using Lipofectamine 2000 reagent (Invitrogen). The supernatant was harvested at 48 h.

Infection of microglia culture models

Cultured adult pMG, MDMi, oMG, and SV40 cells (1×10^5) were infected with 10 ng (p24 Gag) of HIVbalLuc (HXB-2Balgp160Luc) or HIVbalGFP (NL4-3Balgp160GFP). The virus was washed away the next day, and cells were cultured in their respective culture medium for 13–15 days without medium refreshment. The supernatant was collected 2–3×/week. Luminescence was measured using the NanoGlo® Luciferase Assay System (Promega) according to the manufacturer's protocol. Experiments were carried out in duplicate or triplicate. Graphs were generated with GraphPad Prism version 8.3.0 (GraphPad Software) and depict the mean and range.

RNA isolation and quantitative PCR

RNA was isolated from adult primary microglia, MDMi, SV40, CD4 + T-cells, and monocytes using the RNeasy kit (Qiagen, the Netherlands) including the DNase treatment according to the manufacturer's protocol. RNA isolation and downstream gene expression analysis were done in duplicate from 4 (pMG, monocytes) or 5 (CD4 + T cells, MDMi)

different donors, except for SV40. cDNA synthesis was performed with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. qPCR was done in a 7900 Real Time PCR System (Applied Biosystems) with the following cycle conditions: 95 °C for 10 min, 40 cycles at 95 °C of 15 s, and 60 °C for 60 s. Per reaction, 5 µl SYBR green PCR Master Mix (Roche; Life Technologies Corporation, Grand Island, NY), 1 µl primer mix (2 pmol/ml), and 5 ng RNA were added up to a final volume of 10 µl. Primer sequences are listed in Supplementary Table 1. Transcript levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) appeared to be most stable and were used for normalization. Quantification was done by raising 2 to the power of the negative CT values, and absolute expression was then calculated by dividing the CT values of the samples with GAPDH and then multiplying by 10.000. Median, interquartile range, and standard deviation were calculated for each gene using GraphPad Prism version 8.3.0 (GraphPad Software). Z-values were calculated for each gene by subtracting the mean and then dividing by the standard deviation. A heatmap was generated using GraphPad Prism version 8.3.0 (GraphPad Software).

RNA sequencing

The transcriptomic phenotype can be efficiently profiled by sequencing the RNA content of bulk tissues containing up to millions of cells (i.e., bulk RNA-seq; Gawad et al. 2016; Stegle et al. 2015). Cell-type specificity is arguably a criticism when sequencing heterogeneous bulk tissues that contain a multiplicity of cell types but can be achieved by focusing sequencing capacities on one specific cell type. To evaluate transcriptomic similarity across microglia models, we, therefore, selected published microglia model system-specific RNA-seq datasets of adult pMG (Gosselin et al. 2017; Ormel et al. 2018, 2020), cultured pMG (Gosselin et al. 2017; Lopes et al. 2020; Ormel et al. 2018), fetal pMG (Douvaras et al. 2017), monocytes (Gosselin et al. 2017; Ormel et al. 2020), MDMi (Ormel et al. 2020), iPSC-derived microglia (Brownjohn et al. 2018; Douvaras et al. 2017), and organoid-derived microglia (Ormel et al. 2018); integrated these data into one dataset; and subsequently analyzed it in R v4.0.3. We selected these datasets in June 2020.

Data pre-processing

Where raw counts were available, genes with less than one count per sample were removed from the analysis. Then, counts were normalized to log counts per million (logCPM) using the cpm command from edgeR v3.14.0 (Robinson et al. 2009) with setting prior counts to 1 and taking the logarithmic. Subsequently, the normalized datasets were merged. Only healthy and unstimulated

samples were included. The final dataset consisted of 129 samples from 12 datasets containing 7 distinct microglia model systems. Sample and gene outlier detection was performed using interquartile range measures on Pearson correlation with outliers being defined as 3 standard deviations above or below the mean. No samples or genes were detected as outliers. To account for technical bias leading to dataset differences, surrogate variable- and principal component-based correction approaches were applied using the *sva* v3.20.0 (Leek et al. 2012) and *limma* v3.28.14 (Smyth 2005) packages. Post hoc evaluations of the correction approaches by leveraging k-means clustering, unsupervised hierarchical clustering, and PCA, however, showed no sample clustering based on cell-type identity. Instead, no data correction was performed as the validity of this approach was supported through inter-dataset similarity of monocyte and ex vivo primary microglia samples as indicated on PCA and unsupervised hierarchical clustering (see results). Plots were generated using the *ggplot2* v3.3.2 package.

PCA, k-means clustering, and unsupervised hierarchical clustering

Pearson correlation was performed using the default *rcorr* function from the *Hmisc* v4.4.1 package. PCA was executed on samples using the *prcomp* function, scaling and centering the data prior. k-means clustering of samples was performed with the *kmeans* function using the Hartigan–Wong algorithm. The optimum number of clusters was calculated using the *fviz_nbclust* function from the *factoextra* v1.0.7 package. Hierarchical clustering was performed with the *pheatmap* v1.0.12 package using Euclidean distances.

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Declarations

Conflict of interest The authors declare no competing interests.

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