STEPHANIE GUMBS

INVESTIGATION OF THE CENTRAL NERVOUS SYSTEM AS A VIRAL RESERVOIR FOR HIV

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Onderzoek naar het centrale zenuwstelsel als een viraal reservoir voor HIV

(met een samenvatting in het Nederlands)

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Mas mi siña, mas mi sa.
Mas mi sa, mas sabí mi ta!

The more I learn, the more I know.
The more I know, the smarter I am.

- Roland Colastica

Dediká na mi mayornan:
Elton & Maureen Gumbs

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CHAPTER 1

General Introduction

Introduction

Since the emergence of the human immunodeficiency virus (HIV) in the 1980s, this virus has claimed the lives of 36.3 million people with an estimated 37.7 million people currently living with HIV [1]. Advances in antiretroviral therapy (ART), have transformed an HIV prognosis from a death sentence into a chronic disease by effectively suppressing HIV replication below the limit of detection. However, once HIV is integrated into the host genome, none of the antiretroviral drugs can eradicate the infected cells or inhibit the production of new infectious virus from these infected cells [2,3]. Continued viral suppression, therefore, requires lifelong adherence to ART to prevent new rounds of infection, referred to as *de novo* infection, and rebound viremia caused by the persistence of latently infected cells within cellular and anatomical reservoirs [4,5]. The achievement of HIV cure thus relies on the ability to either eradicate these latently infected cells or permanently block the production of new infectious virus and *de novo* infection [6].

Defining a viral reservoir

The term "HIV-reservoir" is widely used to describe cells and tissues that continue to harbor integrated intact proviral DNA that can form replication-competent virus, despite years of suppressive ART. Due to the high error rate of reverse transcription, however, most integrated proviruses contain premature stop codons, deletions, and mutations and are therefore defective [7] [Figure 1]. To date, CD4+ T cells represent the largest and most well-characterized viral reservoir, however, latent HIV has been found in unique cell types across various anatomical compartments in the body, including the lymph nodes, gut-associated lymphoid tissue, bone marrow, lung, and the central nervous system (CNS) [5]. To be considered a replication-competent HIV reservoir, cells have to meet the following criteria: (a) they must harbor integrated proviral DNA and have a long lifespan or the ability to undergo (homeostatic) proliferation; (b) they must possess molecular mechanisms to suppress viral replication, as well as establish and maintain latent infection, and (c) upon reactivation, they should be capable of producing new replication-competent viral particles that can reseed infection [4,8,9].

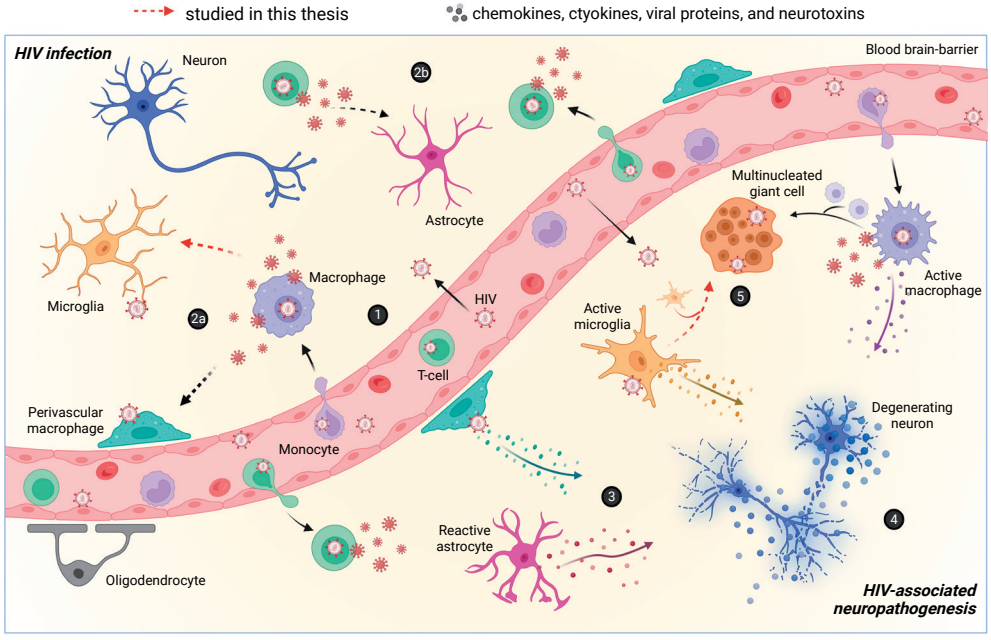

Figure 1: Composition of the HIV reservoir and potential CNS target cells. The panel above depicts the main HIV target cells in the blood and the potential target cells of HIV in the CNS, including the HIV receptors expressed by each cell type. The lower panel represents the HIV reservoir comprised of latently infected cells with intact or defective integrated HIV provirus. Intact provirus can be non-inducible transcriptionally silent, or replication-competent, producing new HIV virions upon reactivation. Defective provirus can produce HIV transcripts and viral proteins but due to large genetic mutations are unable to encode replication-competent viruses. Created with BioRender.com

HIV infection in the CNS

HIV has been detected in the CNS as early as 8 days post estimated infection [10]. Viral entry into the CNS is proposed to be facilitated by circulating HIV-infected CD4+ T cells and monocytes transmigrating into the CNS [10–12] [Figure 2]. Once inside the CNS monocytes differentiate into macrophages. In post-mortem brain studies conducted on untreated or virally suppressed individuals, HIV DNA is most frequently found in microglia and perivascular macrophages [13–17]. These cells have a long half-life of approximately 3 months (macrophages) up to 4.2 years (microglia); however, unlike macrophages, which do not divide and require repopulation from bone marrow-derived monocytes, microglia are a self-sustaining population with a low turnover rate which would allow HIV to persist in the brain for a long time [18,19]. Microglia and perivascular macrophages have been found to express both the CD4 and the CCR5 receptor, making them susceptible to productive HIV infection [Figure 1]. Furthermore, these cells were also observed to be resistant to the cytopathic effects of HIV and express several transcriptional repressors that might be involved in the establishment and maintenance of HIV latency [19,20].

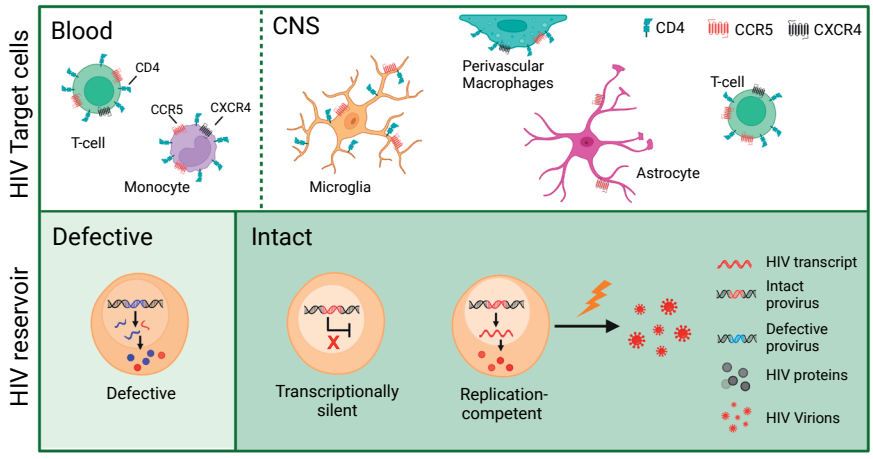

Figure 2: HIV CNS neuroinvasion and the onset of neuropathogenesis: (1) HIV enters the CNS through transmigration across the blood-brain barriers as a cell-free virion or within HIV-1 infected CD4+ T cells or monocytes that differentiate into macrophage. Once inside the CNS, (2a) HIV is proposed to infect microglia and perivascular macrophages, (2b) and to a lesser extent, astrocytes. (3,4) Upon activation, these cells secrete numerous pro-inflammatory chemokines and cytokines, HIV-1 viral proteins and neurotoxins leading to neuronal damage and death. (5) Activated microglia and macrophages also fuse together to form multinucleated giant cells, commonly found in late chronic infection. Created with BioRender.com

Apart from CNS-specific immune cells, the CNS is also routinely supplied with peripheral immune cells, including CD8+ and CD4+ T cells trafficking into the CNS as part of immune surveillance or in response to viral antigens. Considering that CD4+ T cells are the main target cell and reservoir of HIV in the periphery, they most likely also support HIV replication in the CNS and contribute to viral persistence [25]. Altogether, microglia, perivascular macrophages, and CD4+ T cells meet two out of the three criteria (a,b) of an HIV reservoir, making them promising cellular reservoirs for HIV in the CNS. However, whether these cells can fulfill the last criterion (c) and contribute to rebound viremia is yet to be determined.

Research on the CNS as an HIV reservoir has been a major challenge due to the inaccessibility of brain tissue in living subjects. As an alternative to post-mortem brain biopsies, cerebrospinal fluid (CSF) is commonly used to examine the HIV population in the CNS in living subjects and has provided compelling evidence for local viral replication in the brain. This includes the emergence of CSF escape, defined as the detection of HIV in the CSF of ART-treated individuals with an undetectable viral load in plasma, as well as the detection of CNS-specific viral strains that are genetically distinct from the viral population in the plasma, referred to as CNS compartmentalization [26–29]. However, conclusive evidence of HIV replication within CNS resident cells, such as microglia, macrophages, and CD4+ T cells, has yet to be demonstrated

During early infection, HIV variants circulating in the blood mainly use the CCR5 coreceptor and require a high surface density of CD4 for efficient cell entry (R5 T cell-tropic), making CD4+ memory T cell the preferred target cell. However, since CD4+/CCR5+ T cells in the CNS are limited, it is hypothesized that HIV evolves to infect alternative target cells in the CNS [Figure 1]. Considering that microglia and perivascular macrophages are the most promising HIV target cells in the CNS, it is hypothesized that HIV adapts to the lower CD4 density found on these cells relative to that on T cells (R5 macrophage-tropic) [25,30,31]. In the late stages of disease, a switch to X4-tropism may be observed (X4 T cell-tropic) that is associated with accelerated CD4+ T cell depletion and disease progression [32–35].

Furthermore, the persistence of HIV in the CNS contributes to a prolonged neuroinflammatory state leading to neuronal apoptosis and the development of a spectrum of cognitive impairments collectively termed HIV-associated neurocognitive disorder (HAND) that despite suppressive ART still affects 20-50% of the HIV-infected population [36–38]. The driving force behind HAND is still poorly understood but is proposed to be a multifaceted causal event of continued immune dysregulation due to low-level virus production and/or replication and cytotoxic viral proteins [39–41] [Figure 2]. Overall, there is overwhelming evidence that the CNS has the potential to serve as a viral reservoir for HIV and should be taken into consideration when developing new cure interventions.

Measuring the HIV reservoir

Accurate quantification and characterization of the size, genetic composition, and decay kinetics of the HIV reservoir carrying replication-competent HIV are essential for understanding HIV pathogenesis and the development and monitoring of cure interventions. Measurement of the replication-competent HIV reservoir has proven to be extremely difficult due to the large diversity of proviral sequences and a large number of replication-defective proviruses [42].

Numerous assays have been developed that measure different aspects of the HIV reservoir. Among these are quantitative viral outgrowth assays (QVOA) used for quantifying the replication-competent latent reservoir and PCR-based assays to assess the number of intact versus defective proviruses (e.g., IPDA) [42,43]. In HIV-infected individuals on suppressive ART, latently infected peripheral CD4+ T cells carrying intact provirus were observed to decay at a faster rate than T cells containing non-intact or defective provirus [44–46]. Although defective proviruses can produce viral proteins that contribute to neuronal damage and neuroinflammation, they are not considered part of the replication-competent viral reservoir [40,47]. Overall, it is believed that the true size of the replication-competent reservoir lies somewhere between the overestimated value of the IPDA and the underestimated value of the QVOA.

CNS culture models to study neuroHIV

Understanding the pathobiology of the CNS reservoir and how HIV latency is established and maintained is essential for the investigation of associated neuropathogenesis and the development of novel therapeutic and cure strategies that target this reservoir. HIV research on the CNS, however, has long been hampered by the difficulty in obtaining fresh human brain tissue, and the lack of *in vitro* culture models that can accurately represent the CNS on a phenotypic and transcriptomic level. Since microglia are thought to be the main cellular reservoir in the CNS, HIV research on the CNS is typically conducted on microglial culture models [Figure 3].

Until recently, microglia research was typically conducted on primary microglia (pMG), microglial cell lines, and monocyte-derived microglia (MDMi) [48]. Recent technological advancements in stem cell research have enabled the functional modeling of the human brain through the differentiation of induced pluripotent stem cells (iPSC) into a variety of CNS cells, including microglia (iPSC-MG), astrocytes, neurons, and 3D cerebral organoids [49,50]. However, the extent to which these culture models can mimic microglial cells *in vivo* in terms of morphology, phenotype, and transcriptome is still being investigated. Furthermore, without a good reference for (latently) infected cells in the CNS, it remains a challenge to develop CNS culture models that are representative of HIV infection *in vivo*.

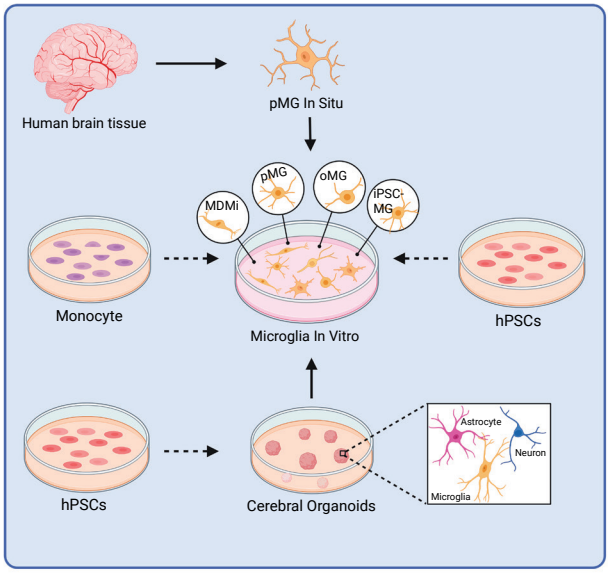

Figure 3: Human microglial cultures models to study neuroHIV. NeuroHIV research can be conducted on primary microglia isolated from fresh human brain tissue (pMG), microglia differentiated *in vitro* from monocytes (MDMi) or induced pluripotent stem cells (iPSC-MG) and within iPSC-derived cerebral organoid with an innate microglia population (oMG). Created with BioRender.com

Targeting the HIV reservoir

To date, HIV cure has only been achieved in a few patients through a stem cell transplant from a CCR5Δ32 donor [51–54]. These donor cells lack the expression of the CCR5 coreceptor, thereby rendering the recipient resistant to CCR5-using HIV variants. Stem cell transplants, however, are extremely riskful and their success rate is highly dependent on finding compatible CCR5Δ32 donors making it unsuitable for HIV cure on a large scale.

Alternative HIV cure interventions are based on the eradication or permanent proviral silencing of all HIV reservoirs by (i) reactivating latent reservoirs, in conjunction with a vaccine, antibody, and/or cellular immunotherapy to facilitate the subsequent elimination of reactivated cells ("shock and kill"), (ii) inducing a state of deep-latency with latency promoting agents to permanently prevent HIV gene transcriptional activation ("block and lock"), or (iii) genome editing of latently infected cells to directly excise integrated proviral DNA (gene therapy) [55]. Unfortunately, none of these cure strategies have proven to effectively reduce the size of the HIV DNA reservoir in HIV-infected individuals. Ex vivo studies also showed that most of the latency reversal agents (LRAs) used in the shock-and-kill approach only reactivate latent provirus in a small subset of latently infected cells [56,57]. In addition, using either of the above strategies to target the CNS reservoir should be carefully chosen as reactivating and killing or genetically modifying infected CNS cells could exacerbate HAND and have disastrous neurocognitive effects.

Aim of this thesis

Scope

The scope of this thesis is to elucidate the underlying mechanisms of HIV infection in the central nervous system (CNS), with a specific focus on microglial cells, to gain a better understanding of the CNS as a viral reservoir for HIV.

Hypothesis

We hypothesize that microglia are the primary target cells for HIV in the CNS, which persist during suppressive antiretroviral therapy and can therefore serve as a cellular HIV reservoir.

Approach

We will use an *in vitro* cerebral organoid model to determine HIV target cells in the CNS and post-mortem human brain tissue to examine HIV genomic DNA integrity in microglial and non-microglial cells after years of suppressive antiretroviral therapy. Furthermore, we will interrogate a variety of human microglial culture models for HIV research on the CNS, as well as examine the genetic and phenotypic characteristics of the viral population as present in paired CSF and plasma samples, both in CD4+ T cells and primary microglia.

Main research questions

- Which human microglial culture models are suitable for the study of HIV?
- How does the viral population in the CSF differ from that in the plasma?
- What are the target cells for HIV in the CNS?
- Is HIV in the CNS replication competent?
- Does HIV reside in the CNS during suppressive antiretroviral therapy?

Thesis Outline

The main objective of this thesis was to investigate whether the central nervous system can function as a viral reservoir for HIV persistence during treatment. First, in **chapter 2,** we interrogated different human microglial culture models on their microglia transcriptome signature and their susceptibility to productive HIV infection, to determine which culture model is suitable for HIV research. Next, in **chapter 3**, we investigated genetic compartmentalization and phenotypic differences between CSF- and plasma-derived viral variants, isolated from viremic individuals not on treatment. Then, in **chapter 4**, we determined the genomic integrity of the proviral DNA in microglial (CD11b+) and non-microglial (CD11b-) cells isolated from a virally suppressed individual. Hereafter, in **chapter 5** we investigated potential HIV target cells in the CNS and described for the first time the use of microglia-containing cerebral organoids as a tool for *in vitro* HIV research on the CNS. Finally, we thoroughly discussed the impact of the shock and kill cure strategy on the CNS reservoir in **chapter 6**.

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CHAPTER 2

2

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 [1]. However, ART neither eliminates HIV that persists in a latent state nor suppresses HIV expression and production from cellular reservoirs in the body [2]. 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) [3–7]. 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 [5,8–10]. 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 [7,11–13]. 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 [14–17]. 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 [18,19]. Viral analyses showed that the CNS cells are predominantly infected by macrophage-tropic HIV-1 variants that utilize the CCR5 coreceptor and require a low density of the CD4 receptor for efficient cell entry and infection (R5 M-tropic) [20–22]. 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 [23]. 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 [24]. 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 [25–28]. Microglia can be isolated by generating a cell suspension of the brain tissue followed by subsequent further microglia-specific enrichment techniques [29–32]. 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 [33,34]. Adding factors such as GM-CSF and IL-34 partly but not fully prevents this loss of phenotype [33]. 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 [35,36]. 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 [37]. Notably, a recent study has reported that some of the circulating HMC3-based cell lines are impure, as the cells are of rat origin [35].

The natural plasticity of monocytes has been exploited in vitro to differentiate these cells towards dendritic cells and macrophages [38,39]. These differentiated cells have been extensively used to study HIV biology [40–46]. 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 [47–49], extracellular matrix [50], or serum-free medium [51–54] 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 [55,56]. 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) [57–59]. First, iPSCs are induced to a hematopoietic lineage and into erythro-myeloid or hematopoietic progenitors via stimulation with simple growth fac- tor cocktails (minimally BMP4, VEGF, SCF, followed by IL-3, M-CSF). Subsequently, microglial differentiation is achieved either by co-culture with neural cells [60–63], by application of neural precursor conditioned media [64], or by addition of cytokines secreted from neurons and astrocytes to the culture media to mimic their presence [61,65–67].

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 cyto-architecture, cell diversity, and transcriptional profile [68–71]. 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 [70–72]. 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 [73]. Other CNS-microglia co- and tri-culture approaches include co-culturing iPSC-derived microglia with cerebral organoids [61,65,74–77], or adding iPSC-derived astrocytes and neurons to iPSC-derived microglia [60,63,78,79].

Comparison of microglia morphology

In the human adult brain, a variety of morphological forms is seen, with differences between gray and white matter [80–82]. 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 [83] (Fig. 1a). These processes are highly dynamic and used for the active surveillance of the surroundings of the cells [84,85]. 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 [86]. 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 [84,85,87].

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 [29,88–90] (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 [37,91,92] (Fig. 1c). Similar morphology was observed in the novel microglial cell line hμglia clone 20 (C20) [35]. 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 primary and a few secondary branches [47,48,50,52,54] (Fig. 1d).

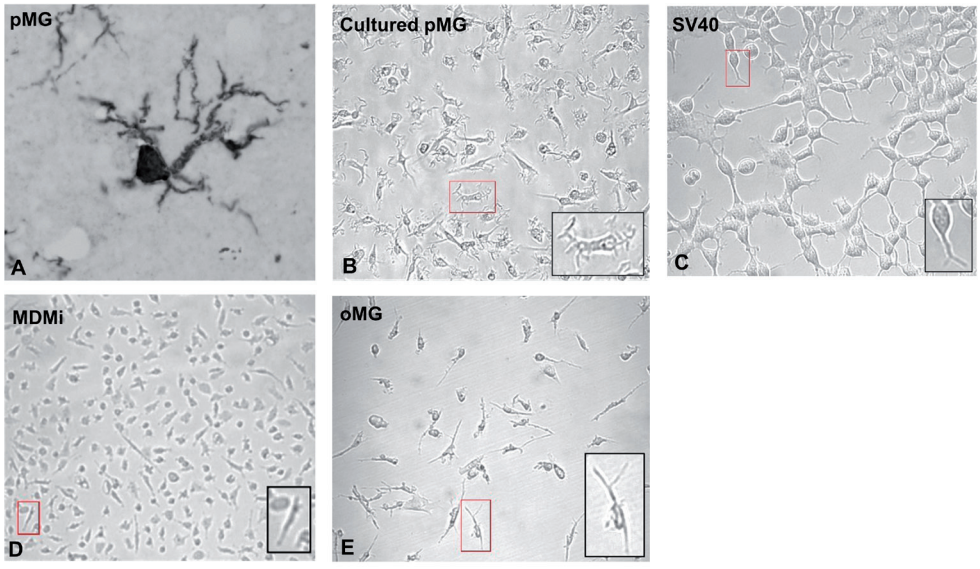

Figure 1. Morphology of human primary microglia and four different microglial in vitro culture models. A IBA-1 stained microglia in DAB- stained human brain sections of GFM at 40 × magnification. **B–E** Phase-contrast images of microglial culture models: adult primary microglia at 7 days post isolation **B**, SV40 microglial cell line **C**, monocyte-derived microglia **D**, and organoid-derived microglia E. Magnification = 10 × **D** and 20 × **B, C, E**

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 [61,63–66,75]. oMG exhibits an elongated or amoeboid-shaped cell body with a few secondary branched processes [73]. Upon isolation and culture, oMG has a spindle shape with a few processes from which some 2nd-degree fine spines emerge [73] (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 [86,93]. 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 [94–96]. However, these immune functions are shared with other myeloid cells in the CNS including non-parenchymal macrophages and infiltrating macrophages from the periphery [97,98]. Several studies reported that microglia and macrophages have different functions during or following injury [99–103]. 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 more CNS-relevant substrates such as synaptosomes, neural progenitor cells, apoptotic neurons, and myelin [29,35,50,53,62–67,73,104,105]. Direct comparison to cultured pMG revealed a similar phagocytic ability with MDMi and oMG, whereas SV40 had limited phagocytosis [50,53,73].

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-1β, and TNF-α [31,106–109]. To compare the models with each other, secretion of IL-6, IL-1β, 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 secre tion of IL-6 and IL-1β varied from modest and moderate in the cell lines MDMi and iPSC-MG to significant in oMG and cerebral organoids [49,62,64,65,73,74,110]. Direct comparison to cultured pMG revealed a significantly higher inflammatory response (IL-6 and IL-1β) in oMG [73].

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-1β, LPS, IFNγ, dexamethasone, IL-4
Human microglia cell lines	Easy	0	None	Globular and spindle-shaped cells with short processes	Phagocytosis: pHrodo-labeled synaptosomes, live neural progenitor cells, dead neuronal cells, Aβ42 Inflammation response to: TNF-α, IL-1β, IFNγ, 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 *S. aureus,* latex beads, zymosan particles Inflammation response to: LPS, IL-6, dexamethasone
iPSC-derived microglia	Intermediate	25-74	CellBIND, Primaria, Matrigel, poly-L-ornithine, fibronectine, gelatin, none	Amoeboid shaped cell body with several 1st and 2nd degree branched processes	Phagocytosis: zymosan-coated microbeads, *Escherichia coli/S.aureus* particles, fibrillar beta-amyloid, tau oligomers and synaptosomes Inflammation response to: IL-1β, IFNγ, 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

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 [33,73,111,112] and identified a specific gene signature for adult and fetal microglia. This includes markers such as AIF1, TMEM119, P2RY12, CX3CR1, CSF1R, and TREM2 [33,55,73,113–120] (Table 2).

Table 2: Overview of microglial-enriched markers

Marker (gene)	Name	Celltype	Function	Reference
TMEM119	Transmembrane Protein 119	Microglia	uncertain	(Bennett et al., 2016; Satoh et al., 2016)
P2RY12	Purinergic Receptor P2Y12	Microglia	Purinergic receptor required for microglia chemotaxis in response to CNS injury	(Butovsky et al., 2014; Bennett et al., 2016)
CSF1R	Colony Stimulating Factor 1 Receptor	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	(Erblich et al., 2011; Nandi et al., 2012; Chitu et al., 2016)
CX3CR1	C-X3-C motif chemokine receptor 1	Microglia and other myeloid lineage cells	Chemokine receptor critical in controlling microglia numbers, synaptic pruning, and functional brain connectivity	(Jones, Beamer and Ahmed, 2010)
TREM2	Triggering Receptor Expressed On Myeloid Cells 2	Microglia and other myeloid lineage cells	Regulatory protein involved in microglia activation and phagocytosis of apoptotic neurons	(Colonna, 2003; Neumann and Takahashi, 2007)
AIF1	Ionized Calcium-Binding Adapter Molecule 1	Microglia and other myeloid lineage cells	Cytoplasmic protein involved in microglia motility, membrane reorganization and phagocytosis	(Imai et al., 1996; Sasaki et al., 2001)

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 [33,121]. This mature microglia signature, therefore, seems to be much dependent on the CNS environment [33]. Accordingly, lower expression levels of CX3CR1, P2RY12, and TMEM119 were generally reported in the microglial cell lines [108,122], MDMi [49,53,108,122] and oMG [73] 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 [33,123], fetal pMG [66], iPSC-MG [66,75], MDMi [49], monocytes [33,49], and oMG [73] to that of uncultured adult pMG [33,49,73] 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. [124].

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. [33] 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 [73].

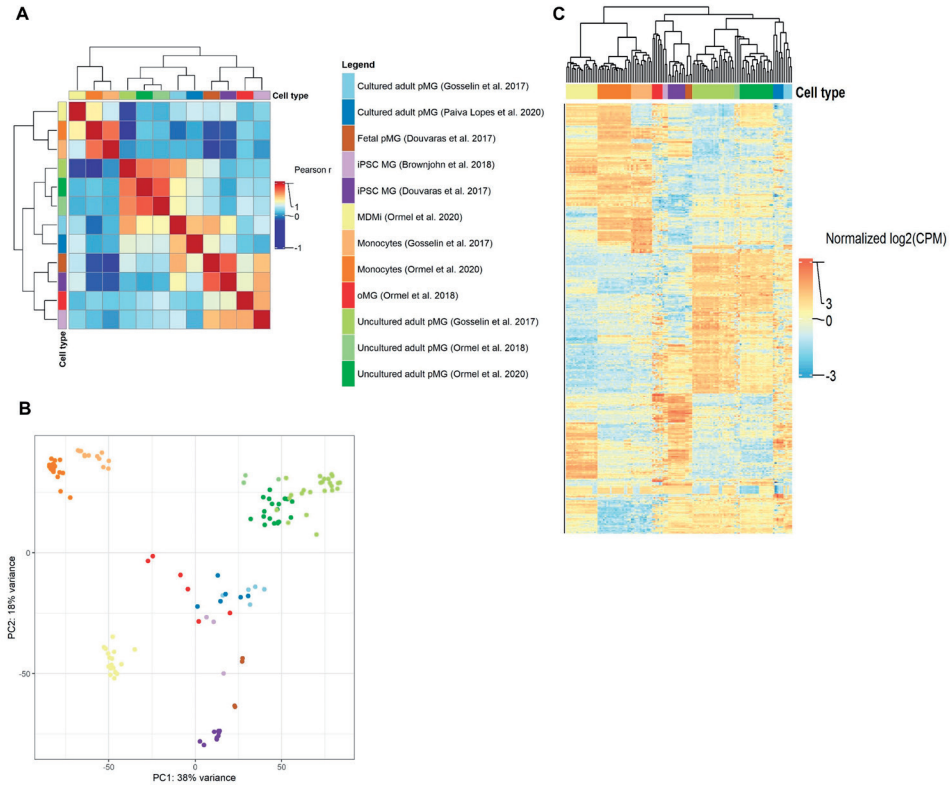

Figure 2. Gene expression analysis of microglia culture models on the 500 most variable genes. Legend shows color coding for cell type. **A** Heatmap depicting the Pearson r correlation effect sizes between cell types based on the 500 most variable genes. Clustering dendrogram is based on Euclidean distances. **B** PC plot depicting cell type distances based on expression variance in the 500 most variable genes. Clustering dendrogram is based on Euclidean distances. **C** Heatmap of log2(CPM) expression values for the 500 most variable genes depicted for each cell type.

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. [124]. 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 iPSC-MG by Brownjohn et al. [75], 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 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 coreceptor 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. [33] and iPSC-MG from Brownjohn et al. [75], but considerably higher than MDMi, cultured pMG from Lopes et al. [123], and iPSC-MG from Douvaras et al. [66] (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. [73] 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 coreceptor. 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 oMG had a lower TRIM5 expression compared to the other models. Expression of SAMHD1 was lower in cultured adult pMG [123] and higher in iPSC-MG [75] compared to that in uncultured adult pMG.

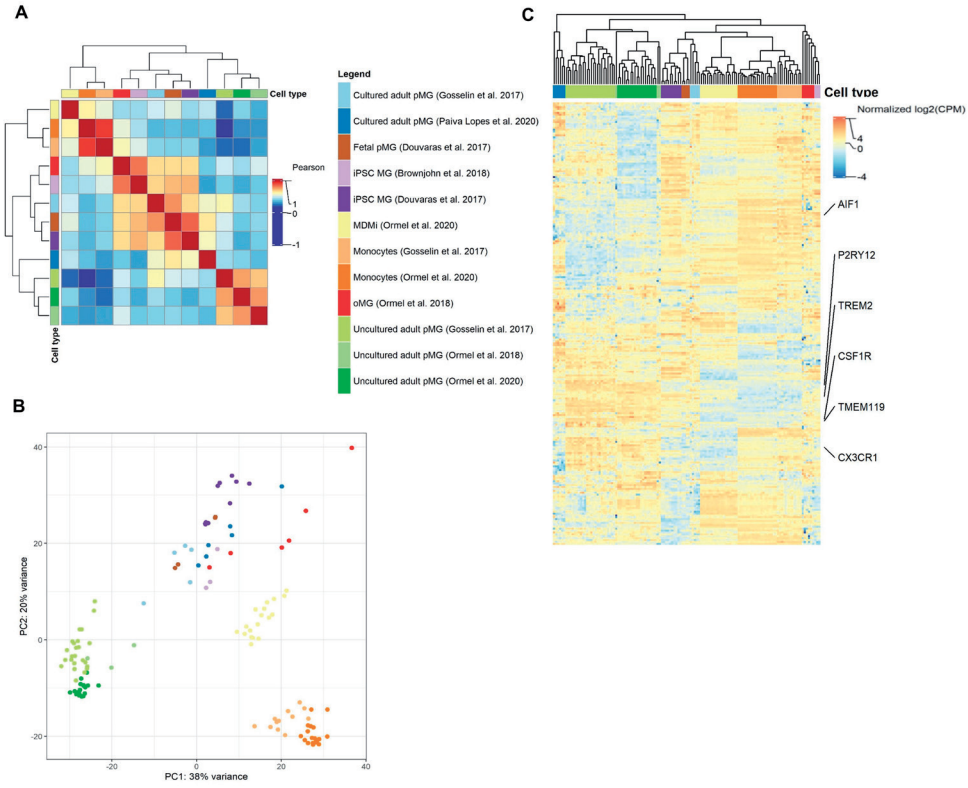

Figure 3. Gene expression analysis of microglia culture models on a microglia-specific core signature. A Heatmap depicting Pearson r correlation effect sizes between the cell types based on microglia core gene expression. Clustering dendrogram is based on Euclidean distances. Clustering dendrogram depicts Euclidean distances. **B** PC plot depicting cell type similarities based on expression variance within microglia core genes. **C** Heatmap of log2(CPM) values for microglia core genes extracted from (Patir et al., 2019).

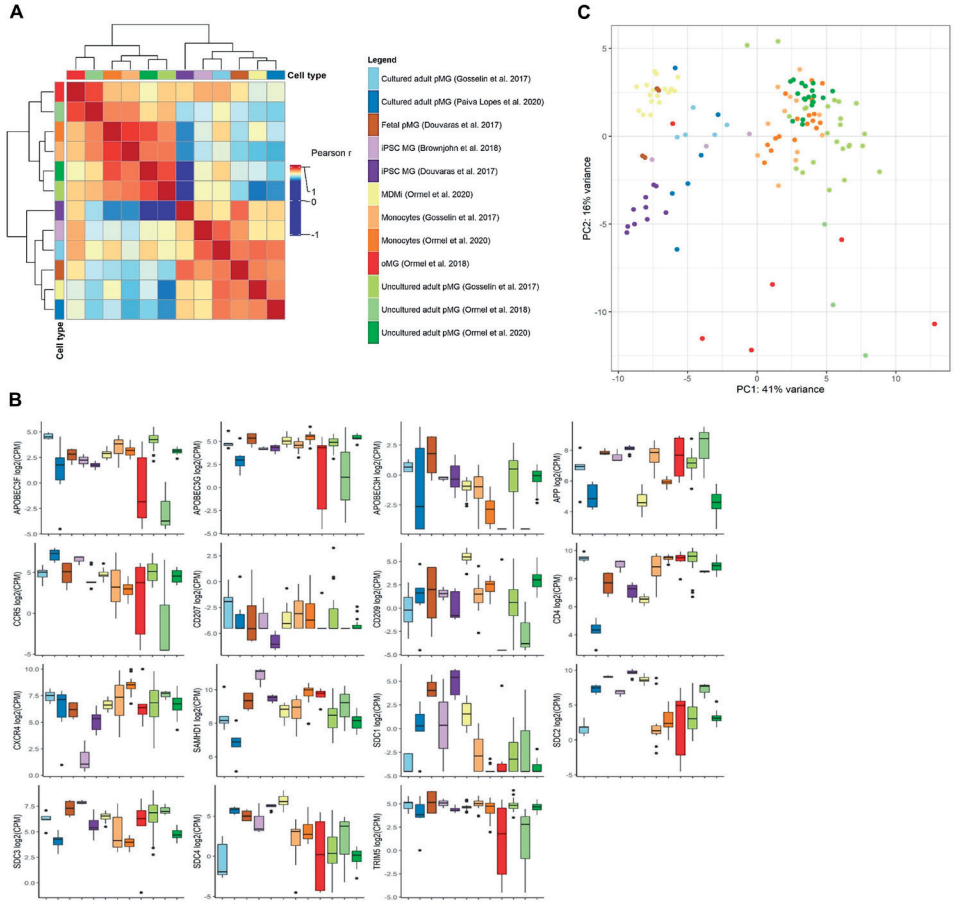

Figure 4. Gene expression analysis of microglia culture models on HIV-relevant genes. A Heatmap of Pearson r for between each cell type (cluster distances are Euclidean). **B** Boxplot of log2(CPM) for selected HIV genes. **C** PC plot depicting cell type distances based on expression variance within selected HIV genes.

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) [125–127]. 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 [125]. 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) [127]. 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 [127]. 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 [126]. 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 [126].

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 [128]. 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 [129]. 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) [129]. This being said, viral strain compartmentalization has been found between different brain regions (frontal lobe, occipital lobe, and parietal lobe) [130].

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 [131]. 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) [131]. The evidence of low copies of HIV RNA in some cases suggests either spontaneous viral reactivation or ongoing low level replication despite suppressive cART [129,131].

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

As mentioned earlier, viruses detected in the CNS are predominantly R5 M-tropic [20,21,132]. 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}) [35,133–145]. 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 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 72h [141,146], 75% at day 5 [35], and about 90% at day 6 post infection [141]. Moreover, HIV-infected microglia formed giant multinucleated syncytia that accumulated in the cultures over time and correlated with peaks in HIV capsid (p24) levels [140,141,144]. This cytopathic effect is reminiscent of microglial nodular lesions observed in the brains of HIV-positive individuals with HAND and AIDS [147,148], among which are the HIV-positive individuals with HIVE in the previously mentioned studies by Trillo-Pazos et al. [127] and Thompson et al. [126]. Furthermore, addition of CCR5 inhibitor maraviroc on day 1 post infection blocked the increase in infection, indicating spread of HIV infection in these cultures [141]. 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}) [135,142].

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}) [47,48,53,79,122,137,149,150]. 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 [137]. 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 [122]. 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 [53].

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	(14) (Lee et al., 1993; Strizki et al., 1996; Ghorpade et al., 1998; Albright et al., 1999, 2000; Schuenke and Gelman, 2003; Huang et al., 2011; Mamik and Ghorpade, 2014; Tatro et al., 2014; Zenón et al., 2015; Asahchop et al., 2017; Castellano, Prevedel and Eugenin, 2017; Cenker, Stultz and McDonald, 2017; Garcia-Mesa et al., 2017)	(Olah et al., 2012; Rustenhoven et al., 2016; Zhang et al., 2016; Mizee et al., 2017)
Human microglia cell lines	SV40		IBA1, TREM2, CD11b, CD68		(Chiavari et al., 2019)
	hμglia		CD68, P2RY12, CD11b	(4) (Alvarez-Carbonell et al., 2017; Garcia-Mesa et al., 2017; Ingram et al., 2020; Rai et al., 2020)	(Garcia-Mesa et al., 2017)
	HMC3, CHME3/5, C13NJ		CD68, CD11b, CD45, IBA1, CX3CR1	(18) (Chugh et al., 2007; Mishra, Chhatbar and Singh, 2012; Wires et al., 2012; Jadhav, Krause and Singh, 2014; Zenón et al., 2015; Lisi et al., 2016; Samikkannu, Atluri and Nair, 2016; Ambrosius et al., 2017; Campbell et al., 2017; Malikov and Naghavi, 2017; Campbell et al., 2019; Chai et al., 2017; Delaney et al., 2017; Tomitaka et al., 2018; Rai et al., 2020; dos Reis et al., 2020; Francis et al., 2020; Ingram et al., 2020)	Janabi et al., 1995; Dello Russo et al., 2018)
Monocyte-derived microglia	MDMi		HLA-DR, IBA1	(2) (Leone et al., 2006; Cherrier et al., 2011)	(Leone et al., 2006)
	iMG		CX3CR1, HLA-DR, CD45		(Ohgidani et al., 2014)
	iMG		TMEM119, P2RY12, PU.1		(Sellgren et al., 2017, 2019)
	MMG		IBA1, CD11b, CD45, HLADRLOW, P2RY12, CD68	(2) (Rawat and Spector, 2017; Rawat, Teodorof-Diedrich and Spector, 2019)	(Rawat and Spector, 2017)
	ML	astrocytes	CD11b, TREM2, IBA1		(Noto et al., 2014)
	MDMi		IBA1	(1) (Bertin et al., 2012)	(Bertin et al., 2012)
	MDMi		P2RY12, CSF1R, TREM2	(1) (Rai et al., 2020)	(Ryan et al., 2017)
	iMG		TREM2, HLADR		(Ormel et al., 2020)
	M-MG		CX3CR1		(Etemad et al., 2012)

Table 3: Overview of the characteristics of all the microglial *in vitro* culture models (continued)

Microglia Model	Name	Co-culture	Microglia markers	HIV Research Citations (n) [a]	Reference
iPSC-derived microglia	pMGLs		CD45, IBA1, P2RY12, TMEM119		(Muffat et al., 2016)
	iPSC-MG		CD11b, CX3CR1, IBA1, P2RY12, TMEM119		(Douvaras et al., 2017)
	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)
	3D cortical organoids + iPSC-derived microglia	Neurons, astrocytes	N.D.		(Brownjohn et al., 2018)
	3D BORG + iPSC-derived microglia	Neurons, astrocytes, oligodendrocytes	N.D.		(Abud et al., 2017)
	hBORG + HMC3 cell line	Neurons, astrocytes	N.D.	(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

[a] Research articles of HIV infection studies with human lab strains

Productive HIV infection of MDMi is also shown in other in vitro studies [47,48,122]. 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 [122]. 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 [79]. As observed for the pMG also, the HMC3 cell line [149] and MDMi [47] 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.

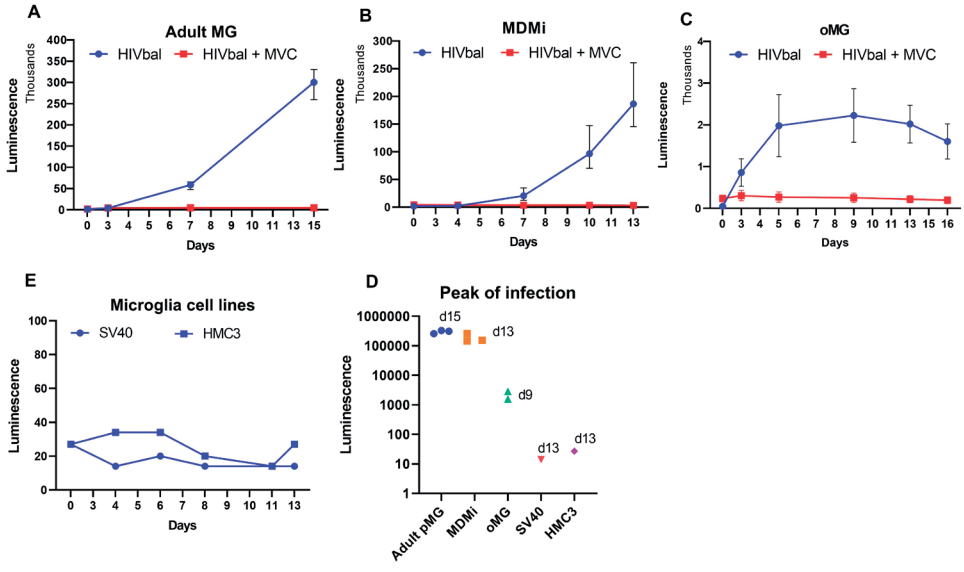

Figure 5. HIV infection and virus production in microglial culture models. Adult primary microglia **A**, MDMi **B**, oMG **C** and microglial cell lines SV40, HMC3 **E** were infected with 10ng (p24 Gag) HIVbal with a luciferase tag and cultured for the indicated days. Supernatant was collected post-infection on the indicated days and virus production was measured with luminescence. **D** Peak infection day of each culture model.

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 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.

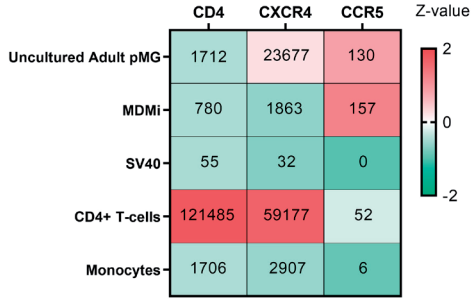

Fig. 6 Gene expression of major HIV receptors in in vitro culture models. Median (IQR) gene expression of CD4, CXCR4 and CCR5 in primary microglia (pMG), monocyte-derived microglia

(MDMi), SV40 microglial cell line, CD4+ T cells and monocytes assessed by RT-PCR and normalized to the reference gene ACTB. All cells are color-coded according to their Z-value (color bar on the right-hand side).

Discussion

Microglia are thought to constitute the main viral reservoir for HIV in the CNS [23], 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 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-1β, 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.

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 cluster 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 microenvironment 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

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 [33], 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 physical factors from the complex CNS microenvironment and lack of intercellular communication with other CNS cell types [55,121]. 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 [60,63,78,79], incorporation into 3D cerebral organoids [61,65,74–77], or transplantation of iPSC- derived hematopoietic progenitors into humanized mice [65,67,151–153]. 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 [151–153]. 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 [55,65,154–156] or the use of neural progenitor cell conditioned medium [64].

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 coreceptors in iPSC-MG compared to cultured pMG [122]. 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} [48,53,122,138,142,157]. 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%) [126,127,129,131]. 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} [122]. 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 [23]. 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 [35]. 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 [146]. Another study by Ryan et al. showed a reduction in viral production after culturing HIV-infected iPSC-MG with IPSC-derived neurons and astrocytes [79]. 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.hersenbank.nl). Informed consent was obtained from all donors. Human microglia were isolated and cultured according to the protocol described before [158]. In short, a mechanical and enzymatic dissociation with DNAse 1 (200 μg/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 200µl microglia medium (RPMI 1640 (Gibco Life Technologies, USA) supplemented with 10% FCS, 1% penicillin–streptomycin (Gibco Life Technologies, USA) and 100ng/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. [36]. 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. [49]. 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, 2mM l-glutamine, 1% penicillin–streptomycin, 25% ACM, 10ng/ml M-CSF, 10 ng/ml GM-CSF, 20ng/ ml TGFβ, 12.5ng/ml IFNγ, and 100ng/ml IL-34 (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 [73]. Three-dimensional organoids were dissociated with enzymatic dissociation using papain (18.6U/ml, Worthington, LK003176) and DNAse 1 (337U/ml Worthington, LK003170), followed by microglia isolation using CD11b+ MACS (Miltenyi Biotec, Germany) according to the protocol published before [73]. 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 (HxB2ΔENVluc). pHXB2PS is derived from pHXB2WT [159], which expresses the full-length HIV-1 sequence HXB2 (9719 bp, GenBank accession number 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 [160]. 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 envelop 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'-GGT CAG GGA GTC TCC ATA GAA TGG AGG-3' and HIV-R-end-rev1 5'-GCA CAC AAC GCG TGA AGC ACT CAA GGC AAG CTT TAT TGA GGC -3', followed by a nested PCR with the primers gp160-fw 5'-TAG TAG TAGCASYAA TCA TCG CAA TAG TTG TGTGG-3' and gp160-rv 5'-CTC GTC TCA TTC TTT CCC TTACMKCAG GCC ATCC-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 (NL4-3Balgp160GFP), 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 Gene-JET Plasmid Miniprep Kit (Thermo Scientific). All HIV constructs were verified by nucleotide sequencing.

Replication competent viral stocks were generated by transfecting HEK293T cells with the chimeric infectious plasmids (HxB2Balgp160Luc and NL4-3Balgp160GFP) using Lipofectamine 2000 reagent (Invitrogen). The super- natant was harvested at 48 h.

Infection of microglia culture models

Cultured adult pMG, MDMi, oMG, and SV40 cells (1×10^5) were infected with 10ng (p24 Gag) of HIVbalLuc (HXB2Balgp160Luc) 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 Nano- Glo® 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 15s, and 60 °C for 60s. Per reaction, 5µl SYBR green PCR Master Mix (Roche; Life Technologies Corporation, Grand Island, NY), 1µl primer mix (2pmol/ ml), and 5ng RNA were added up to a final volume of 10µl. 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 (Graph- Pad 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; [161,162]). 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 [33,49,73], cultured pMG [33,73,123], fetal pMG [66], monocytes [33,49], MDMi [49], iPSC-derived microglia [66,75], and organoid-derived microglia [73]; 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 [163] 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 [164] and limma v3.28.14 [165] 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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CHAPTER 3

3

Characterization of HIV variants from paired CSF and Plasma samples in primary microglia and CD4+ T cells

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Abstract

Despite antiretroviral therapy (ART), HIV persistence in the central nervous system (CNS) continues to affect a large portion of the HIV-infected population through a wide range of cognitive impairments. Upon disease progression, CCR5-using T cell-tropic viruses transmigrating from the blood into the CNS, are hypothesized to evolve into CCR5-using macrophage-tropic viruses that have an enhanced ability to infect cells with low CD4 surface expression such as perivascular macrophages and microglia. We examined HIV-1 RNA concentration, coreceptor usage, and the prevalence of CSF compartmentalization in paired CSF and blood samples obtained from 19 adults not on treatment. Full-length envelope CSF- and plasma-derived reporter viruses were generated from 3 subjects and phenotypically characterized for viral tropism in human primary CD4+ T cells and primary microglia. Median HIV RNA levels were significantly higher in plasma than in CSF (5.01 vs. 4.12 log10 cp/mL; p=0.004) and coreceptor usage was mostly concordant for CCR5 across the paired samples (n=17). Genetically compartmentalized CSF viral populations were detected in 2 subjects, one with and one without neurological symptoms. All viral clones had an R5 T cell-tropic phenotype; however, CSF-derived viruses were more efficient at infecting primary microglia. In addition, we observed an intermediate macrophage-tropic phenotype, for 3 CSF and 1 plasma viral clone. Overall, these data provide supporting evidence for the evolution of the Env protein in the CNS and the susceptibility of primary microglia to productive HIV infection.

Keywords: Microglia • HIV • HIV-associated neurocognitive disorder • Neuropathogenesis • T cells • Deep sequencing • Compartmentalization

Introduction

Despite antiretroviral therapy (ART), HIV persistence in the central nervous system (CNS) continues to affect a large portion of the HIV-infected population resulting in a wide range of cognitive impairments [1]. The onset and progression of HIV-associated neurocognitive disorder (HAND) is believed to be multifactorial, including continued immune dysregulation and residual chronic inflammation in response to low-level virus production (or replication) and cytotoxic viral proteins [2,3].

During early infection, R5 T cell-tropic viruses, characterized by their ability to efficiently enter CD4+ T cells but not macrophages and microglia, represent the majority of the viral population [4]. Upon disease progression, genetically distinct viral populations have been found in the CSF and brain tissue of both untreated and virally suppressed individuals, irrespective of the presence of neurological disorders [5–7]. While extensive research has been conducted on the genetic compartmentalization between the CNS and blood compartment, their phenotypic characteristics remain poorly understood. Previous studies have examined the CD4 entry phenotype of CNS- and plasma-derived pseudotyped viruses using monocyte-derived macrophages and/or the Affinofile cell line, on which CD4 and CCR5 surface expression can be differentially induced [8,9]. Contrary to the R5 T cell-tropic plasma-derived viruses, CNS-derived viruses were R5 Macrophage-tropic (M-tropic), referring to their enhanced ability to infect cells with low CD4 surface expression such as macrophages [10–13]. Accordingly, HIV DNA and/or RNA within the CNS are mostly found in perivascular macrophages and microglia, which have low CD4 surface levels [14].

Although the Affinofile cell line is commonly used for entry tropism analysis, this model system is derived from a T-cell line and therefore cannot fully represent the entry determinants for primary microglia, such as attachment receptors, endocytosis mechanisms, and microglia-specific restriction factors. Therefore, it remains to be determined whether macrophage-tropic HIV variants have the same entry advantage for microglia as they do for low CD4 Affinofile cells and monocyte-derived macrophages. In this study, we characterized HIV-1 variants isolated from paired CSF and plasma samples, obtained from viremic HIV-infected individuals without antiretroviral treatment. Genetic CNS compartmentalization was assessed using three methods, Wright's measure of population subdivision (Fst), Nearest-neighbor statistic (Snn), and the Slatkin-Maddison test (SM) [15]. Furthermore, we examined the phenotype of CSF- and plasma-derived reporter viruses in CD4+ T cells, as well as human primary microglia. To our knowledge, this is the first study to combine genetic characterization with phenotyping in a human primary blood and CNS cell.

Methods

Design and study population

For this cross-sectional study, paired CSF-plasma samples were collected from stored samples in the period 2001-2016 from patients in care at the University Medical Center Utrecht and participating in the Dutch ATHENA observational cohort. Paired samples were obtained for clinical diagnostic purposes [Table S1]. A total of 19 subjects, with and without neurological symptoms, had sufficient material for virological analysis and were included in the study. The CSF of neuroasymptomatic patients was collected to exclude neurosyphilis, as part of the standard clinical procedure. Paired samples were defined as CSF and plasma-EDTA (or serum) obtained within 7 days from each other. All patients had detectable HIV RNA in plasma at the time of lumbar puncture and were ART-naïve or off-treatment at the time of sampling [Table 1 and S1].

HIV RNA and plasma CD4 count analysis

HIV RNA levels in plasma and CSF were determined by an ultrasensitive viral load assay with a reported cut-off value of 50 copies/ml (Amliprep/COBAS Taqman HIV-1 assay, Roche).

Next-generation sequencing and coreceptor prediction

Viral RNA was isolated according to the method developed by Boom et al [boom 1990]. The HIV-1 envelope V3 region was amplified by RT-PCR (Titan One Tube RT-PCR kit, Roche) followed by a nested PCR (Expand High Fidelity PCR System, Roche), according to the manufacturer's protocol. Please refer to supplementary Table 2 for a list of the primers used. PCR products were purified with the Qiaquick PCR purification kit (Qiagen). Library preparation was done using a Nextera-XT DNA Library Preparation and Index kit (Illumina, USA) according to the manufacturer's instructions. The resulting libraries were normalized and pooled. Sequencing was performed on an Illumina MiSeq platform using the MiSeq Reagent Kit v2 for 500 cycles. After aligning the sequence reads of each subject with the consensus sequence of their respective subtype, reads that overlap the entire V3 region were isolated and trimmed. Unique V3 sequences with a prevalence of >1% in the population were used for HIV-1 coreceptor tropism. Coreceptor usage was predicted with the Geno2pheno[coreceptor] algorithm for deep sequences with the recommended False-positive rate (FPR) cut-off value for deep V3 sequencing of 3.5% [16]. It is predicted that an FPR value below 3.5% indicates an X4 virus, whereas a value above 3.5% indicates an R5 virus.

Compartmentalization analysis

Genetic compartmentalization was determined for each subject based on deep-sequenced V3 sequences using three methods: Wright's measure of population subdivision (Fst) [17], Nearest-neighbor statistic (Snn) [18], and the tree-based Slatkin-Maddison test (SM) [19].

All three methods were conducted with the HyPhy software [20]. For the two distance-based methods (Fst and Snn), the Tamura-Nei 93 algorithm was applied along with a bootstrap value and permutation test of 10,000. For the tree-based SM test, multiple sequence alignments of the HIV *env* V3 regions were performed using the MAFFT software [21], a Nearest Neighbor Joining Tree was constructed using MEGA version 11.0.11 [22], and the Kimura 2-parameter substitution model was applied. Compartmentalization was evaluated using the nearest-neighbor statistic (Snn) [18], applying 10 000 permutations, implemented using the HyPhy software package [20]. CSF viral populations were defined as either compartmentalized (cp), if all three tests were significant ($p < 0.01$), or equilibrated (eq) if statistical significance was not reached ($p > 0.01$) [15].

Generation of HIV viral clones

The HIV-1 envelope (gp160) region was amplified by RT-PCR (Superscript IV Reverse Transcriptase Kit, Invitrogen) followed by a nested PCR (Platinum Taq Superfi PCR Master Mix, Invitrogen), according to the manufacturer's protocol. Please refer to supplementary Table 2 for a list of the primers used. Envelope amplicons were introduced into a HxB2 gp160deletion vector with a luciferase reporter gene (HxB2ΔENVluc), previously described in [23], using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). We used the same vector carrying either the gp160 sequence of JRCSF (R5 T-tropic), YU-2 (R5 M-tropic) or BaL (R5-tropic) as controls. HEK293T cells were transfected with the chimeric plasmids using lipofectamine 2000 reagent (Invitrogen). After 48h, the supernatant containing replication-competent virus was harvested and stored at −80 °C until further use. p24 was determined with an ELISA p24 assay (Aalto Bioreagent, Dublin, Ireland).

HIV Infection in primary microglia and CD4+ T cells

Fresh postmortem adult human brain tissue was provided by the Netherlands Brain Bank (NBB). The isolation of primary microglia was conducted according to the protocol described previously with some minor modifications for human brain tissue [24]. Primary microglia were infected overnight with 10ng (p24Gag) virus per 100.000 cells after which medium was replaced and cells were cultured for 14 days in microglia medium without medium refreshment.

PBMCs were isolated from peripheral blood obtained from healthy donors by Ficoll-Paque density gradient. CD4+ T cells were subsequently isolated through negative selection with the CD4+ T Cell Isolation Kit (Miltenyi Biotec 130-096-533), according to the manufacturer's protocol. Before infection, CD4+ T cells were stimulated for 2 days in culture medium (RPMI 1640 (Gibco Life Technologies) with 10% Fetal Bovine Serum, 1% penicillin-streptomycin (Gibco Life Technologies) and 20U/mL IL-2 (Invitrogen)) supplemented with Phytohaemagglutinin (5µg/mL). CD4 infection was performed in Duplo for 3 hours with 10ng (p24 Gag) virus per 100.000 cells in Eppendorf's placed on a tube rotator. For the

Maraviroc experiment, CD4+ T cells were treated with 100nM MVC for 1h before infection. Following infection, medium was fully replaced with culture medium and CD4+ T cells were cultured for 14 days in 96-wells plates without medium refreshment.

Luminescence

Supernatant was collected 2-3 times per week and luminescence was measured according to the manufacturer's protocol with the Nano-Glo® Luciferase Assay System (Promega). The graphs were created with GraphPad Prism version 8.3.0.

Statistical analysis

All data were analyzed with GraphPad Prism version 8.3.0. Descriptive statistics were used to compare the characteristics between the paired plasma and CSF samples. The nonparametric Wilcoxon signed rank test is used to compare groups with paired data, including the differences in FPR. Differences within continuous variables (e.g., viral load) compared to categorical data (e.g., neurological symptoms) were performed by using the Mann-Whitney U test. A spearman's rho rank correlation is used to determine the association of HIV-RNA levels.

Results

Clinical characteristics

Paired plasma and CSF samples were collected from 19 adult subjects enrolled in the Dutch ATHENA observational cohort, of which 10 were diagnosed with neurological complications. Subjects were mainly infected with Subtype B, except for subject 6 (subtype CRF02_AG) and subject 25 (CRF12_BF), and were ART naïve or off treatment at the time of sampling. Majority of the study population was male, with a mean age of 47 years and a mean CD4+ T-cell count of 312 cells/μL. The clinical and virological characteristics of each subject can be found in Table 1 and Table S1.

Median HIV RNA levels were significantly higher in plasma than in CSF (5.01 vs. 4.12 log10 cp/mL; p=0.004) and moderately correlated with each other (Pearson r = 0.42; p = 0.04) [Figure 1A]. A similar pattern in virus concentration was also observed in the neurosymptomatic subjects (5.22 vs. 4.25 log10 cp/mL; p =0.05) but not in the neuroasymptomatic subjects [Figure 1B,C]. A comparison of the CSF and plasma RNA levels between the neurosymptomatic and neuroasymptomatic subjects, however, showed no significant difference [Figure 1D,E].

Table 1: Clinical, virologic and phylogenetic characteristics of the subject population

Subject ID	Disease state (CDC) [a]	CD4 [b]	Origin	HIV VL [c]	FPR Range [d]	Tropism [e]	Fst	Snn	SM	CSF Compart. [g]
1	A2	209	CSF	3.95	30.1 - 73.3	R5	0.20	0.02	0.302	eq
			Plasma	5.65	30.1 - 37.1	R5				
2	A2	373	CSF	3.21	17.3	R5	N/A	N/A	N/A	N/A
			Plasma	5.01	17.3 - 46.8	R5				
3	unknown	549	CSF	4.72	76.2 - 95.2	R5	0.66	0.91	1	eq
			Plasma	5.88	68.6 - 95.2	R5				
4	unknown	400	CSF	3.13	12	R5	N/A	N/A	N/A	N/A
			Plasma	3.15	12 - 33.7	R5				
6	C3	16	CSF	4.88	100	R5	0.15	<0.0001	0.09	eq
			Plasma	5.34	4.8 - 99.2	R5				
7	B2	246	CSF	3.72	26.2 - 78.1	R5	0.70	0.38	0.33	eq
			Plasma	4.31	48.7 - 72.1	R5				
8	unknown	10	CSF	5.28	7.8 - 44.2	R5	0.33	0.67	0.26	eq
			Plasma	5.35	4 - 81	R5				
10	A1	705	CSF	4.43	83 - 97	R5	0.84	0.80	0.24	eq
			Plasma	5.1	83 - 97	R5				
12	A2	375	CSF	4.72	6.3 - 20.4	R5	0.21	0.59	0.1	eq
			Plasma	4.49	10.5 - 20.4	R5				
13	unknown	30	CSF	5.21	74.6 - 91.2	R5	<0.0001	<0.0001	0.01	cp
			Plasma	6.04	35.1 - 53.7	R5				
14	A2	469	CSF	3.54	71.1	R5	0.54	0.34	0.59	eq
			Plasma	4.27	71.1 - 90.7	R5				
16	C3	83	CSF	4.12	72 - 94.6	R5	0.72	0.86	0.6	eq
			Plasma	5.1	76 - 94.6	R5				
17	A1	424	CSF	4.38	2.5 - 5	X4	0.70	0.01	0.04	eq
			Plasma	3.17	1.7 - 55.1	X4				

Table 1: Clinical, virologic and phylogenetic characteristics of the subject population (continued)

Subject ID	Disease state (CDC) [a]	CD4 [b]	Origin	HIV VL [c]	FPR Range [d]	Tropism [e]	Fst	Snn	SM	CSF Compart. [g]
18	A1	574	CSF	2.87	15 - 46.8	R5	0.44	0.33	0.13	eq
			Plasma	4.56	15 - 46.8	R5				
19	A1	387	CSF	4.77	16 - 87	R5	<0.0001	<0.0001	<0.0001	cp
			Plasma	4.18	0.2 - 90.3	X4				
20	A0	139	CSF	3.25	64 - 70.8	R5	0.27	0.53	1	eq
			Plasma	3.71	56.1 - 74.4	R5				
21	A2	399	CSF	3.74	30.1 - 52.1	R5	0.29	0.92	0.16	eq
			Plasma	3.78	30.1 - 52.1	R5				
25	A0	362	CSF	3.06	25.2	R5	N/A	N/A	N/A	N/A
			Plasma	5.88	25.2	R5				
27	C3	185	CSF	4.95	38.8 - 86.2	R5	0.56	0.73	0.38	eq
			Plasma	5.73	8.5 - 86.2	R5				

Footnotes:

a HIV disease stage according to the CDC 1993 Revised Classification System for HIV Infection (PMID: 1361652)

b estimated plasma CD4 cells/µl, value determined by test value closest to sampling time of pair

c VL, viral load; estimated HIV-RNA (log10 copies/ml) determined by test value closest to sampling time of pair

d FPR, False-positive rate; lowest and highest FPR detected in CSF and plasma based on V3 amplicons (Miseq sequencing) with the geno2pheno algorithm

e Geno2pheno coreceptor prediction; R5: virus predicted to use the CCR5 coreceptor. X4: virus predicted to use the CXCR4 coreceptor

f Comparative genetic analyses of viral populations in blood plasma and cerebrospinal fluid using three statistical analyses: Wright's measure of population subdivision (Fst), Nearest-neighbor statistic (Snn) and the Slatkin-Maddison test (SM). Genetic compartmentalization was statistically significant at P values <0.01

g Characteristics of the HIV viral population in the CSF compartment (compart): Cp (compartmentalized), if all three tests were significant ($p < 0.01$), or equilibrated (Eq) if statistical significance was not reached ($p > 0.01$)

N/A.: V3 sequences identical in CSF or identical between CSF and plasma

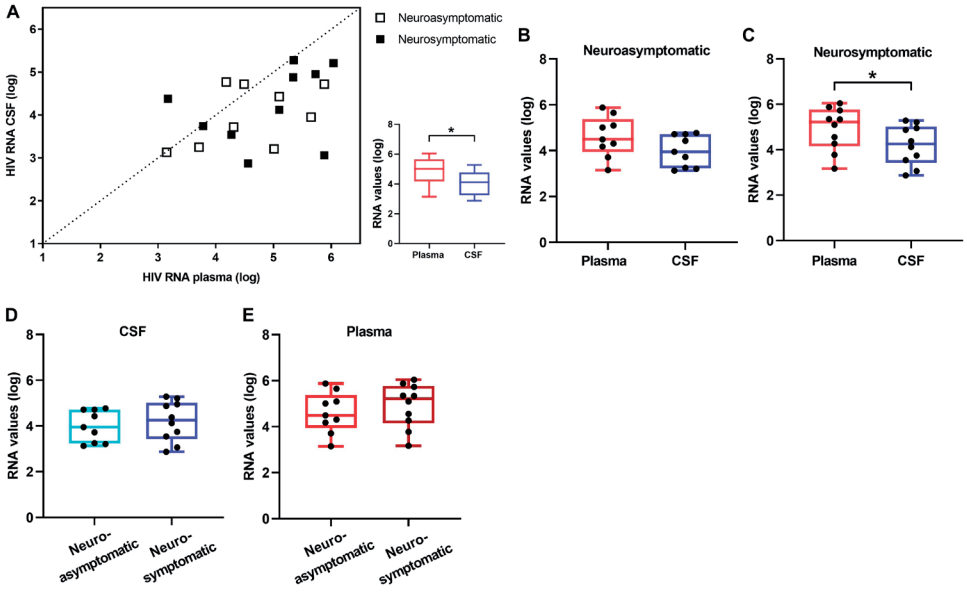

Figure 1. Relationship of HIV-1 RNA levels (log 10cp/mL) measured in paired plasma and CSF samples in neuroasymptomatic and neurosymptomatic subjects. (A) Plot depicts the correlation between HIV RNA CSF and HIV RNA plasma for all subjects. Black dashed line represents line of identity. **(B, C)** Boxes depict median HIV RNA levels and IQR, measured in plasma (red) and CSF (blue) in neuroasymptomatic and neurosymptomatic subjects. **(D, E)** Boxes depict CSF (blue) and plasma (red) median HIV RNA levels and IQR between neuroasymptomatic and neurosymptomatic subjects. * = Statistically significant ($p < 0.05$) determined by the nonparametric Wilcoxon signed rank test.

Analyses of CNS compartmentalization

Genetic compartmentalization between CSF and plasma HIV variants was determined based on the V3 region in the viral envelope (env) gene. CSF viral populations were defined as either compartmentalized (cp), if all three compartmentalization analyses (Fst, Snn, SM) were significant ($p < 0.01$), or equilibrated (eq) if statistical significance was not reached ($p > 0.01$) [15] (Table 1). Most of the subjects (89%) had equilibrated viral populations in their CSF and plasma. Significant genetic CNS compartmentalization was detected in two subjects, 13 and 19. Subject 13 had advanced disease (CD4 count 30 cells/µl) and had mild neurological symptoms, namely balance disturbances and peripheral weakness. In contrast, subject 19 had less advanced HIV infection (CD4 count 387 cells/µl) and was neurologically asymptomatic, suggesting that CNS compartmentalization is not always associated with neurological symptoms.

CSF-derived viral variants can efficiently enter CD4+ T cells, with a modest enhancement for viral entry in low-CD4 expressing primary microglia

Coreceptor usage was mostly concordant across the paired samples, with 17 out of 19 pairs predicted to consist exclusively of CCR5-using viral strains in both compartments [Table 1]. Subjects 17 is predicted to have CCR5- and CXCR4-using viral strains in both plasma and CSF, whereas subject 19 is predicted to have CXCR4- and CCR5-using viruses in the plasma but only CCR5-using virus in the CSF. In addition to being the only subject with X4-using virus in the CSF, subject 17 was also the only subject diagnosed with severe HAND (HIV encephalopathy) and one of the subjects with a CSF > plasma HIV RNA discordance (1.21 log10 copies/mL). A comparison of the lowest FPR values in plasma and CSF revealed no correlation or significant difference [Data not shown].

Once inside the CNS, HIV DNA is primarily detected in perivascular macrophages and primary microglia [14]. We investigated the entry phenotype of the CSF and plasma HIV variants from compartmentalized (cp) neuroasymptomatic subject 19 and two equilibrated (eq) subjects, subjects 8 and 27, with neurological symptoms and low CD4 count (10 and 185 cells/ul, resp.) [Table S1]. First, we generated CSF- and plasma-derived luciferase reporter viruses using the full-length envelope (*Env*) gene amplified from CSF and plasma. For each subject, we obtained a diverse mixture of viral clones with different FPR values between the CSF and plasma [Figure 2]. These viral clones were phenotypically characterized for viral entry into CD4+ T cells (high CD4 surface levels) and primary microglia (low CD4 surface levels), the main HIV target cells in the blood and CNS. Considering that CD4 surface expression levels on CD4+ T cells and microglia are likely to differ between donors, we used up to 3 different donors and three lab strains as a control for infection: two R5 M-tropic virus (Bal and YU-2) and one R5 T-tropic virus (JRCSF).

For subjects 19 (cp) and 27 (eq), we observed no significant difference between the ability of the CSF and plasma viruses to infect CD4+ T cells [Figure 2B, C]. In subject 8 (eq), we observed a ≥10-fold higher infectivity with CSF clones 4 and 6, compared to the plasma viruses [Figure 2A]. Interestingly, this infectivity was also substantially higher than CSF clone 1 which had the same FPR value, suggesting that there are other determinants outside of the V3 loop that can greatly affect cell entry. Furthermore, treatment with the CCR5 inhibitor maraviroc (MVC), supported coreceptor prediction and significantly inhibited T-cell infection by the R5-predicted CSF and plasma viruses, whereas the X4-predicted plasma viruses of subject 19 were resistant to MVC inhibition [Figure 2D-F]. R5-predicted CSF clone 4 of subject 8, despite having a high FPR value of 28.8, was also greatly resistant to MVC inhibition (≥60%), suggesting that this clone can potentially use both coreceptor for cell entry [Figure 2D].

Lastly, phenotyping of the viruses in low CD4-expressing primary microglia revealed that most CSF and plasma clones could not efficiently enter these cells, although CSF-derived

viral clones were overall better than the plasma clones [Figure 3]. The enhanced ability of CSF clones, to infect microglia was more pronounced in compartmentalized subject 19 [Figure 3B]. While both BaL and YU-2 are R5 M-tropic viruses, BaL was isolated from infant lung tissue [25], whereas YU-2 was isolated from brain tissue [26] and hereby potentially more representative of the neurotropic viruses in the CNS. From this perspective, we compared the infection of the viral clones to YU-2 and found that each subject had one CSF clone with an intermediate phenotype, defined as ≥50% of YU-2 infection, for cell entry in microglia [Figure 4]. In subject 27, we also found one plasma clone with this intermediate entry phenotype. The plasma clone had a higher FPR than the CSF clone with a similar intermediate phenotype (71.1 vs. 58.6), suggesting two separate virus populations possibly originating from different low CD4- expressing cells.

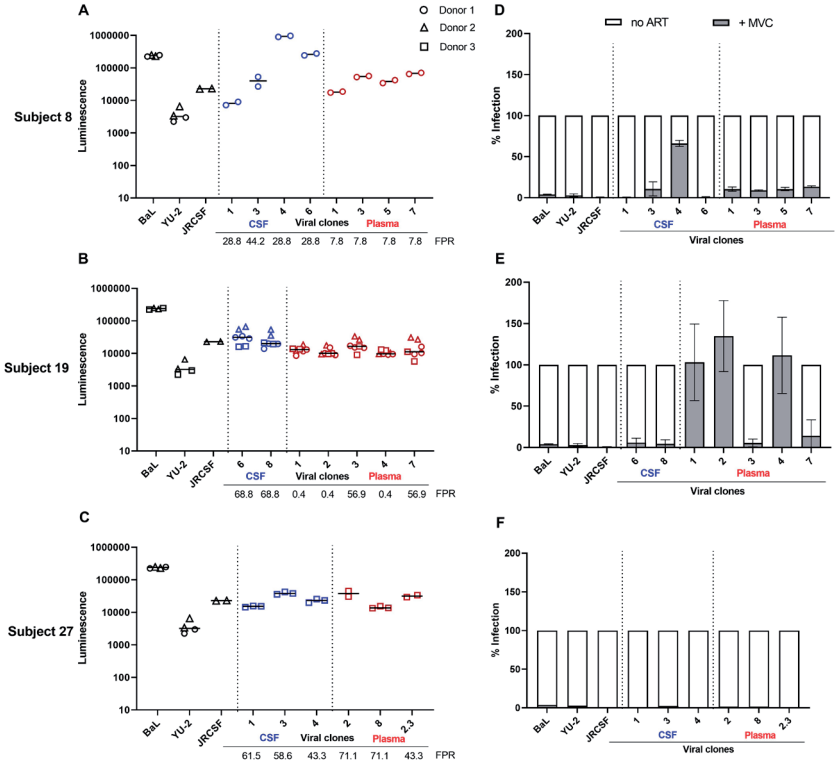

Figure 2. CSF- and plasma-derived viruses can efficiently infect and replicate in CD4+ T cells. **(A-C)** CD4+ T cells were infected with 10ng (p24 Gag) CSF- or plasma-derived virus generated from subjects 8, 19 and 27. Scattered dot plots depict luciferase activity measured in supernatant collected on day 14 post-infection, whereas horizontal black lines represent median luminescence. **(D-F)** CD4+ T cells were untreated (no ART) or treated with 100nM Maraviroc (MVC) prior to infection with 10ng (p24 Gag) CSF- or plasma-derived virus generated from subjects 8, 19 and 27. Bar graphs depict the maximum infection measured on day 14 post-infection with (grey) and without (white) treatment with MVC. Black error bars depict standard errors of the means. Lab strains and viruses derived from plasma (red) or CSF (blue) are separated by vertical dotted lines.

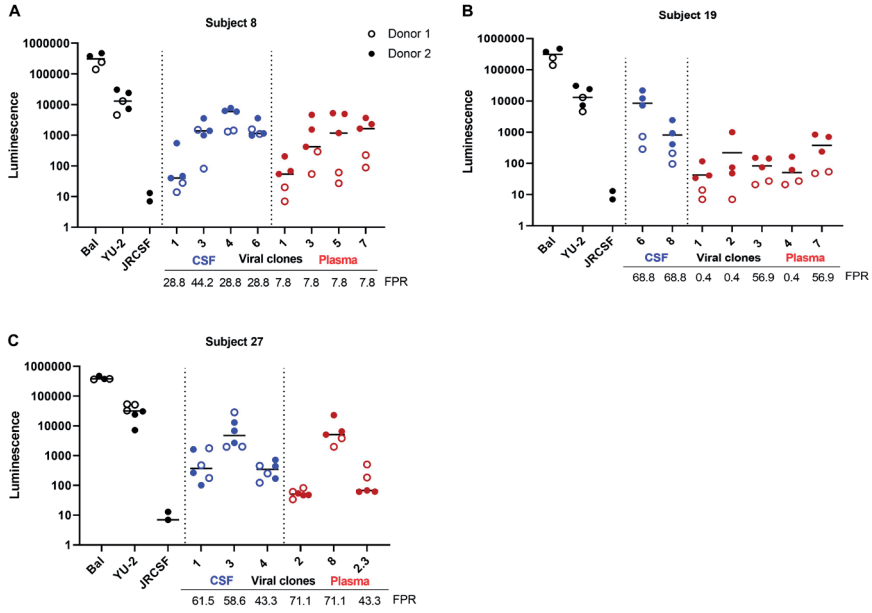

Figure 3. CSF-derived viruses have a modest enhancement for infecting primary microglia. **(A-C)** Primary microglia were infected with 10ng (p24 Gag) virus generated from subject 8, 19 and 27. Graphs represent luciferase activity measured in supernatant collected on Day 17 post-infection. Black line depicts the median luminescence measured. Lab strains and viruses derived from plasma or CSF are separated by vertical dotted lines.

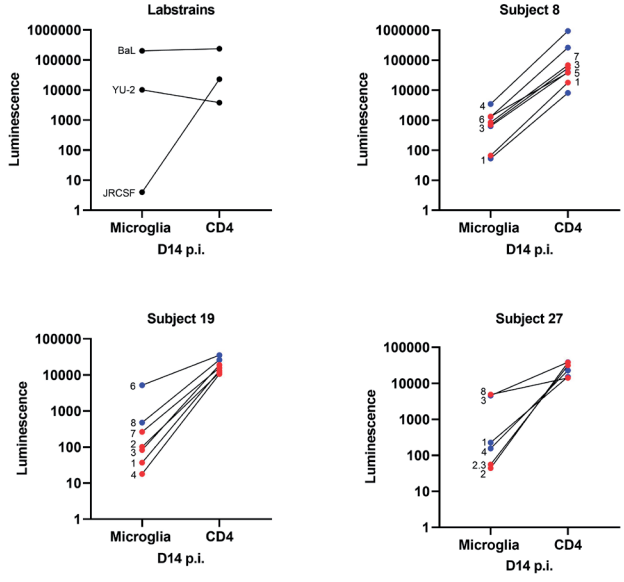

Figure 4. Majority of the CSF and plasma viral population displayed the typical R5 T cell-tropic phenotype. Graphs represent luciferase activity measured in supernatant collected on Day 14 post-infection (D14 p.i.) in primary microglia and CD4+ T cells. Lab strains are depicted in black, CSF-derived viruses in blue and plasma-derived viruses in red.

Discussion

With up to 43% of the HIV-infected population still affected by lasting HIV-associated neurological impairments despite viral suppression with ART, research on the neuropathogenesis of HIV remains essential [27]. In this study, we report, for the first time, that patient-derived replication-competent HIV variants can infect and replicate in human primary microglia. Notably, HIV replication was more efficient in primary CD4+ T cells.

HIV RNA can be detected in the CSF as early as 8 days post estimated infection, however, RNA levels in CSF are generally lower than in plasma [28]. Within our study population, HIV RNA levels were significantly lower in CSF than in plasma, however, 16% (n=3) of the subjects (subjects 12, 17, 19) had higher virus concentration in CSF than in plasma. Among the three subjects, only subject 17 had neurological symptoms (HIV encephalopathy). A recent multicenter study reported that up to 30% of treatment-naïve patients with HIV-associated dementia (HAD) had CSF to plasma HIV RNA discordance [29]. The detection of higher levels of HIV RNA in CSF than in plasma suggests compartmentalized viral production and/or replication in CNS resident cells. We found a genetically compartmentalized viral population in the CSF in 2 subjects (subjects 13 and 19).

Within the first two years of infection, CSF compartmentalized variants are predominantly R5 T-tropic and associated with clonal amplification and the presence of elevated CSF pleocytosis [30]. As the disease progresses in the advanced stage to HAD, both compartmentalized R5 T-tropic and R5 M-tropic CSF viral populations can be detected [12]. R5 M-tropic viruses were more genetically diverse indicative of viral replication in CNS resident long-lived cells. Notably, while subjects 13 and 19 both had compartmentalized R5-using CSF viruses, only subject 13 was neurologically affected, suggesting distinct viral tropism [12,31], namely R5 M-tropic (subject 13) and R5 T-tropic (subject 19). In addition, an X4-using viral population was found in the CSF of subject 17 who was diagnosed with HIV encephalopathy. As the only subject with severe HAND, it remains to be determined whether the prevalence of X4-using virus in the CNS is associated with the progression of neurological disease.

In the CNS, HIV is primarily detected in perivascular macrophages and primary microglia, both of which express the CCR5 coreceptor [14]. In this study, we phenotypically characterized CSF- and plasma-derived viral clones from compartmentalized subjects 19 and equilibrated subjects 8 and 27 for their ability to infect and replicate in CD4+ T cells and primary microglia. Characteristic of both M- and T-tropic viruses, all viral clones were able to effectively infect high CD4-expressing T cells with no major differences between the CSF and the plasma viruses. Treatment with MVC confirmed productive infection and corroborated the prediction of both X4 and R5-using viruses in the plasma of subject 19 and

revealed a possible dual-tropic viral population in subject 8. Interestingly, we also observed an enhanced infection of the X4-using viruses following treatment with MVC, suggesting that treatment of CD4+ T cells with MVC increases their susceptibility to X4-using viral infection possibly due to cell activation [32,33].

In line with previous studies on monocyte-derived macrophages and Affinofile cells [10–13], CSF-derived viral clones were overall more efficient at infecting low CD4 expressing primary microglia than the plasma-derived clones, despite differences among donors. This infection, however, never reached the level of the R5 M-tropic lab strains Bal and YU-2, and therefore did not meet the criteria for an M-tropic phenotype. R5 T cell-tropic viruses found in the CSF are presumed to originate from infiltrating infected CD4+ T cells or are potentially produced by resident CD4+ T cells in the brain parenchyma [4,12]. Nonetheless, we observed an intermediate phenotype, defined as ≥50% of YU-2 infection, for several CSF clones (one in each subject) and one plasma clone. Other than CSF and plasma [9,30,34], viruses with an intermediate entry phenotype, determined by low CD4 Affinofile cells and/or monocyte-derived macrophages, have also been detected in peripheral tissues, such as the colon, lungs, and lymph nodes [10]. Due to the relatively invasive nature of CSF collection, longitudinal samples were not obtained, therefore we were not able to determine whether this intermediate phenotype represents an evolutionary intermediate on the path to macrophage tropism. A very recent paper by Woodburn et al. reported that patient-derived M-tropic HIV Env proteins confer an entry advantage over T cell-tropic Envs when infecting primary microglia [35]. In line with our study, infection of primary microglia with an R5 T cell-tropic virus with an intermediate M-tropic phenotype approached but did not reach the infection level of the M-tropic viruses.

It is hypothesized that the enhanced ability of M-tropic viruses to utilize low CD4 surface expression for viral entry is marked by an increased Env: CD4 affinity, enhanced sensitivity to sCD4 inhibition, and other subtle changes in the trimer conformation of the Env protein. Several studies have reported a variety of substitutions in the envelope gene found to be associated with M-tropic CNS-derived viruses, such as N283 in the CD4 binding site (CD4bs) [36], a conserved amino acid in the V1 loop [37], and the loss of an N-linked glycosylation site at 386 [38,39]. However, none of these genetic mutations were conserved across different studies.

Furthermore, although the limited HIV infection observed in the microglial cells is primarily due to the inability of the CSF- and plasma-derived R5 T-tropic viruses to enter the cells, our infection assay has two noteworthy limitations. First, we used the Env protein rather than full-length viral clones. While the envelope protein is the major determinant for coreceptor usage and CD4 binding, we cannot completely rule out the possibility of viral evolution outside of the Env gene that contributes to the macrophage phenotype. Second, we used

a cell-free virus infection assay which might not fully represent the modes of microglia infection *in vivo*. Non-M-tropic viruses were recently shown to productively and efficiently infect macrophages through Env-dependent cell-cell fusion with infected CD4+ T cells [40]. The Envs expressed on infected T cells also showed enhanced interaction with the CD4 and CCR5 receptors and were less dependent on the surface density, compared to the cell-free virus-associated Envs. However, the infection of microglial cells *in vivo* through cell-to-cell fusion with infiltrating infected CD4+ T cells is yet to be demonstrated. In addition, the limited infection of primary microglia observed with both M- and T-tropic viruses can also be attributed to the host-restriction factors expressed in microglia, such as Sp3 protein and C-EBPγ, that function as transcriptional repressors [41].

We were able to derive significant and compelling evidence that supports the CNS as a viral reservoir for HIV. Among these findings is the detection of a genetically distinct CSF viral population, indicating viral replication in the CNS, as well as the detection of CSF-derived viral clones that exhibit a modestly enhanced ability to enter primary microglia, a characteristic well known to be associated with macrophage-tropism. In this small convenience sample, we were not able to study a relation between clinical symptoms and CNS diversification. Ultimately, the evidence of viral replication and evolution in the CNS highlights the importance of including the CNS in the development of new cure strategies designed to target HIV reservoirs.

Supplementary Materials is available via Surfdrive: Table S1: Neurological diagnosis and ART history of the subject population; Table S2: PCR primers sets for the amplification of HIV V3 and Env gene. https://surfdrive.surf.nl/files/index.php/s/VGGFNezafbbxn5p

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Institutional Review Board Statement: Fresh postmortem adult human brain tissue was provided by the Netherlands Brain Bank (NBB). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of VU Medical Center (VUMC, Amsterdam, The Netherlands). Paired CSF and plasma samples were obtained as part of the AIDS Therapy Evaluation in the Netherlands (ATHENA) cohort which includes patients by an opt-out principle.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

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CHAPTER 4

Detection of Intact HIV proviral DNA in the CNS of a virally suppressed HIV-infected individual

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Abstract

There is compelling evidence that the central nervous system (CNS) can function as a viral reservoir for HIV, including the detection of HIV DNA in CNS myeloid cells of virally suppressed individuals. The question remains, however, whether the DNA detected is intact and capable of producing a replication-competent virus that could fuel rebound viremia. Using the intact proviral DNA assay (IPDA), we examined the genomic integrity of proviral HIV DNA in CNS cells isolated from three different brain regions of a virally suppressed individual on long-term ART. Full-length envelope reporter viruses were generated from HIV variants from the frontal lobe and phenotypically characterized in CD4+ T cells. An estimated 13% of the total detectable proviral DNA in the brain was intact and was more prevalent in the microglia-enriched (0.52%) than the microglia-depleted fraction (0.19%). Phenotypic characterization revealed efficient viral entry and replication in CD4+ T cells for all viral clones via the CCR5 HIV coreceptor. This study provides evidence that intact HIV proviral DNA can reside in both microglial and non-microglial cells, which can potentially be reactivated and contribute to rebound viremia following ART interruption or cessation.

Keywords: HIV • latent reservoir • IPDA • Central nervous system • Microglia

Introduction

Following infection, HIV-infected individuals are subjected to a lifetime of antiretroviral therapy (ART) to continuously suppress viral replication and prevent viral rebound from hidden anatomical and cellular HIV reservoirs established early during infection [1–3]. Any successful effort to eradicate these latently HIV-infected cells will require a thorough understanding of the molecular mechanisms and the size of the replication-competent reservoir.

The quantitative viral outgrowth assay (QVOA) has gained wide acceptance as the "gold standard" for the measurement of the replication-competent viral reservoir [3]. However, the QVOA is costly and greatly underestimates the size of the replication-competent reservoir due to suboptimal induction (<10%) of all replication-competent proviruses in vitro [4,5]. In addition, the QVOA relies on the isolation of a large number of viable cells, which is relatively straightforward from blood specimens, but is much more challenging from tissues, particularly the CNS, which is only available post-mortem in limited quantities. As an alternative, the recently developed intact proviral DNA assay (IPDA) uses two strategically chosen amplicons placed within the HIV-1 packaging signal (Ψ) and *env* gene to discriminate between intact and defective proviruses, irrespective of their transcriptional status [6]. The IPDA gives an accurate upper limit on the replication-competent reservoir size, but the actual frequency of latently infected cells responsible for rebound viremia is believed to lie between the values reported by the QVOA and the IPDA [7].

The largest and best characterized viral reservoir, to date, are the resting CD4+ T cells. However, HIV has been detected in a variety of cell types throughout the body that can potentially serve as a reservoir for HIV [8]. Technological advancements in the last decade have provided compelling evidence for the existence of latently infected cells in the central nervous system (CNS) [9–12]. In particular, HIV DNA has been detected in 1-10% of CD68+ microglia/macrophage cells [13–15] and, most recently, also in a very small subset of astrocytes (0.4-5.2%) [16]. Yet, a critical question remains as to whether the DNA detected is intact and capable of producing a replication-competent virus that could fuel rebound viremia.

In this study, we used the IPDA to examine the genomic integrity of the proviral DNA isolated from CNS cells from post-mortem brain tissue of a virally suppressed individual enrolled in the Manhattan HIV Brain Bank. In addition, we phenotypically characterized the *env* gene in CD4+ T cells to determine its replication competence.

Methods

Study participant

Fresh human brain tissue of Donor MHBB773 was provided by the Manhattan HIV Brain Bank, a member of the National NeuroAIDS Tissue Consortium (MHBB, U24MH100931), under protocol number R588. MHBB operates using protocols under the supervision of the Icahn School of Medicine at Mount Sinai (ISMMS) Institutional Review Board. Written informed consent was obtained from the subject, or their primary next-of-kin, before collecting and using autopsy tissues for medical research.

Human Brain processing and DNA isolation

Fresh human brain tissue was mechanically and enzymatically dissociated to obtain a single cell suspension, followed by a Percoll gradient to remove myelin and red blood cells as previously described [28]. The enrichment of microglia was achieved using CD11b+ MACS (Miltenyi Biotec 130-093-634) according to the manufacturer's protocol. Flowthrough containing CD11b- cells was collected and is termed "microglia-depleted" fraction. DNA was isolated from microglia-enriched (CD11b+) and microglia-depleted (CD11b-) cell fractions using the DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol.

Intact proviral DNA assay

Genomic DNA was isolated using the DNeasy Blood and tissue kit (Qiagen), according to the manufacturer's guidelines. HIV intact and defective proviral DNA was quantified using a multiplex ddPCR assay, targeting both the Ψ (psi) region and part of the Env region [6]. Total psi and Env copies were combined and considered as the total defective proviral DNA. The DNA shearing Index (DSI), as well as the total number of copies/1x10^6 cells, was computed using the RPP30 household gene. HIV copies were corrected for this DSI and normalized to the number of input cells. HIV and RPP30 reactions were conducted independently and in parallel, for which an input of 500ng and 20ng DNA was used respectively. Used primer and probe sequences (5′-> 3′) could be found in Table S1. Cycle conditions were performed according to the manufacturer's protocol and assay prescriptions, with an exception of the annealing temperature at 55°C [6]. Analysis of the results was done with Quantasaft version 1.7.4, for which replicate wells were combined before analysis. The pie charts were generated with GraphPad Prism 8.3.0 (Graph-Pad Software).

Generation of recombinant viral clones

To generate the recombinant FRONT viral clones, we first amplified the HIV-1 envelope (gp160) gene using a 5-fold serial dilution to obtain single viral clones. Amplification was conducted by RT-PCR (Superscript IV Reverse Transcriptase Kit, Invitrogen) followed by a Nested PCR (Platinum Taq Superfi PCR Master Mix, Invitrogen), according to the manufacturer's protocol. Please refer to Table S2 for a list of the primes used. Envelope

amplicons were introduced into a HxB2 gp160deletion vector with a luciferase reporter gene (HxB2ΔENVluc), previously described in [29], using the NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). We used the same vector carrying either the gp160 sequence of YU-2 (R5 M-tropic) or BaL (R5-tropic) as controls. Chimeric infectious plasmids were transfected into HEK293T cells using Lipofectamine 2000 reagent (Invitrogen) to generate viral stocks. The supernatant containing replication-competent virus was harvested at 48h post-transfection and stored at 80 °C until further use. p24 was determined with an ELISA p24 assay (Aalto Bioreagent, Dublin, Ireland).

CD4+ T cell isolation and HIV infection

PBMCs were obtained from a healthy donor by Ficoll-Paque density gradient centrifugation of peripheral blood, after which CD4+ T cells were collected through negative selection with the CD4+ T Cell Isolation Kit (Miltenyi Biotec 130-096-533) according to the manufacturer's protocol. CD4+ T cells were stimulated for 2 days in culture medium (RPMI 1640 (Gibco Life Technologies) with 10% Fetal Bovine Serum, 1% penicillin-streptomycin (Gibco Life Technologies), and 20U/mL IL-2 (Invitrogen) supplemented with Phytohaemagglutinin (5µg/mL). CD4+ T cells were infected in Duplo with 10ng (p24 Gag) virus per 100.000 cells for 3 hours in Eppendorf's placed on a tube rotator. Maraviroc was administered to CD4+ T cells 1h before infection at a concentration of 100nM. After viral incubation, cells were washed 3 times with PBS and cultured in 96-wells plates in culture medium for 14 days without medium refreshment.

Luminescence

Supernatant was collected 2-3 times per week and luminescence was measured with the Nano-Glo® Luciferase Assay System (Promega) according to the manufacturer's protocol. The graphs depict the mean and range of each viral clone and were generated with GraphPad Prism 8.3.0 (Graph-Pad Software).

Results

Study participant

Human post-mortem brain tissue was obtained from a 65-year-old male, enrolled in the Manhattan HIV Brain Bank, on long-term ART with an undetectable viral load (<50cp/mL) for at least four years before he died. Unfortunately, as an "off-study" patient, no cognitive or blood-brain barrier assessments were performed.

Detection of intact HIV DNA within CNS cells

Microglia, macrophages, and CD4+ T cells are the only cells in the CNS that express both the CD4 and the CCR5 receptor required for HIV entry, whereas astrocytes and neurons

express either the CXCR4 or CCR5 coreceptor but lack the expression of CD4 [9]. To determine whether these CNS cells harbor intact proviral DNA, we performed the IPDA on the microglia-enriched (CD11b+) and the microglia-depleted (CD11b-) cell fraction isolated from 3 different brain regions, frontal lobe (FRONT), occipital lobe (OCC) and subventricular zone (SVZ).

Intact proviruses accounted for 13% of all detectable proviral DNA in the brain and were approximately 2.8-fold higher in the microglia-enriched than the microglia-depleted fraction [5,187 vs. 1,865 copies/million cells] [Figure 1]. Within the three different brain regions, the occipital lobe had the most intact proviruses whereas defective proviruses were more prevalent in the frontal lobe. In the subventricular zone, intact proviruses were only detected in the microglia-enriched fraction.

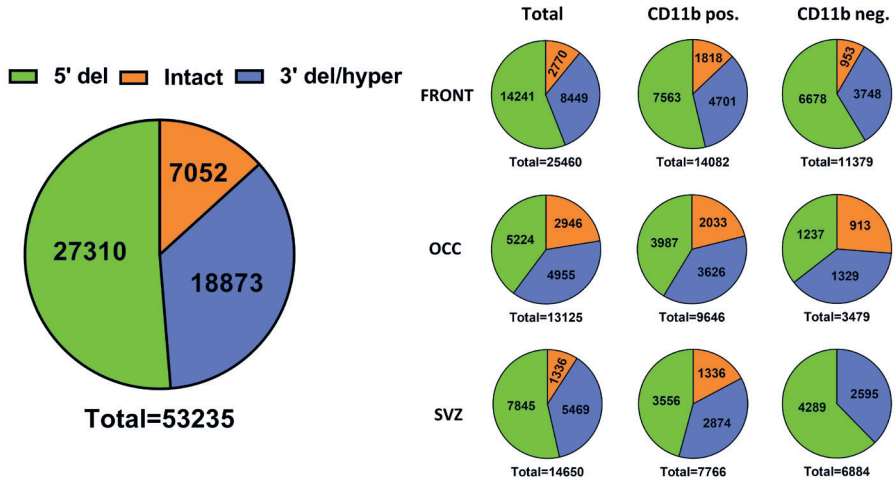

Figure 1: IPDA on CNS cells. Left pie chart depicts the sum of all intact and defective proviruses detected. Right pie chart depicts the number of proviruses detected per brain region within the total (total), microglia-enriched (CD11b pos.) and microglia-depleted (CD11b neg.) cellular fractions per million cells. Values within the left pie charts represent the number of intact and defective proviruses detected per fraction per million cells in each brain region. The total number of proviruses detected per million cells is written below each chart. FRONT = frontal lobe, OCC = occipital lobe, SVZ = subventricular zone

Intact proviral DNA in the CNS can produce replication-competent virus

Following the detection of intact proviral DNA, the key question is whether this proviral DNA produces replication-competent virus. To gain insight into the replication competence of the proviruses, we introduced the full-length *env* gene, amplified from the viral DNA from the FRONT microglia-enriched fraction, into our replication competent HXB2ΔENV luciferase vector and phenotypically characterized these viruses in CD4+ T cells. All viral

clones were able to productively infect and replicate within CD4+ T cells up to the last day of the experiment (day 14 post-infection) [Figure 2]. Furthermore, the infection could be greatly inhibited by Maraviroc, a CCR5 inhibitor, indicating that viral entry was predominantly through the CCR5 receptor.

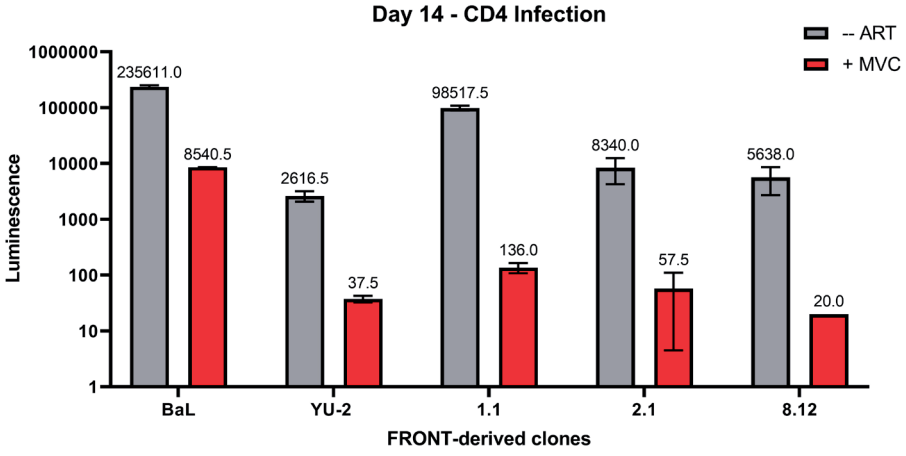

Figure 2: Replication competence of FRONT-derived viral clones in CD4+ T cells. Graph depicts the luminscence measured on day 14 post-infection from 2 lab strains (BaL, YU-2) and 3 viral clones (1.1, 2.1, 8.12) generated from the microglia-enriched (CD11b+) fraction of the frontal lobe (FRONT). CD4+ T cells were untreated (-ART; grey) or treated with Maraviroc (+MVC; red) during infection.

Discussion

To successfully eradicate or permanently silence the HIV reservoir, it is imperative to have a thorough understanding of where the anatomical reservoirs are hidden, the frequency of replication-competent latently infected cells within these reservoirs, and the molecular mechanisms established in these cells to maintain latency. Previous post-mortem brain studies have detected proviral DNA in microglia, perivascular macrophages, and astrocytes of virally suppressed individuals without neurological deficits [16–18], however, whether this proviral DNA is intact and encodes for replication-competent virus remained unanswered.

This study reports, for the first time, evidence of intact proviral DNA in the CNS of an HIV-infected individual on long-term ART. In line with previous post-mortem studies, proviral DNA was predominantly found in microglial cells, with a prevalence rate of 3.2%, of which about 0.5% was intact. Interestingly, intact proviral DNA was also detected in the microglia-depleted cell fraction of the frontal and occipital lobes (0.10 and 0.09%), but not in the subventricular zone. The latter is most likely due to the low cell input, which was 2.9 and 4-fold lower than the OCC and FRONT cell fractions, and did not reach the limit of detection.

The intact proviruses detected in the frontal and occipital lobe microglia-depleted fractions could have originated from residual microglia, CNS resident CD4+ T cells, and/or astrocytes [19; JO Narcís, personal communication, July 15, 2022]; however, there is limited evidence that astrocytes can support productive infection [16,20,21]. In contrast, R5 T cell-tropic compartmentalized HIV variants are often detected in the CSF, with little or no ability to infect low CD4-expressing cells such as microglia and macrophages, providing compelling evidence for viral replication and persistence in CNS resident CD4+ T cells [22,23].

Next, we phenotypically characterized the *env* gene using our HXB2-luciferase vector and found that all viral clones were capable of infecting CD4+ T cells through the CCR5 receptor and complete the HIV replication cycle. This suggests that upon interruption of ART, intact proviral DNA in microglial cells are able to produce new replication-competent viruses in the CNS that may contribute to rebound viremia.

This being said, the interpretation of IPDA results should be done with the technical limitations in mind. Importantly, despite the ability of the IPDA primer/probe set to identify and exclude 97% of defective proviruses, approximately 30% of the intact proviruses are believed to still contain small defects not overlapping IPDA amplicons [6]. Thus, the true frequency of intact proviral DNA within these CNS cells *in vivo* is most likely around 70% of the predicted IPDA values. Furthermore, cure interventions primarily aim to eliminate or permanently silence cells carrying intact proviral DNA, however, translationally competent defective proviruses can express viral proteins associated with numerous adverse effects in the CNS and should ideally be included in cure interventions [24–27].

Collectively, the evidence of intact and defective proviruses in the CNS points towards the presence of latently infected cells in the CNS that can potentially fuel viral rebound upon ART interruption and should be considered as a target in HIV cure interventions.

Supplementary Materials are available via Surfdrive: Table S1: Primer sets of IPDA; Table S2: PCR primers sets for the amplification of HIV Env gene.
https://surfdrive.surf.nl/files/index.php/s/VGGFNezafbbxn5p

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CHAPTER 5

Characterization of HIV-1 Infection in Microglia-Containing Human Cerebral Organoids

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Abstract

The achievement of an HIV cure is dependent on the eradication or permanent silencing of HIV latent viral reservoirs, including the understudied central nervous system (CNS) reservoir. This requires a deep understanding of the molecular mechanisms of HIV's entry into the CNS, latency establishment, persistence, and reversal. Therefore, representative CNS culture models that reflect the intercellular dynamics and pathophysiology of the human brain are urgently needed in order to study the CNS viral reservoir and HIV-induced neuropathogenesis. In this study, we characterized a human cerebral organoid model in which microglia grow intrinsically as a CNS culture model to study HIV infection in the CNS. We demonstrated that both cerebral organoids and isolated organoid-derived microglia (oMG), infected with replication-competent HIVbal reporter viruses, support productive HIV infection via the CCR5 coreceptor. Productive HIV infection was only observed in microglial cells. Fluorescence analysis revealed microglia as the only HIV target cell. Susceptibility to HIV infection was dependent on the co-expression of microglia-specific markers and the CD4 and CCR5 HIV receptors. Altogether, this model will be a valuable tool within the HIV research community to study HIV–CNS interactions, the underlying mechanisms of HIV-associated neurological disorders (HAND), and the efficacy of new therapeutic and curative strategies on the CNS viral reservoir.

Keywords: Microglia • HIV • HIV-associated neurocognitive disorder • Neuropathogenesis • Central nervous system • Organoid • Matrigel

Introduction

HIV enters the central nervous system (CNS) early during infection mainly through infected monocytes or CD4+ T lymphocytes and, to a lesser extent, as viral particles crossing the blood-brain barrier (BBB) [1–3]. Despite this modern era of antiretroviral therapy (ART), characterized by the suppression of HIV replication, roughly 50% of treated HIV-infected individuals are afflicted with a range of cognitive impairments, collectively termed HIV-associated neurocognitive disorders (HAND) [4,5]. The onset and progression of HAND are still unknown but hypothesized to be multifactorial, including continued immune dysregulation and residual chronic inflammation in response to viral persistence and production, and the ensuing accumulation of cytotoxic viral proteins [6–8].

Once it crosses the BBB, HIV mainly infects microglia and, to a lesser extent, macrophages that express both the CD4 and the CCR5 coreceptors required for productive HIV infection [9]. HIV DNA and/or RNA have been detected in 1–10% of microglia and macrophages in both untreated and virally suppressed individuals who died with and without (severe) HAND [10–14]. HIV DNA has also been detected in the astrocytes (0.4–5.2%) of virally suppressed individuals; however, whether astrocytes support productive HIV infection remains controversial, as they do not express the CD4 receptor [15,16]. Instead, HIV entry into astrocytes is proposed to occur via receptor-mediated endocytosis or direct cell-to-cell contact with CD4+ infected T cells [16–20]. Neurons, however, are presumed to be overall resistant to HIV infection. Furthermore, microglia and, to a lesser extent, astrocytes have a long lifespan and can undergo cell division, which enables them to function as a stable, long-term HIV reservoir in the CNS [16,21,22].

To achieve an HIV cure, we need to eradicate or permanently silence all HIV viral reservoirs, including the CNS. Hence, we need a thorough understanding of the molecular mechanisms of HIV CNS entry, latency establishment, viral persistence, HIV-induced neuropathogenesis, and reactivation of the latent virus. However, due to ethical and technical restrictions, neuroHIV research has been predominantly confined to the examination of postmortem brain tissue and two-dimensional (2D) CNS culture models, such as primary cultures from human brain tissues and in vitro-differentiated CNS cells [23–27]. Alternatively, non-human primates (NHP) and genetically modified mouse models have been used. Considering HIV-1 does not infect NHPs and rodents, recapitulating the human disease requires genetic manipulation of the host or HIV, thereby making it more difficult to translate these animal studies to the in vivo scenario [28–33].

Recent advancements in stem cell technologies have enabled researchers to functionally model a diverse range of human organs. Cerebral organoids are self-organized, three-dimensional (3D) cell aggregates that mimic the brain's cytoarchitecture and the molecular

composition of the developing human brain [34–36]. Cerebral organoids have been used to model several neurodevelopmental disorders [37,38] and neurotropic infectious diseases such as Zika (ZIKV), human cytomegalovirus (CMV), herpes simplex virus (HSV), and, more recently, SARS-CoV-2 [39,40].

However, as cerebral organoids are neuroectoderm-derived, a major limitation has been the lack of microglia due to their distinct developmental origin from the mesoderm lineage. Several researchers attempted to rectify this deficiency by introducing microglia into cerebral organoids [41–44]. Alternatively, we have successfully generated cerebral organoids in which microglia grow intrinsically [45]. Given the critical role of the CNS in latency persistence and the limitations of current in vitro culture models, cerebral organoids are quickly gaining interest in the HIV research community. Very recently, Dos reis et al. [44] presented an HIV-infected cerebral organoid model that supports productive viral infection by introducing HIV-infected human primary microglia and immortalized HMC3 microglial cell lines to their 3D cerebral organoid model. In this study, we characterized HIV infection in a cerebral organoid model in which microglia developed intrinsically [45] and related this to the infection of organoid-derived microglia (oMG) and primary human microglia (pMG).

Materials and Methods

Generation of 3D Human Microglia-Containing Cerebral Organoids

Induced pluripotent stem-cell (iPSC) lines (OH1.5, OH2.6 and OH3.1) were generated from human fibroblasts isolated from skin biopsy samples obtained from 3 healthy donors and have been described before [45,46]. The generation and characterization of the iPSC cell lines were performed by the MIND facility of the UMC. Three-dimensional cerebral organoids were differentiated from the iPSC cell lines, as described before [45]. After matrigel embedment, organoids were transferred to a petri dish and kept in organoid differentiation medium without retinoic acid (RA). Four days later, the medium was supplemented with retinoic acid, and the Petridishes were placed on a belly dancer shaker (stand 4; IBI Scientific BDRLS0001). No changes were made in the composition of the neural induction or organoid differentiation media. Organoids were qualitatively selected for downstream experiments following the previously described guidelines [47].

Generation of 2D Human Cerebral Organoid Dissociates

To assess the susceptibility of cells within the organoids to HIV infection, we first dissociated the 3D organoids to 2D organoid dissociates according to the protocol published by Janssens et al. [48] with some minor modifications. In short, organoids were dissociated using accutase (Innovative Cell Technologies, AT104, San Diego, CA, USA) and plated on matrigel-coated 6-well plates. Organoids were either dissociated separately (single method)

or together in a pool of 2–3 organoids (pooled method). After centrifugation, the pellet was collected in organoid differentiation medium with RA and 1 mL organoid suspension was plated per well. After plating, 2D cultures were maintained for 6 days, and 50% of the medium was changed every 2–3 days.

Isolation and Culture of Primary and Organoid-Derived Microglia

Fresh postmortem adult human brain tissue was provided by the Netherlands Brain Bank (NBB). All subjects gave their informed consent for inclusion before they participated in the study. Primary microglia were isolated according to the protocol described before with some minor modifications for human brain tissue [49]. Organoids were dissociated into a single-cell suspension by enzymatic dissociation using papain (18.6 U/mL, Worthington, LK003176, Columbus, OH, USA) and DNAse 1 (337 U/mL, Worthington, LK003170) according to the protocol published before [45].

Microglia enrichment was achieved by positive selection for CD11b expression, using magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Primary microglia (pMG) or organoid-derived microglia (oMG) were cultured in poly-L-lysine hydrobromide (PLL)-coated 96-well plates (1×10^5 cells/well) in microglia medium (RPMI 1640; Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% FCS, 1% penicillin-streptomycin (Gibco Life Technologies, USA) and 100 ng/mL IL-34 (Miltenyi Biotec, Germany)).

Viral Preparation and HIV Infection

The HxB2(Balgp160) luciferase reporter viruses (HxB2Luc and HxB2Balgp160Luc) and the NL4-3Balgp160 GFP reporter virus (NL4-3Balgp160GFP) were generated, as described before [26,50]. HEK293T cells were transfected with the infectious plasmids (HxB2Luc, HxB2Balgp160Luc, and NL4-3Balgp160GFP) using lipofectamine 2000 reagent (Invitrogen). Supernatant containing replication-competent virus was harvested 48h post-transfection and stored at −80°C until further use. p24 was determined with ELISA p24 assay (Aalto Bioreagent, Dublin, Ireland).

pMG and oMG were infected with 10ng (p24 Gag) HIVbal (HXB2Balgp160Luc) or HIVbalGFP (NL4-3Balgp160GFP). The medium was fully replaced the next day and cells were cultured in microglia medium for 13–15 days without medium refreshment. For maraviroc (MVC) treatment, pMG and oMG were treated with 200nM Maraviroc (MVC) for 1 h before infection. The next day, after medium replacement, cells were cultured in microglia medium with 100nM MVC for 13–15 days without medium refreshment. To analyze new rounds of infection, 100nM MVC was added to the culture medium 3 days post-infection. All experiments were carried out in duplicate for each condition.

The 2D organoid dissociates and 3D organoids were infected overnight with 100ng (p24 Gag) HxB2 (HxB2Luc), HIVbal, or HIVbalGFP. The next day, the medium was fully replaced and culture was continued in organoid differentiation medium with RA. For MVC and Raltegravir (RAL) treatment, 2D organoid dissociates and 3D organoids were treated with 200 nM MVC or 400 nM RAL for 2 h before infection. Following medium replacement, culture was continued in organoid differentiation medium with RA with 100nM MVC or 200nM RAL. To maximize viral infection, half of the medium was refreshed only 1x per week. All experiments were carried out in duplicate or triplicate. As a negative control for virus capture and release, we also generated empty Matrigel droplets using the same method as we use for embedding cerebral organoids: 30uL droplets of matrigel (Corning, 356234) were made on indented parafilm and placed in the incubator (37°C, 5% CO_2) for 30 min to solidify. Matrigel droplets were then transferred to a 24-well plate and infected with the same protocol as for the 3D organoids.

Luminescence and Immunofluorescence

For luminescence measurements, supernatant was collected 2–3x per week and measured using the Nano-Glo® Luciferase Assay System (Promega) according to the manufacturer's protocol. Graphs were generated with GraphPad Prism version 8.3.0 (GraphPad Software) and depict the mean and range.

For immunostaining, 2D organoid dissociates were washed with PBS and fixed with 4% PFA for 2h at RT. 3D organoids were washed with PBS and fixed with 4% PFA overnight at 4 °C, followed by 30% sucrose solution incubation for 2 days at 4 °C. The 3D organoids were then embedded in tissue tek (VWR, 25608-930) and sectioned at a thickness of 20µM using a cryostat (Leica CM3050S). The 2D organoid dissociates and 3D organoids were stained according to the protocol described before [45]. See Supplementary Table S1 for antibodies used in this study. Images were obtained with a Zeiss Axio-Scope A1 or Fluoview FV1000 confocal microscope.

Gene Expression Analysis with Real-Time PCR

RNA isolation was performed with the RNeasy kit (Qiagen, Hilden, The Netherlands), including DNAse treatment according to the manufacturer's protocol. Downstream gene expression analyses were conducted in duplicate from 2 different donors (pMG) or 3 different iPSC lines (2D organoid dissociates and 3D organoids). cDNA synthesis and qPCR were performed as described before [26]. Primer sequences are listed in Supplementary Table S2. Absolute gene expression levels were determined (2ΔCT) and normalized to the reference gene Beta-actin (ACTB). Graphs were generated using GraphPad Prism version 8.3.0 (GraphPad Software).

Single-Cell Sequencing of Cerebral Organoids with SORT-Seq

To further assess the expression of HIV receptors in cerebral organoids at the single-cell level, we made use of an available cerebral organoid single-cell dataset (Kübler in preparation) focused on the inflammatory responses of cerebral organoids. Cerebral organoids generated from OH1.5 and OH2.6 were dissociated at week 9 of differentiation. Viable single cells were FACS sorted based on 7AAD (Dead/alive) and CD45 expression (microglia) into 384-well plates, called cell capture plates, which were ordered from Single Cell Discoveries, a single-cell sequencing service provider based in the Netherlands. Each well of a cell capture plate contains a small, 50nl droplet of barcoded primers and 10µL of mineral oil (Sigma M8410). After sorting, plates were immediately centrifuged, snap-frozen, and shipped on dry ice to Single Cell Discoveries, where single-cell RNA sequencing was performed according to an adapted version of the SORT-seq protocol (Muraro et al. [51] with primers described in van den Brink et al. [52]). Cells were heat-lysed at 65°C followed by cDNA synthesis. After second-strand cDNA synthesis, all the barcoded material from one plate was pooled into one library and amplified using in vitro transcription (IVT). Following amplification, library preparation was performed following the CEL-Seq2 protocol [53] to prepare a cDNA library for sequencing using TruSeq small RNA primers (Illumina). The DNA library was paired-end sequenced on an Illumina Nextseq™ 500, high output, with a 1 × 75 bp Illumina kit (read 1: 26 cycles, index read: 6 cycles, read 2: 60 cycles).

During sequencing, Read 1 was assigned 26 base pairs and was used to identify the Illumina library barcode, cell barcode, and UMI. Read 2 was assigned 60 base pairs and used to map to the reference transcriptome Homo sapiens hg19 (including mitochondrial genes) with BWA-MEM [54]. Data was demultiplexed, as described in Grün et al. [55]. Mapping and generation of count tables were automated using the MapAndGo script [56]. Unsupervised clustering and differential gene expression analysis was performed with the Seurat R toolkit [57,58].

Briefly, we merged each plate's count matrix into a single Seurat object and then applied stringent quality control (QC) metrics to filter out cells. Before QC, we removed ERCC spike-in genes and genes that were expressed in less than five cells. Based on the distribution of UMI and gene counts per cell, we removed 950 cells with less than 1.000 UMI counts and 400 gene counts. Raw counts were log-normalized with the NormalizeCounts function. Normalized counts were corrected for variance from log UMI count, cell line, days in vitro, and plate covariates using the ScaleData function. Using the corrected count matrix, we constructed a shared nearest-neighbor graph with the FindNeighbours function using the first 14 principal components. We calculated clusters based on the Leiden algorithm [59] with the FindClusters function (resolution = 0.6, iterations = 20). tSNE plots were calculated with the RunTSNE command. Marker genes for each cluster were identified with the Wilcox rank sum test using the FindAllMarkers function. Clusters were annotated with cell-type identity by calculating gene-set enrichment odds ratio and median log-fold change of

cell-type gene sets per cluster. Gene sets were extracted from organoid studies by Kanton et al. and Quadrato et al. [60,61]. We used a microglia core signature list by Patir et al. [62] to identify organoid-derived microglia.

Results

Cerebral Organoids Contain Microglia, Astrocytes, and Neurons

Human iPSCs were differentiated into microglia-containing cerebral organoids according to the protocol previously described [45]. These organoids contain intrinsically grown microglia (Iba1), astrocytes (S100b), and neurons (Tuj1) and express specific genes for microglia (*AIF1, TMEM119, P2RY12, CX3CR1, CSF1R, TREM2*), astrocytes (*GFAP, ALDH1L1*), and neurons (*MAP2, NEUN, TBR2*) (Figure 1A,B). Single-cell RNA sequencing confirmed the presence of cell clusters enriched with markers for microglia, neurons, astroglia, endothelial cells, oligodendrocytes, and a variety of CNS progenitor and precursor cells (Figure 1C). As we have previously shown by bulk RNA-seq analysis on oMG [45], we show that the microglia cluster from the single-cell analysis exhibits a consistently high expression of microglia signature genes [62], including markers that are often not expressed on other microglial culture models, such as *TMEM119* [26] (Figure 1D).

Next, we assessed the expression of the main HIV receptors (*CD4, CXCR4 and CCR5*) required for viral entry. *CD4* expression was detected on microglia, whereas CXCR4 was mostly detected on non-microglia cell clusters, including astroglia, neuronal/forebrain, and dorsal/ventral progenitors, in line with other cerebral organoid scRNA-seq datasets [60,61] (Figure 1E). *CCR5* expression was detected on a small fraction of microglial cells and not on other cell clusters, suggesting that without targeted sequencing approaches, SORT-seq reaches a detection threshold concerning this gene.

Organoid-Derived Microglia Support HIV Infection via the CCR5 Receptor

Microglia grown intrinsically within organoids (oMG) have been extensively characterized and reported to resemble primary microglia at the whole-transcriptome and functional level [45]. To determine whether oMG are susceptible to HIV infection, we isolated microglia from 3D cerebral organoids generated from two iPSC lines (OH1.5 and OH3.1) and infected them with 10 ng (p24 Gag) of CCR5 M-tropic HIV-1 HxB2Balgp160 Luciferase reporter virus (HIVbal). Using the same experimental conditions, viral infections were performed in human primary microglia (pMG). Virus production was measured in the form of luminescence released over time. oMG were found to support HIV infection and production; however, contrary to pMG that show a continuous increase in virus production up to day 14, oMG reached peak infection on day 6 (Figure 2A,B). This may be explained by the higher sensitivity to cell death we observed in oMG compared to primary microglia (Supplementary Figure S1).

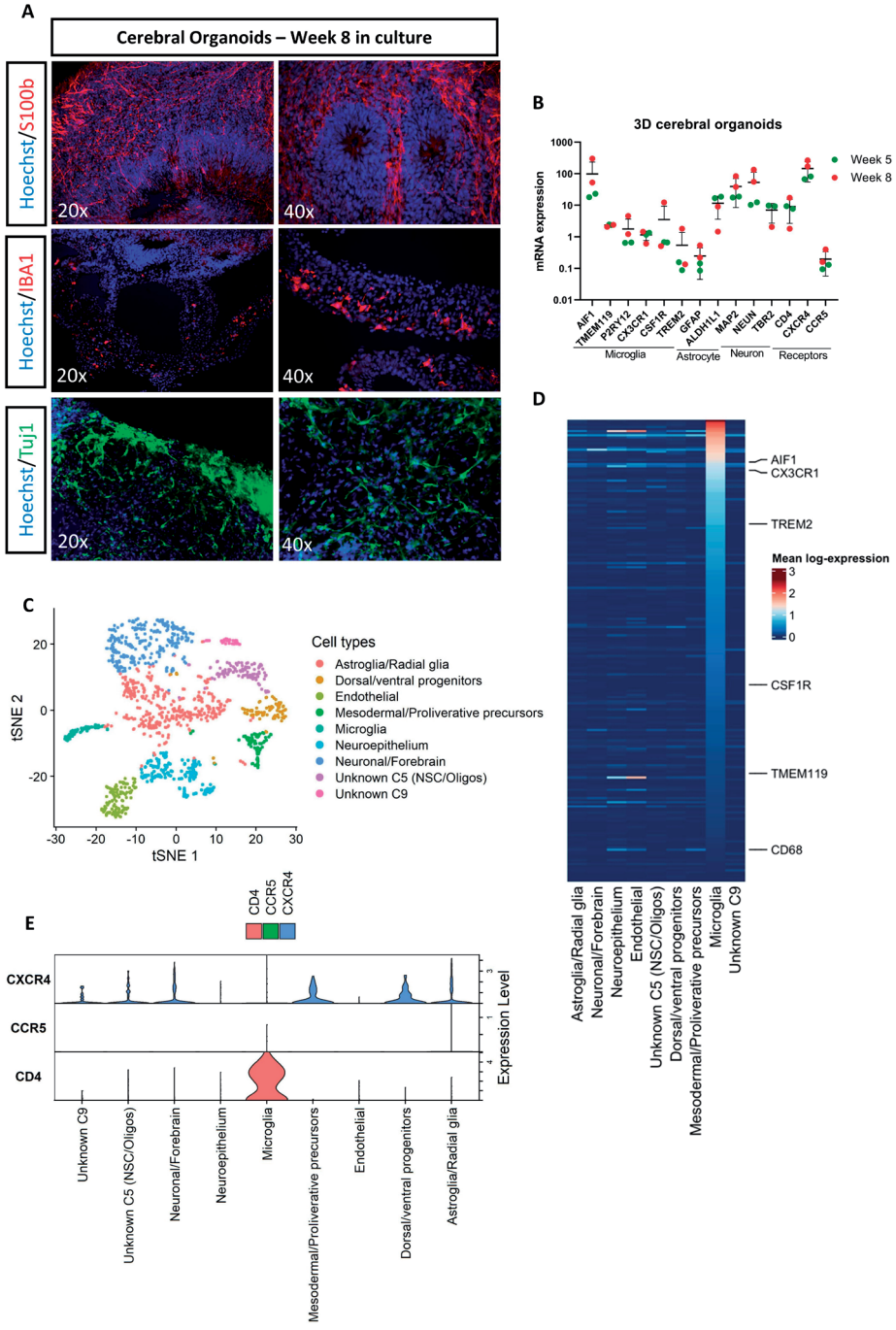

Figure 1. Cerebral organoids exhibit microglia, astrocyte, and neuronal cells and express HIV receptors. (A) Double immunostainings of S100b (astrocyte), IBA1 (microglia), and Tuj1 (neurons) combined with nuclear staining Hoechst after 8 weeks in culture. Representative pictures of

cerebral organoids from iPSC line OH1.5 are shown. Magnification of 20x and 40x was used. **(B)** mRNA expression levels of microglia, astrocyte, and neuron-specific markers and HIV receptors were assessed by qRT-PCR. Gene expression was normalized to the reference gene ACTB. The means ± standard errors of the means are shown. **(C-E)** SORT-seq data of LPS-stimulated organoids after 9-10 weeks in culture. **(C)** Annotated clusters on tSNE plot. Cluster 5 (C5) was only partially annotated. Cluster 9 (C9) was not annotated. **(D)** Heatmap of Patir et al. (2019) microglia signature gene expression across each cluster. **(E)** Violin plots of CD4, CCR5, and CXCR4 gene expression. Expression levels shown are log-normalized and covariate corrected.

The addition of Maraviroc (MVC), a CCR5 inhibitor, before infection (Day-1) restricted viral infection in both oMG and pMG, indicating specific viral entry via the CCR5 coreceptor (Figure 2A,B). Interestingly, the addition of MVC on day 3 post-infection showed no viral suppression, suggesting that the increase in luminescence observed in pMG and oMG is due to continued viral production, as opposed to new rounds of infection after day 3. Furthermore, susceptibility of oMG to HIV infection was confirmed by the detection of GFP+ oMG following infection with 10ng (p24 Gag) of HIV-1 NL4-3Balgp160 GFP reporter virus (HIVbalGFP) (Figure 2C). The distribution of intracellular GFP protein and fluorescence intensity of GFP+ oMG was similar to GFP+ pMG (Figure 2D).

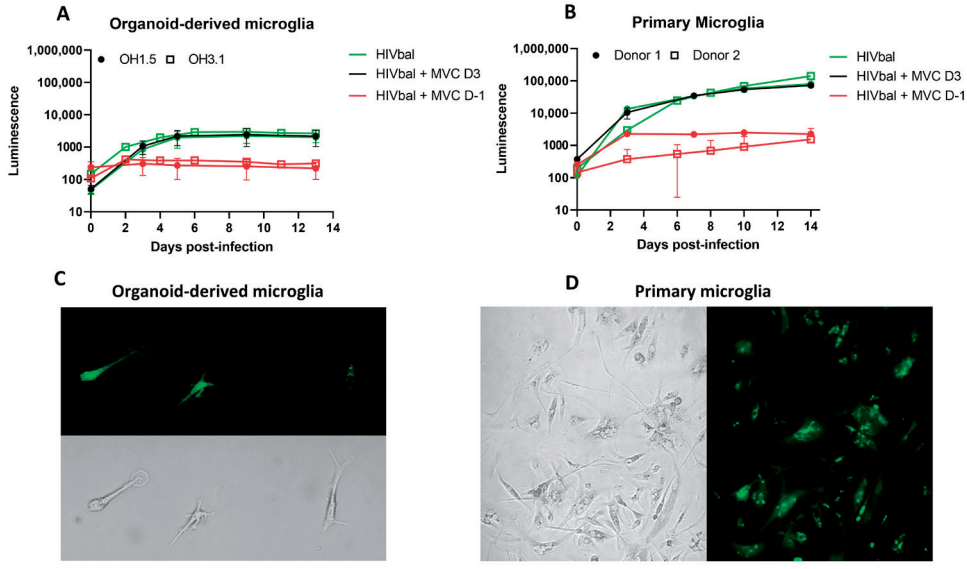

Figure 2. Primary microglia and organoid-derived microglia support productive HIV infection. **(A)** Organoid-derived microglia and **(B)** primary microglia were infected with HIVbal and treated with MVC (200 nM) pre-infection (D-1) and 3 days post-infection (D3). Supernatant was collected at each timepoint and analyzed for luciferase activity. The means ± standard errors of the means are shown. Representative pictures of **(C)** organoid-derived microglia (OH1.5) and **(D)** primary microglia infected with HIVbalGFP, taken in culture after 9 (oMG) and 11 days (pMG) of infection. All pictures were taken with a magnification of 20x.

2D Organoid Dissociates

After confirming HIV infection of oMG, we investigated whether HIV infection is supported in the microenvironment of the cerebral organoid. First, we enzymatically dissociated 3D organoids to 2D organoid dissociates, which were subsequently kept in culture for 7 days to re-establish cellular interactions in 2D culture [48]. Infection of 2D organoid dissociates with 100 ng (p24 Gag) HIVbal showed viral infection and continuous virus production up to day 14 that could be inhibited by the addition of MVC or Raltegravir (Ral) before infection (Day-1) (Figure 3A). Infection of 2D organoid dissociates with the CXCR4 tropic HIV-1 HxB2-luciferase reporter virus (HxB2) was not supported. Ormel et al., 2018 [45], reported higher numbers of ramified microglia and the expression of mature microglial markers in 3D organoids cultured for 5 weeks compared to 3 weeks; therefore, we evaluated whether the susceptibility to HIV infection is influenced by the developmental stage of the organoid by generating a timeline starting from week 3 and up to week 9 in culture. Interestingly, we observed higher viral production in week 3 and 6 compared to week 7, 8, and 9 (Figure 3B).

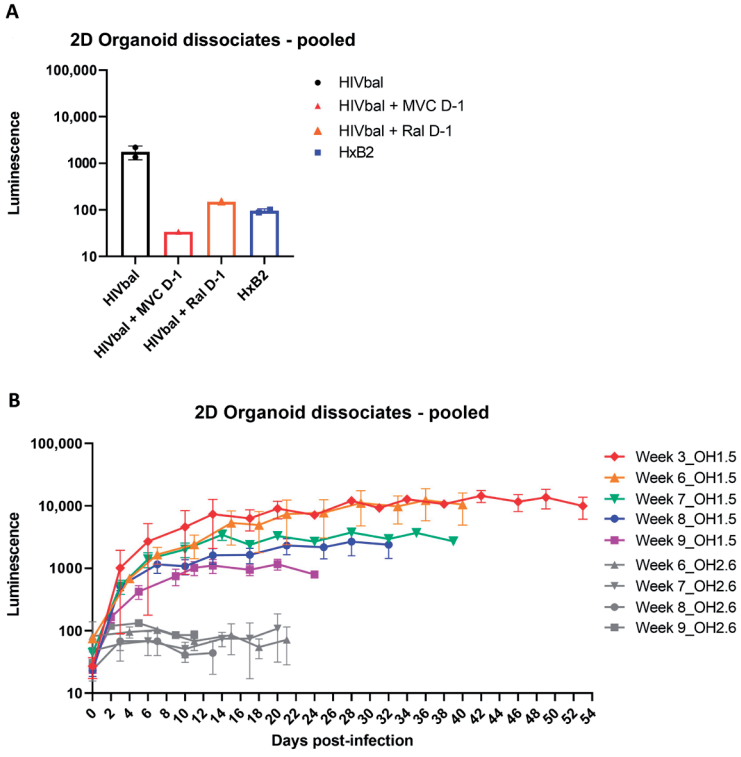

Figure 3. Two-dimensional organoid dissociates support productive HIV-1 infection. (A) Week 5 2D organoid dissociates were infected with HIVbal and HxB2 and treated with MVC (100 nM) and Ral (200 nM) pre-infection (D-1). Bar graphs represent luciferase activity measured in supernatant collected on Day 14, post-infection. **(B)** 2D organoid dissociates starting from week 3 up to week 9 were infected with HIVbal. Cerebral organoids were derived from iPSC line OH1.5 or OH2.6. Supernatants were collected at each timepoint. Both graphs depict the means ± standard errors of the means.

Gene expression analysis revealed a higher expression of *AIF1*, *CD4*, and *CCR5* in week 3 and week 6 organoid dissociates, whereas the expression of astrocyte- and neuron-specific markers was generally similar to the organoid dissociates of week 7, 8, and 9 (Figure 4). This suggests that the susceptibility of 2D organoid dissociates to HIV infection is dependent on the prevalence of microglia and the expression of *CD4* and *CCR5*. Accordingly, 2D organoid dissociates generated from the OH2.6 iPSC line were not susceptible to HIV infection, most likely due to the low expression of *CD4* and *CCR5*, despite having a high expression of microglia-specific markers (Figures 3B and 4).

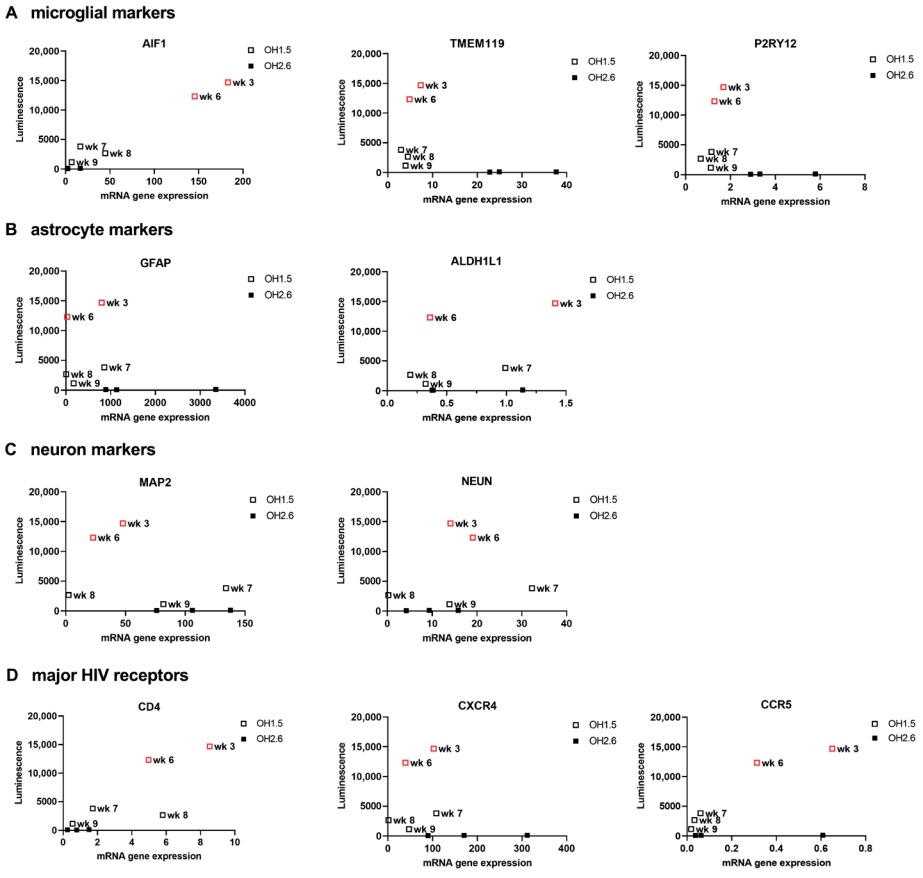

Figure 4. mRNA expression levels of microglia, astrocyte, and neuron markers and HIV receptors in 2D Organoid dissociates. (A) Microglia, **(B)** astrocyte, **(C)** neuron markers, and **(D)** HIV receptor expression levels were assessed by qRT-PCR and plotted against the highest luminescence value of corresponding 2D organoid dissociates. Red squares depict the organoid dissociates with the highest luminescence values. Cerebral organoids were derived from iPSC line OH1.5 or OH2.6. Gene expression was normalized to the reference gene ACTB.

Next, we sought to enhance viral infection in these 2D organoid dissociates through minor modification of the dissociation protocol, originally described by Janssens et al., 2019 [48]. Briefly, we dissociated each organoid separately, instead of a pool of three organoids, then plated the cell suspension of each organoid in a Matrigel-coated 6-well plate so that each well contained the cell suspension derived from one organoid. With this new dissociation protocol, referred to as single dissociation, we were able to increase viral infection by 10-fold (Figure 5A). Despite the optimized protocol, 2D organoid dissociates from the OH2.6 iPSC line did not support HIV infection, highlighting the importance of choosing the right iPSC line for generating cerebral organoids that are susceptible to HIV infection. To further confirm HIV infection of OH1.5-derived 2D organoid dissociates, and to determine the target cells of HIV, we infected 2D organoid dissociates with HIVbalGFP. GFP was detected exclusively in microglia (Iba1+) within 4 days of infection, indicating that, within the 2D cerebral model system, microglia are the only HIV target cells (Figure 5B).

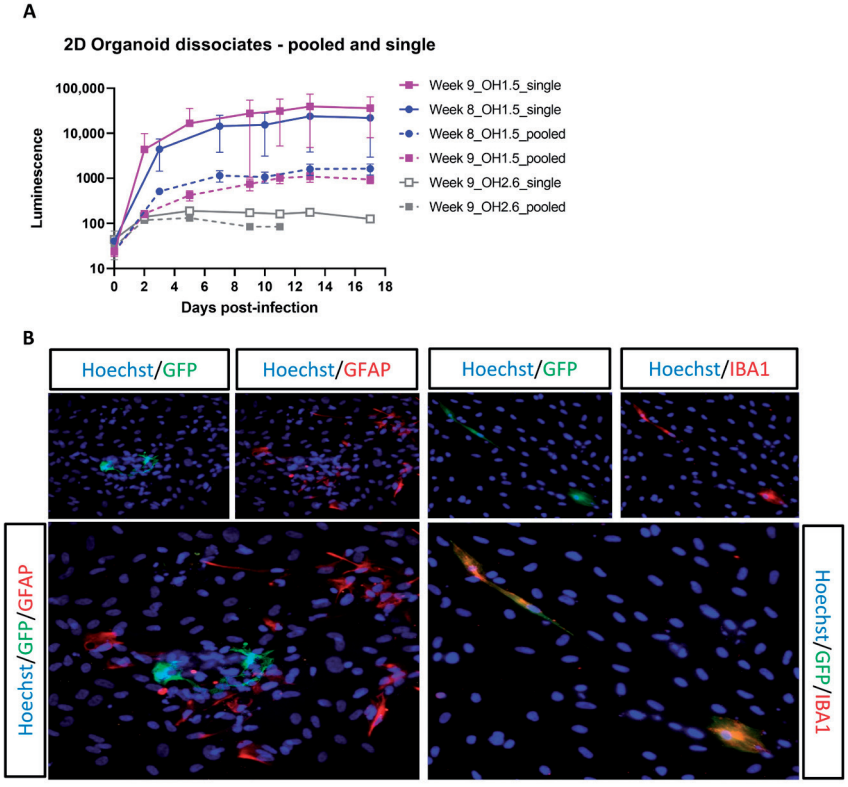

Figure 5. Optimization of 2D organoid dissociates HIV infection. (A) 2D organoid dissociates (OH1.5 and OH2.6), dissociated via the pooled and single method, were infected with HIVbal. Supernatant was collected at each timepoint and analyzed for luciferase activity. The means ± standard errors of the means are shown. (B) Double immunostainings of GFP combined with GFAP (astrocyte) and IBA1 (microglia) at day 7 post-infection of OH1.5 2D organoid dissociates with HIVbalGFP. All pictures were taken with a magnification of 20x.

3D Cerebral Organoids

Following the successful infection of the 2D organoid dissociates, we proceeded to infect 3D cerebral organoids with HIVbal, starting at week 5 up to week 9. Contrary to the 2D organoid dissociates that showed continuous virus production, peak infection was reached within the first week of infection and steadily decreased in the following weeks, except for week 5 and week 7 organoids (Figure 6A). A similar infectivity and infection pattern was unexpectedly also observed in the OH2.6 iPSC line, despite not being susceptible to HIV infection in the 2D organoid dissociates.

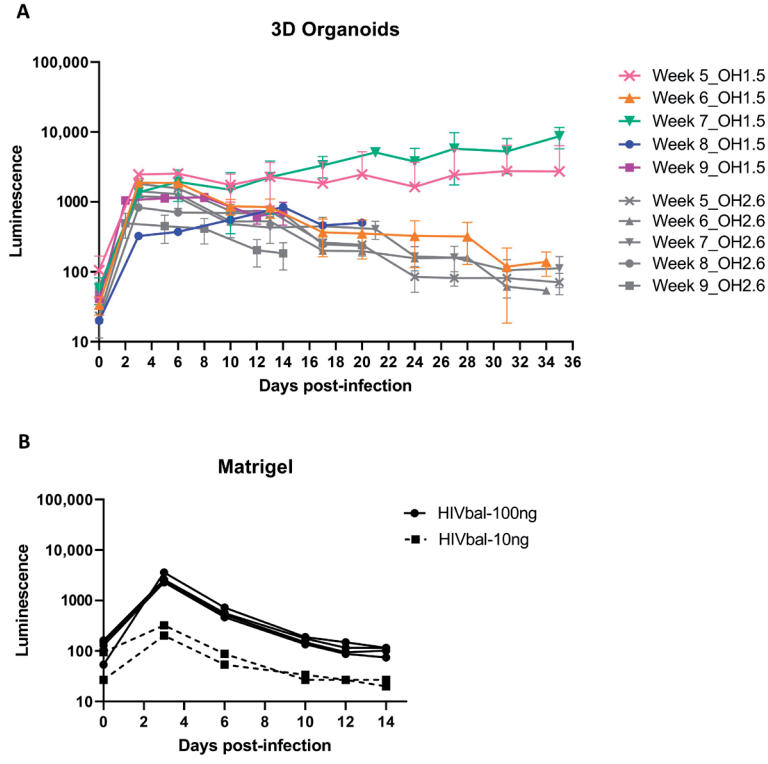

Figure 6. HIV infection of 3D organoids. (A) Three-dimensional cerebral organoids, starting from week 5 up to week 9, were infected with 100 ng (p24 Gag) HIVbal. Cerebral organoids were derived from iPSC line OH1.5 and OH2.6. **(B)** Matrigel droplets were infected with 10 ng and 100 ng (p24 Gag) HIVbal. Supernatant was collected at each timepoint and analyzed for luciferase activity. The means ± standard errors of the means are shown.

To get a better understanding of the luminescence observed from these OH2.6-derived organoids, we investigated whether Matrigel, used for organoid embedment, has any effect on the release of the luciferase protein into the culture medium. Infection of Matrigel droplets with HIVbal resulted in the release of luminescence over time, which was consistent

with the luminescence pattern observed after infection of 3D organoids (Figure 6B). This suggests that, except for week 5 and week 7 organoids, the luminescence measured was most likely due to luciferase diffusion from the Matrigel and not a result of productive viral infection. Accordingly, *CD4* and *CCR5* gene expression was exclusively found in week 5 and week 7 organoids (Figure 7). Week 7 organoids also showed high expression of *AIF1*. Taken together, this highlights that caution should be taken when using the release of luciferase in the culture medium as a measurement for viral production.

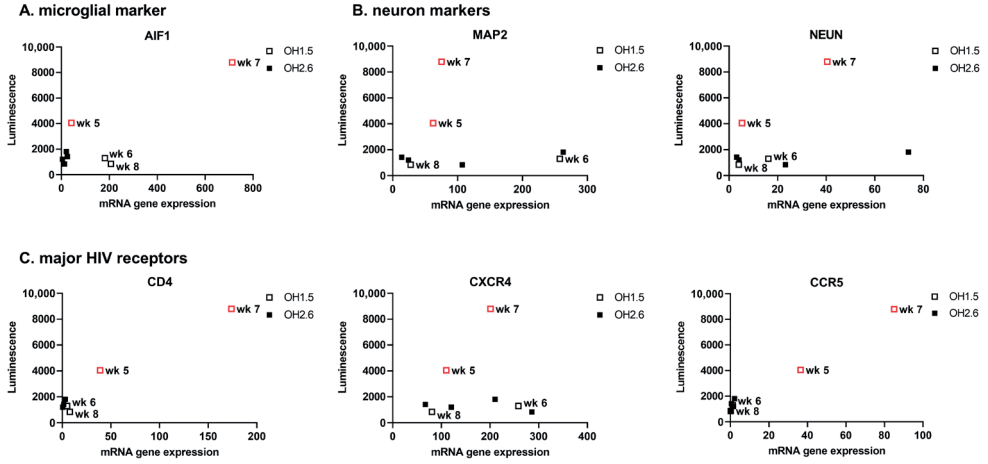

Figure 7. mRNA expression levels of microglia and neuron markers and HIV receptors in 3D Organoids. (A) Microglia and **(B)** neuron markers and **(C)** HIV receptor expression levels were assessed by qRT-PCR and plotted against the highest luminescence value of corresponding 3D organoid. Red squares depict 3D organoids with continued luminescence release. Cerebral organoids were derived from iPSC line OH1.5 and OH2.6. Gene expression was normalized to the reference gene ACTB.

Lastly, we investigated whether the microglia grown within the organoids can mimic the multinucleated pathology of HIV-infected microglia often observed in human postmortem brain tissue. Three-dimensional cerebral organoids were infected with HIVbalGFP. GFP+ cells were found exclusively in microglia (Iba1+) within 3 days of infection, further confirming that microglia are the only target cells of HIV in the 3D organoid model system (Figure 8A). Interestingly, GFP+ cells also had the characteristic multinucleation observed in postmortem human brain tissue (Figure 8B).

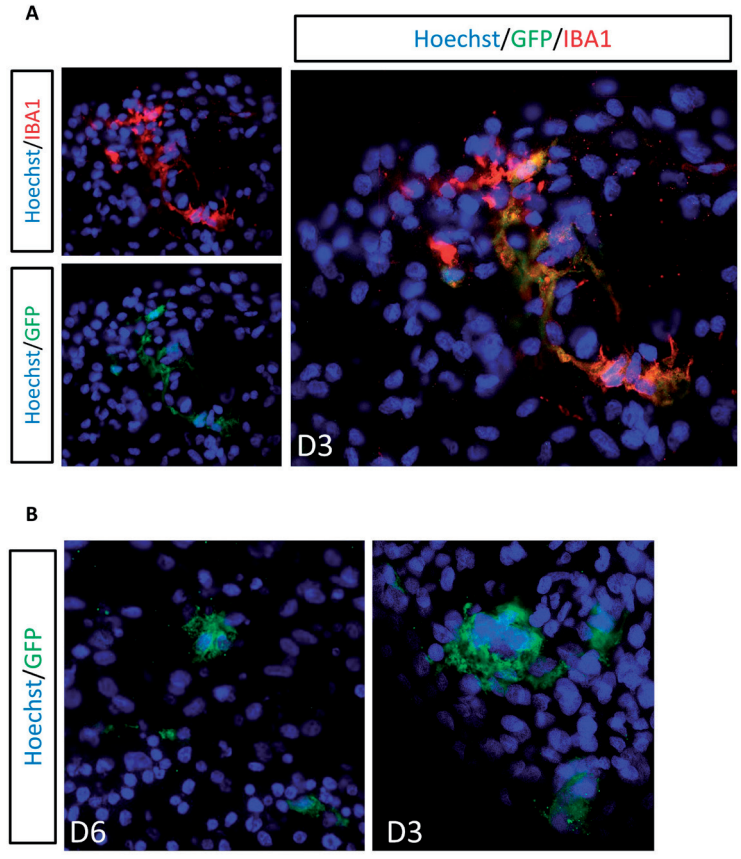

Figure 8. Microglia are the only HIV target cells. (A) Double immunostainings of 3D cerebral organoids (OH1.5) with GFP combined with IBA1 (microglia) at day 3 post-infection with NL4-3Balgp160GFP. **(B)** Representative pictures of GFP+ multinucleated microglia (IBA1+) in 3D cerebral organoids at day 3 and day 6 post-infection with HIVbalGFP. All pictures were taken with a magnification of 40x

Discussion

To advance our knowledge on the CNS viral reservoir, having a good human representative CNS culture model is essential to study the underlying mechanisms of HIV-1 CNS infection, persistence, and reversal. Cerebral organoids are proposed to become a powerful tool to model the human CNS and advance NeuroHIV Research, with multiple application possibilities [63]. In this study, we characterized microglia-containing human cerebral organoids in the context of HIV infection. We demonstrated productive HIV infection in organoid-derived microglia, 2D organoid dissociates, and 3D organoids. HIV infection could be successfully inhibited with MVC, indicating that infection is mediated through the CD4 and CCR5 coreceptors. Interestingly, the addition of MVC on day 3 revealed that

infectivity takes place mainly within the first 3 days of infection, followed by continued virus production up to day 6 in oMG and day 14 in pMG. This finding, however, is inconsistent with fluorescence images showing an increase in GFP+ cells in culture over time, suggesting new rounds of infection [9] (Supplementary Figure S2). However, contrary to the continuous virus production and viral spread we observed in cultured pMG, HIV infection in postmortem brain tissue is reported to be focally distributed in about 1 to 10% of CD68+ microglia/macrophage cells, irrespective of ART treatment and/or HAND [10–14]. This sporadic detection of viral genome was also observed in SIV-infected macaque models, with an infectivity of 0.268 and 231 IUPM (infectious units per million) in treated and untreated macaques [64–68]. These findings suggest that HIV infection in the brain is limited and takes place without viral spread. In this regard, the low infection of HIV in oMG, 2D organoid dissociates, and 3D organoids, as compared to cultured pMG, is reflective of the limited HIV-infected microglial population observed in vivo and in non-human primates. Interestingly, within the organoids, we also observed microglia clusters in which all cells were infected as well as uninfected Iba1+ cells that were in close proximity to infected cells, suggesting a limitation in viral spread (Supplementary Figures S3 and S4). Nonetheless, it remains to be addressed whether the decrease in viral production observed in oMG after the first week of infection is due to a higher cell death rate or, potentially, an increased susceptibility to HIV latency compared to pMG. A small number of astrocytes (0.4–5.2%) have been reported to be infected by HIV-1, both in vivo and in vitro, mainly through endocytosis [10,12,15,16,19,69–72]. Within our organoids, HIV infection was only observed in microglia. This is consistent with dos Reis et al., 2020 [44] and Ryan et al., 2020 [27], in which microglia were found to be the only HIV target cells within their human brain organoid (hBORG) and hiPSC-based tri-culture model of neurons, astrocytes and microglia. However, we acknowledge that our experiments were performed on organoids in which astrocytes have not yet fully matured and should be repeated with older organoids with fully matured astrocytes [73]. Furthermore, although the CXCR4 receptor is expressed on neurons and, to a lesser extent, astrocytes, cerebral organoids were not susceptible to the CXCR4-using HxB2 lab strain, most likely due to the lack of co-expression with the CD4 receptor.

In line with this finding, susceptibility to HIV infection with HIVbal was found to be highly dependent on the co-expression of the CD4 and CCR5 receptor genes and the microglia-specific marker AIF1 rather than the maturation of the organoid, suggesting that intrinsically grown microglia are susceptible to HIV infection, irrespective of maturation. This finding, however, highlights an important limitation of the organoid model, which is the variability between organoids from the same batch and across iPSC lines, most likely caused by the self-patterning-based development. Consequently, although OH1.5- and OH2.6-derived organoids were generated with the same protocol and under the same conditions, OH2.6-derived organoids had very limited expression of the HIV receptors and

were not susceptible to HIV infection despite the expression of microglia-specific markers (TMEM119 and P2RY12). Several groups have been able to minimize heterogeneity by substituting Matrigel for polymer-based scaffolds [74], removing Matrigel before spinning culture [75], or the use of miniaturized spinning bioreactors for organoid culture [76,77]. Alternatively, cerebral organoids can also be generated in the presence of exogenous patterning factors to generate specific brain regions with less heterogeneity than unguided protocols [78]. Nonetheless, we highly recommend researchers perform a pilot experiment with cerebral organoids derived from different iPSC lines to determine which iPSC line produces organoids susceptible to HIV infection based on the co-expression of microglia-specific markers and the major HIV receptors, CD4, CXCR4, and CCR5.

A well-known difficulty of the cerebral organoid model is the development and the ensuing limited number of microglia (~1%) within the organoids. As microglia are the main target cell for HIV in the CNS, having a suitable amount of microglia within the organoids is essential. To mitigate this, Xu et al. [79] co-cultured hPSC-derived primitive macrophage progenitors (PMPs) and primitive neural progenitor cells (NPCs) at the onset of 3D organoid formation to generate microglia-containing brain organoids. By controlling the starting number of the PMPs and NPCs, they were able to control the ratio of microglia within the organoids. Alternatively, iPSC-derived microglia [41–43], primary microglia, and the microglial cell line HMC3 [44] have been incorporated into mature cerebral organoids to overcome the lack of microglia differentiation within cerebral organoids during development [26].

In addition to the aforementioned obstacles, we caution researchers of practical limitations when using luminescence as a readout for the viral infection of Matrigel-embedded organoids. We observed that Matrigel withholds viral particles and/or luciferase protein despite generous washing post-infection to remove unbound virus and remnants. Therefore, we highly recommend the use of additional methods, such as p24 ELISA, HIV RNA transcripts, and/or a fluorescently labeled viral vector to validate HIV infection. As more protocols are replacing Matrigel, we believe this will also facilitate the use of luciferase-tagged reporter viruses in the future, although it remains important to also assess the effects of these polymer scaffolds when using a luminescence-based readout system.

The first study to demonstrate the utility of cerebral organoids within HIV research was recently reported by Dos reis and colleagues. In this study, they incorporated HIV-infected HMC3 microglial cell line or human primary microglia into a 3D human brain organoid (hBORG) model that supported virus production and exhibited an increased inflammatory response (TNF-α and IL-1β) [44]. Microglial cell lines have been reported to have large transcriptomic and phenotypic discrepancies with primary microglia, a high proliferation rate, and poor-to-no expression of microglia-specific markers (i.e., CX3CR1, P2RY12, and TMEM119) and the major HIV receptors (CD4, CXCR4 and CCR5) [25,26,80].

Therefore, microglial cell lines are limited in their use for HIV research studies. Furthermore, while infection of microglia before incorporation into the cerebral organoids greatly facilitates infectivity, this does not represent the in vivo scenario, as HIV infection of microglia takes place within the CNS in the presence of other CNS cells. Our study, on the other hand, is the first to characterize HIV infection within microglia-containing cerebral organoids. Although a more in-depth investigation of the key HIV neuropathological features of HAND (such as neuroinflammation) and neurological damage was not in the scope of this study, we observed several multinucleated GFP+ cells within the organoids that resembled the multinucleation observed in HIV-infected iPSC-derived microglia [27] and cultured primary human microglia [9,81,82] and postmortem brain tissue of HIV-infected individuals.

Altogether, despite the current obstacles of 3D cerebral organoids, the model presented in this paper accounts for several of the shortcomings of 2D monoculture models, postmortem brain tissue biopsies, and animal models currently used for HIV CNS research. Furthermore, with ongoing advancements in cerebral organoid generation and culture to mitigate these limitations, cerebral organoids will become a valuable human-representative 3D CNS culture model to advance neuroHIV research. The use of cerebral organoids within the HIV research field will require the controlled induction of the mesoderm lineage to allow microglia differentiation or incorporation of microglia within organoids to ensure the support of HIV-1 infection.

To the best of our knowledge, we are the first to report the productive HIV-1 infection of microglia-containing cerebral organoids. This model system can be used to study the impact of HIV infection on the CNS, gain a better understanding of the neuropathogenesis of HAND and HIV latency in the brain, and facilitate the testing of new therapeutic and curative strategies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/v14040829/s1, Figure S1: Viability of Organoid-derived microglia and primary microglia after HIV infection; Figure S2: Graphical representation of GFP+ distribution in primary microglia infected with HIVbalGFP. Images were taken in culture after 11 days of infection. Magnification of 10x was used; Figure S3: Graphical representation of GFP+ microglial cells within 3D cerebral organoids. (A). Double immunostainings of a 3D cerebral organoid (OH1.5) with GFP combined with IBA1 (microglia) at day 14 post-infection with HIVbalGFP. Magnification of 10x was used. (B). A zoomed-in image of Figure 3A taken at 40x magnification depicting GFP+ /IBA1+ microglia cells; Figure S4: HIV spread within 3D cerebral organoids is limited. Double immunostainings of 3D cerebral organoids (OH1.5) with GFP combined with IBA1 (microglia) at day 14 post-infection with HIVbalGFP. Magnification of 20x was used; Table S1: Antibodies used in this study for immunofluorescence; Table S2: Primer sequences used for qRT-PCR experiments.

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Institutional Review Board Statement: Fresh postmortem adult human brain tissue was provided by the Netherlands Brain Bank (NBB). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of VU Medical Center (VUMC, Amsterdam, The Netherlands). Skin biopsy samples were obtained from healthy individuals for the isolation of fibroblasts and the generation of iPSC in accordance with the Declaration of Helsinki, and the protocol was approved by the Medical Ethical Committee of the University Medical Center Utrecht (UMC, Utrecht, The Netherlands). All subjects gave their informed consent for inclusion before they participated in the study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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CHAPTER 6

6

Shock and kill within the CNS: A promising HIV eradication approach?

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The most studied HIV eradication approach is the "shock and kill" strategy, which aims to reactivate the latent reservoir by latency reversing agents (LRAs) and allowing elimination of these cells by immune-mediated clearance or viral cytopathic effects. The CNS is an anatomic compartment in which (persistent) HIV plays an important role in HIV-associated neurocognitive disorder. Restriction of the CNS by the blood–brain barrier is important for maintenance of homeostasis of the CNS microenvironment, which includes CNS-specific cell types, expression of transcription factors, and altered immune surveillance. Within the CNS predominantly myeloid cells such as microglia and perivascular macrophages are thought to be a reservoir of persistent HIV infection. Nevertheless, infection of T cells and astrocytes might also impact HIV infection in the CNS. Genetic adaptation to this microenvironment results in genetically distinct, compartmentalized viral populations with differences in transcription profiles. Because of these differences in transcription profiles, LRAs might have different effects within the CNS as compared with the periphery. Moreover, reactivation of HIV in the brain and elimination of cells within the CNS might be complex and could have detrimental con- sequences. Finally, independent of activity on latent HIV, LRAs themselves can have adverse neurologic effects. We provide an extensive overview of the current knowledge on compartmentalized (persistent) HIV infection in the CNS and on the "shock and kill" strategy. Subsequently, we reflect on the impact and promise of the "shock and kill" strategy on the elimination of persistent HIV in the CNS.

Keywords: Astrocytes • CNS • Compartmentalization • Eradication • HAND • HIV •Inflammation • Latency • Latency reversal • Microglia • Perivascular macrophages •Persistence • Reservoir • Shock and kill

Abbreviations: ART, antiretroviral therapy; BAF, BRG1/BRM-associated factor; BBB, blood–brain barrier; BET, bromodomain and extra terminal domain; C/EBP, CCAAT/enhancer binding protein; COUP, chicken ovalbumin upstream promotor transcription factor; CRF, circulating recombinant form; CSF, cerebrospinal fluid; CTIP2, COUP-TF interacting protein; DSIF, DRB-Sensitivity Inducing Factor; HAD, HIV-associated dementia; HAND, HIV-associated neurocognitive disorder; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HIC1, hypermethylated in cancer 1; HMAG1, high mobility group AT-hook 1; HMT, histone methyltransferase; HP1, heterochromatin protein 1; iPSC, induced pluripotent stem cell; LRA, latency reversing agent; LSD1, lysine specific demethylase; LTR, long terminal repeat; NELF, negative elongation factor; NFL, neurofilament light chain; NIK, NF-kB inducing kinase; NR4A2, nerve growth factor IB-like nuclear receptor Nurr1; PET, positron emission tomography; PKC, protein kinase C; P-TEFb, positive transcription elongation factor; PWH, people with HIV; RNAPII, RNA polymerase II; SMAC, second mitochondria-derived activator of caspases; SP, SV40-promoter specific factor; TAR, transactivation response element; Tat, transactivator protein; TF, transcription factor; TRBP, TARRNA binding proteins.

Abstract

Introduction

HIV-1, referred to as HIV from this point, is the major cause of AIDS. HIV is one of the major global health challenges, with approximately 38 million people infected.[1, 2] Despite the success of antiretroviral therapy (ART) at suppressing viral replication and reducing AIDS-related morbidity and mortality, HIV cure remains elusive due to the presence of latently infected cells and subsequent rebound viremia after ART interruption or cessation.[3] Consequently, permanently eliminating the replication competent virus without the need for lifelong therapy is the ultimate goal for HIV cure.

HIV can reside in anatomic compartments including the CNS.[4, 5] HIV RNA and/or DNA have been detected in the cerebrospinal fluid (CSF)[6-11] and postmortem CNS (myeloid)-resident cells, such as microglia and perivascular macrophages, in both untreated and virally suppressed individuals.[12-15] The CNS is an immune-restricted anatomic compartment shielded from the periphery by the blood–brain barrier (BBB) with a unique microenvironment consisting of CNS-specific cell types, transcription factors (TFs), and immune surveillance.[5, 16, 17] The adaption and isolated replication of HIV in the CNS, along with the poor penetration of ART across the BBB, give rise to genetically distinct HIV CNS populations, called compartmentalization in both ART naïve and treated individuals.[10, 11, 18, 19]

It is likely that HIV can persist in the brain and has the potential to cause a rebound of viremia upon ART cessation. Therefore, to achieve cure, HIV also needs to be eliminated from the CNS. Multiple strategies are currently designed that aim to eradicate the persistent HIV reservoir.[20] The most studied approach is the "shock and kill" strategy, aimed to reactivate ("shock") the latent reservoir by latency reversing agents (LRAs), which will be subsequently cleared ("kill") by the immune system or by virus-induced cytopathogenicity.[21] Because of the BBB and the presence of genetically diverse compartmentalized viral populations with differences in HIV transcription profiles, reactivation of HIV in the brain by LRAs might be complex, occur with different effectivity or have detrimental consequences on brain functioning.[22-24] In this review, we will provide an extensive overview of (persistent) HIV infection in the CNS and the current knowledge on the "shock and kill" strategy. Subsequently, we will reflect on the impact and promise of the "shock and kill" strategy on the elimination of persistent HIV in the CNS.

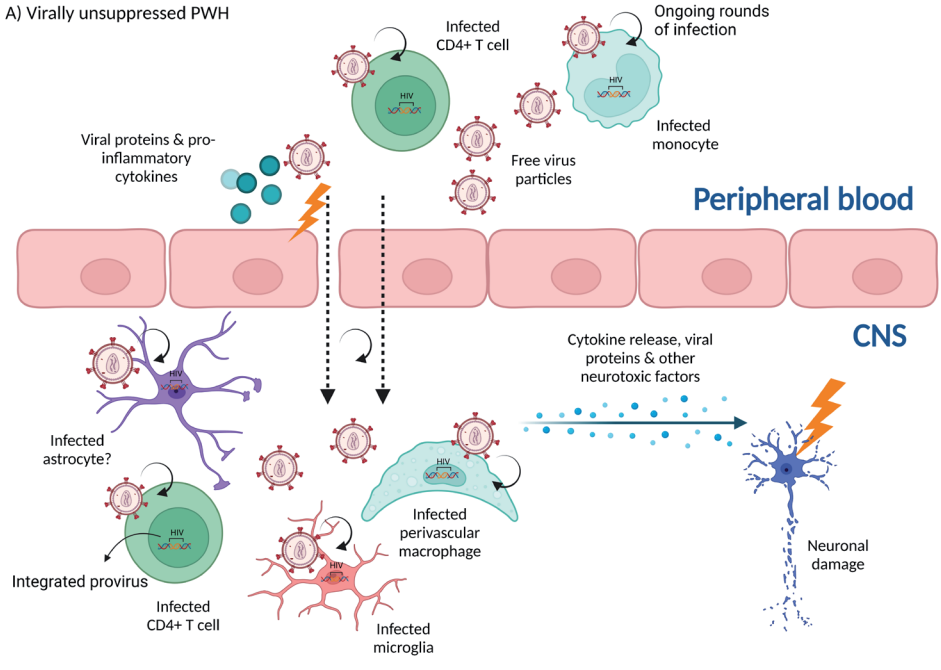
HIV infection and persistence in the CNS

HIV entry in the CNS

HIV causes infection of predominantly $CD4^+$ T cells in lymph nodes at site of transmission, as reviewed earlier.[25] A few days after initial infection, these infected $CD4^+$ T cells start

circulating throughout the body. Particularly high numbers of HIV-infected cells are present in the GALT, contributing to a systemic peak viremia in the first weeks after initial infection.[26] During this systemic peak of viremia, HIV can spread to various other tissues and anatomic compartments,[27, 28] including the CNS.[4, 29]

Exposure of the BBB to the HIV envelope and/or the HIV extracellular transactivator protein (Tat) in the periphery may cause increased permeability of the endothelial cell layer, a down-regulation of the tight junction proteins, all contributing to the penetration of HIV into the CNS.[30-33] Moreover, proinflammatory cytokines and chemokines are secreted in the periphery and in the CNS during HIV infection, making the BBB more permeable[34] (Figure 1A). The most widely accepted mechanisms for the entry of HIV in the CNS are migration of either circulating cell-free virus, or trafficking of HIV-infected CD4$^+$ T cells, and to a lesser extent, infected monocytes crossing the BBB into the CNS[35-38] (Figure 1A). HIV RNA and inflammatory markers can already be detected in the CSF within the first weeks after infection.[6-10] Generally, the viruses in CSF during this early stage of infection are largely derived from blood, which is termed a *noncompartmentalized* or *equilibrated* CSF infection.[39] In line with this observation, it was shown that infected CD4$^+$ T cells could be detected in the CNS of SIV-infected rhesus macaques already 12 days post infection, whereas the number of infected monocytes was limited.[40] These observations are suggestive for migration of infected CD4$^+$ T cells to be the primary mechanism for HIV entry in the brain.



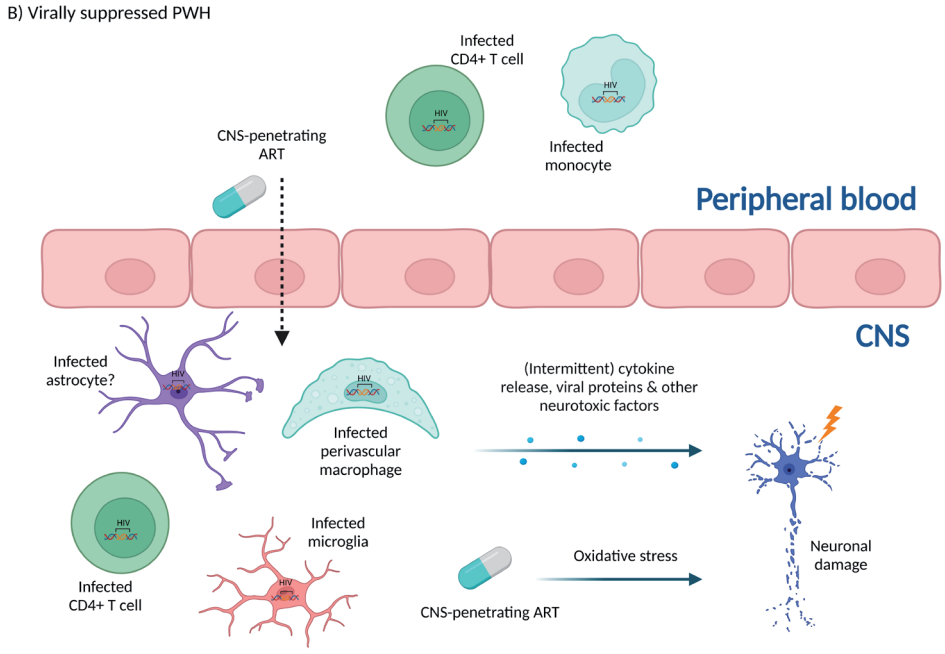


Figure 1 Overview of neuropathogenesis in virally unsuppressed and suppressed PWH. (A) Virally unsuppressed PWH: The exposure of viral proteins and inflammatory cytokines may cause increased permeability of the blood–brain barrier. This can contribute to the entrance of HIV within the CNS via free virus particles, infected CD4+ T cells, or infected monocytes. Subsequently, cells in the CNS can be infected. Ongoing rounds of viral infection occur, within the periphery and the CNS. Consequently, there can be a continuous influx of peripheral virus into the CNS. The presence of virus and viral proteins, release of cytokines and neurotoxic factors might cause neuronal damage and contribute to the development of HIV-associated neurocognitive disorder (HAND). **(B) Virally suppressed PWH:** In suppressed PWH, CNS-penetrating ART can pass the blood–brain barrier. These might cause oxidative stress, which might contribute to neuronal damage in these individuals. Cells in the periphery and CNS can be infected from before the start of therapy, but there are no rounds of ongoing infection. Nevertheless, these infected cells can still produce virus, cytokines, or can be intermittently activated, possibly also contributing to neuronal injury. Created with Biorender.com.

HIV neuropathogenesis

HIV infection can lead to an impairment of neurocognitive function, resulting in HIV-associated neurocognitive disorders (HAND). Because of the large variations seen in clinical symptoms and their severity, HAND is categorized in: asymptomatic neurocognitive impairment, mild neurocognitive disorder, and HIV-associated dementia (HAD).[1] Before introduction of ART, HAND was commonly seen in up to 70% of all patients with AIDS or symptomatic HIV infection[41] of whom 20–30% developed HAD.[42] Within the ART era, HAND is still seen in up to 42% of individuals,[43] but luckily HAD has significantly decreased.[41]

The development of HAND is characterized by pathologic neuronal degradation. Neuroimaging via MRI and computed tomography show atrophy of cerebral regions.[44, 45] Moreover, already soon after infection elevated levels of the biomarker neurofilament light chain (NFL), associated with neuronal injury, can be measured in the CSF.[46] The presence of neuronal loss implies that HIV infection either directly or indirectly leads to neuronal death. Infected cells release viral proteins such as gp120 and Tat, which are neurotoxic and promote neurodegeneration,[47-49] (Figure 1A). Furthermore, CNS immune activation will occur resulting in additional neurotoxicity via neuroinflammation and the release of proinflammatory cytokines, which are also neurotoxic,[9, 50-53] (Figure 1A). Apart from viral factors, HAND was also shown to be increased by the presence of systemic inflammation and the presence of common comorbid conditions such as cardiovascular disease, chronic lung disease and diabetes.[54]

It has been shown that initiation of ART causes a reduction in inflammatory markers in the CSF and brain,[52, 55-57] and reduces but does not completely reverse neuronal injury.[58] Also the incidence of HAND in HIV-suppressed individuals remains high, albeit its severity decreases.[41] This might be caused by ART-induced oxidative stress in neurons, as reviewed in Brew *et al.* and Ghosh *et al.*,[47, 59] (Figure 1B). Furthermore, it is interesting to note that presence of infected cells in the CSF is related to neurocognitive disorders.[60] Moreover, some levels of inflammation markers in CSF were correlated to neurocognitive performance and others not.[55, 60] Also low levels of HIV RNA in CSF most likely caused by viral production from activated cells may contribute to HAND,[60-62] (Figure 1B). Interestingly, activation of cells in the CNS was observed in virally suppressed individuals who underwent a positron emission tomography (PET) scan, of which the activation did correlate to neurocognitive performance.[63] However, it was also shown that not in all individuals experiencing HAND, viral RNA could be detected in the CNS.[62] This could be a timing or stochastic issue due to limitations in frequent, longitudinal sampling of CSF. In some cases, HAND may be caused by ongoing viral replication due to insufficient drug penetration in the CNS, (Figure 1B), as will be discussed in more detail in Section **2.3.2**.

The CNS as a persistent (latent) reservoir of HIV

In order to consider an anatomic compartment as a biologically relevant HIV reservoir, it must fulfill several criteria. First, HIV DNA needs to be present in cells with a long lifespan or which undergo (homeostatic) proliferation. Second, the infected cells should be able to produce new replication-competent viral particles. At last, cells should have mechanisms to suppress viral replication and enable latent infection.[64, 65] There is evidence that the CNS may serve as a (latent) anatomic reservoir of HIV,[4, 5] although the strength of these lines of evidence varies. In the coming paragraphs we will discuss HIV CNS infection, compartmentalization and reservoir formation.

HIV infection and compartmentalization in virally unsuppressed individuals

As indicated earlier in this review, early stages of infection of the brain are characterized by the entry of CD4$^+$ *T cell-tropic* viral variants. Once in the CNS, HIV is exposed to a completely different microenvironment, which also includes brain-specific HIV target cells.[29] As a result, compartmentalized HIV populations, genetically distinct from viral populations replicating in the periphery, can be detected in the first months after infection in the CSF of untreated people with HIV (PWH).[8, 10] The compartmentalization of viral quasispecies in the CSF is preserved throughout infection.[10, 66] The early initiation of ART was shown to limit but not exclude compartmentalization in the CSF.[56] Genetic characterization of the viral quasispecies is largely based on phylogenetic analysis of the highly variable HIV envelope glycoprotein gp120. This viral envelope protein is required for cell entry and determines affinity for the main CD4 receptor and the coreceptors CCR5 and/or CXCR4.[67] Viruses found in the CSF can evolve from R5 *T cell-tropic* viruses,[68] which require expression of the CCR5 coreceptor and high surface density of the CD4 receptor, to Macrophage-tropic (*M-tropic*) viruses, which also require the CCR5 coreceptor, but a low surface CD4 receptor density. As both of these receptors are expressed on CD4$^+$ T cells, both viruses can efficiently infect these cells. *M-tropic* viruses can also infect cells from the macrophage-monocyte lineage, which are also present in the CNS, since these cells express CCR5 but have low CD4 surface expression.[69-72] In vitro adaptation to *M-Tropic* variants demonstrated a lower functionality of the viral envelope gene, with reduced particle infectivity and prolonged entry transitions compared with *T cell-tropic* variants,[73] whereas in vivo characterization of the *M-Tropic* variants only showed increased susceptibility to soluble CD4.[69] Adaptation to *M-Tropic* variants might only occur in immune-privileged sites, however future studies are needed to elucidate the differences between *M-Tropic* and *T cell-tropic* variants.

In the CSF of ART naïve PWH, mostly compartmentalized *R5 T cell-tropic* variants were detected during the first 2 years of infection, after which evolution toward *M-tropic* viruses was observed.[10] In the CSF of unsuppressed individuals in more advanced stages of infection, a mixture of both R5 *T cell-tropic* viral variants and *M-tropic* compartmentalized viral variants could be detected.[68] Moreover, viral isolates obtained from brain autopsies of individuals with severe neurologic symptoms also represented a mixture of *M-tropic and T cell-tropic* viruses.[70] It was observed that the *M-tropic* variants in CSF display a higher genetic diversity and decay slower after start of ART than R5 *T cell-tropic* viral quasispecies.[68] Consequently, it is hypothesized that *M-tropic* quasispecies represent infection of the relatively long-lived CNS-resident cells, whereas the R5 *T cell-tropic* variants represent clonal expansion and possibly an influx from peripheral cells responding to immune activation.[10, 68] This suggests that the CSF is an intermediate compartment in which the periphery, as well as the CNS, contribute to the viral population.[66] Moreover, when comparing drug resistance mutations patterns of viral sequences from paired CSF and plasma samples, independent evolution of the viral quasispecies in the CSF was

observed.[74, 75] Altogether, providing evidence that the CNS serves as an independent site of viral replication in CNS-specific target cells resulting in viral compartmentalization.

HIV infection and compartmentalization in virologically plasma-suppressed individuals
In the large majority of HIV-infected individuals on ART, viral replication is suppressed and the levels of HIV RNA in the CSF are below the level of detection. However, in some individuals with an undetectable plasma viral load, HIV RNA can be detected in the CSF, a phenomenon referred to as CSF escape.[61, 76-79] A minority of the PWH experiencing CSF escape show persistent levels of elevated CSF viremia with undetectable plasma levels in longitudinal studies, which does suggest that persistent viral production or independent replication occurs within the CSF.[11, 80, 81] This might be due to poor drug penetration over the BBB resulting in a limited potential to inhibit viral replication in the CNS, as reviewed before.[82] In line with this explanation, it has been shown that usage of better CNS-penetrating ART reduces CSF escape.[83]

Analysis of the CSF viral escape populations showed genetically diverse viral quasispecies in some individuals.[11, 84] In 1 individual, also the selection of resistance mutations was observed.[11] It is highly unlikely, because of the suppression of HIV replication in the periphery in these individuals, and the association of CSF escape with selection of drug resistance, that these were derived from trafficking peripheral cells.[79] At last, analysis of the CSF viral escape populations showed that they largely consisted of *T cell-tropic* variants,[11, 84, 85] but within 1 individual also *M-tropic* variants were observed at consecutive time points.[11] Given the fact that *M-tropic* HIV viral strains are hardly observed within the periphery, it is likely that these M-tropic viruses originate from the CNS.[79] Generally, this supports the idea that the viral escape as observed in the CSF of these PWH on ART is the result of ongoing viral replication in CNS-resident cells in ART-suppressed individuals.[11, 79]

Apart from persistence of HIV in the CSF as a result of ongoing viral replication due to poorly penetrating antiretroviral drugs, viral latency might also be a mechanism of HIV persistence in the CSF.[64, 65] Latently infected cells are classically defined as cells that carry integrated HIV DNA and are transcriptionally silent, but upon activation produce replication competent virus causing a rebound in viremia when ART is stopped.[2] Their persistence is controlled by the half-life of the infected cells, their ability to proliferate and the epigenetic context of the integrated proviral genome.[2] It is also important to note that the current arsenal of ART cannot prevent viral production from activated cells. It is known that latent cells in the periphery can be activated resulting in viral production, and subsequently can revert back to latency.[86] Interestingly, the viral Tat protein can also be observed in the CSF of ART-suppressed individuals, indicating viral production.[78] However, it is difficult to discriminate viral production from ongoing (low-level) viral replication. In the majority of cases, viral RNA in CSF was detected just once (viral blip) indicative of viral production rather than ongoing

viral replication.[11, 80, 81] Consequently, the presence of viral RNA and Tat might be the result of viral production from intermittently activated latent cells.

HIV reservoir analysis in CNS tissue of unsuppressed and suppressed PWH

Apart from studying viral RNA in the CSF, CNS tissue of PWH can be studied to get insight in viral reservoir formation. Multiple studies have found compartmentalized HIV DNA sequences in CNS tissue as compared with peripheral tissues in virally unsuppressed individuals.[19, 87-90] Moreover, HIV DNA has been detected within CNS-resident cells in postmortem brain material of HIV-unsuppressed individuals.[12-14, 91-93] One study reported that the proviral genome sequences isolated from myeloid cells in the CNS were intact, suggestive of replication-competent virus.[93] This provides evidence that HIV replication occurs within the brain-specific myeloid cells in unsuppressed PWH.

However, in order to gain more insight into latency of HIV in the CNS, tissue of suppressed individuals needs to be studied. Within a study using single-molecule real-time sequencing of HIV isolates in the brain and lymphoid tissues of a virally suppressed individual 7 months before death, compartmentalization of HIV in postmortem brain tissue has been observed.[18] Interestingly, phylogenetic analysis also incidentally showed viral strain compartmentalization between different brain regions, indicating that different compartments exist in the CNS.[18, 87] Furthermore, also in postmortem brain tissue of suppressed individuals, HIV DNA was found in CNS-resident cells, and was reported to be intact.[93] Interestingly, in some of these resident CNS cells, no viral RNA was detected, suggestive for viral latency. Another study showed that directly after cessation of ART, compartmentalized viral populations in the CSF were detected, which were highly distinct from the viral populations present in the paired blood samples.[95] All in all, these data are indicating that the CNS may serve as a viral reservoir. Due to the limited availability of HIV-infected human brain material and paired CSF and plasma samples, further evidence regarding HIV latency in CNS human brain material is still lacking.

HIV cellular reservoirs in the CNS

The data above are highly suggestive for the CNS to be a persistent HIV reservoir according to the criteria discussed in Section **2.3**.[65, 66] Of brain-specific cells, neurons and oligodendrocytes are presumed to be overall resistant to HIV infection and thus do not meet the criteria for an HIV reservoir.[96] Astrocytes are long lived resident innate immune cells of the CNS that also have the ability to proliferate.[59, 97] HIV DNA has been detected in astrocytes in virally unsuppressed PWH,[12-14, 92] but not in virally suppressed individuals.[15] Astrocytes were shown to be susceptible to HIV infection in vitro,[98, 99] however HIV infection was nonproductive.[14, 100, 101] Due to the lack of CD4 surface expression on astrocytes, viral infection or transmission may occur via cell-to-cell contact, receptor-mediated endocytosis or via engulfment of neuronal debris or infected cells.[102-104105] Therefore, astrocytes are not

considered to be a true cellular reservoir of HIV (Figure 1). Nevertheless, it is suggested that they may contribute to HIV-related cell injury, via the production of astrogliosis.[1]

Multiple myeloid cell populations are present in the CNS,[105] and HIV DNA has been detected in microglia and perivascular macrophages in postmortem brain material of both ART-treated and virally unsuppressed PWH.[12, 13, 15, 64, 91, 92] Microglia are tissue-resident macrophages and are part of the human innate immune system. Microglia are able to repopulate themselves throughout life and are thought to have a lifespan of approximately 4 year, whereas perivascular macrophages have a lifespan of months and need replenishment from the bone morrow.[64, 106] Microglia and perivascular macrophages express low levels of CD4, CCR5, and CXCR4 as compared with $CD4^+$ T cells.[107] Therefore, it is likely that HIV DNA detected in the postmortem brains is derived from infection with compartmentalized *M-tropic* viruses. However, HIV reverse transcription has been shown to be inhibited via the expression of the restriction factor SAM domain and HD domain-containing protein 1 in cells of the myeloid lineage.[108] Nevertheless, human primary microglia cultures and in vitro microglial culture models have shown productive HIV infection with *M-tropic* viruses via the CD4 and CCR5 receptor, but did not support infection with *T-tropic* viruses.[107, 109, 110] Moreover, studies with SIV in rhesus macaques and HIV-infected humanized immunodeficient mice show productive infection and suspected latency of replication competent virus in microglia and perivascular macrophages during ART suppression as shown by increase in viral load upon cell stimulation.[23, 111-113] Altogether, based on the cellular characteristics of microglia, the mounting evidence of susceptibility to productive HIV infection, and the presence of HIV DNA, microglia and to a lesser extent perivascular macrophages are thought to be the main HIV reservoir in the brain (Figure 1).

The peripheral $CD4^+$ T cells are the most studied biologic relevant HIV reservoir, in which productive infection and latency is observed.[25] Because of the presence of $CD4^+$ T cells in CSF due to trafficking and tissue-resident $CD4^+$ T cells in the human brain parenchyma,[114] it is likely that $CD4^+$ T cells provide a cellular reservoir for HIV, (Figure 1). However, direct identification of $CD4^+$-infected T cells in the CNS is challenging because of their low frequency in the CSF and the brain.[29] Nevertheless, sequencing envelope genes of viral isolates from CSF and postmortem brain tissues showed compartmentalization of both *M tropic* and *T cell-tropic* viruses.[11, 18, 68] Specifically, the presence of CD26 on virion surfaces derived from individuals with CSF escape was observed, which has been shown to be an indication of *T cell-tropic* virus. Also, virions with CD36 on their surface, as a marker of M-tropic virus, have been observed in these individuals.[85] Altogether, this supports the idea that infected $CD4^+$ T cells are present in the CNS, which implies that also latency in $CD4^+$ T cells might occur in the CNS reservoir.

Culture models for HIV CNS persistence studies

In order to get more insight into the details of HIV persistence and latency in the CNS, in vitro model systems are being used. As microglia are thought to be the main cellular HIV reservoir, in vitro studies of HIV infection in the CNS have been performed on myeloid cell model systems, such as the infected monocyte-derived macrophages.[22, 115] However, the translation of these results toward the CNS is limited, because of the distinct phenotype of microglia compared with macrophages.[115]

Therefore, current in vitro studies of HIV infection in the CNS are mostly done on microglial culture models, as reviewed earlier.[110, 116] Human primary microglia cultures and monocyte-derived microglia are highly susceptible to infection, showing continuous viral production up to 1 month postinfection.[110, 116-118] However, this is not representative of the small population of focally distributed infected microglial cells (1–10%) in the brain,[13, 15, 91, 92] which suggests that important restriction factors might be down-regulated in culture or that infection is greatly affected by surrounding CNS cells and the CNS environment in vivo. In vitro studies on human primary astrocyte cultures consistently report a low (nonproductive) HIV infection through CD4-independent mechanisms.[102-104] Although this low infectivity (1–3%) is reminiscent of the small infected population in the brain, it is still unclear whether and how astrocytes become infected in vivo in the absence of the CD4 receptor. Long-term culture (>120 days) of primary microglia and astrocytes have led to viral latency suggesting that these primary cells retained at least some of the genes and/or restriction factors required for the induction of latency in vivo,[104, 117] such as the proapoptotic protein Bim, which surprisingly was up-regulated in latently infected macrophages.[117] However, the technical challenges of obtaining fresh brain tissue and the limited number of cells postisolation complicates the use of primary cells for down-stream HIV (latency) analysis. To circumvent this, primary microglia have been immortalized to generate microglial cell lines such as HMC3, SV40 and Hμglia.[107, 119] We do not recommend the use of these cell lines for HIV infection studies as there is no to limited support of HIV infection and large transcriptomic discrepancies with primary microglia.[107, 110, 116, 120] Alternatively, latently HIV-infected clones derived from the hμglia cell line show great promise as a model for the initial assessment of HIV latency reversal in microglia, although future studies will need to improve on the high levels of spontaneous progressive HIV reactivation observed in order to better recapitulate the suspected in vivo scenario.[121, 122]

Recent technologic advancements in stem cell research have enabled the generation of a variety of CNS cell types including cerebral organoids and opened unique opportunities to study host-virus interactions in the CNS.[123] HIV infection of induced pluripotent stem cell (iPSC)-derived microglia and organoid-derived microglia, decreased after the 1st week of infection, resembling HIV infection in vivo.[109, 110, 116] 2D coculture models incorporating iPSC-derived microglia with iPSC-derived astrocytes and/or iPSC-derived neurons and

3D cerebral organoids can be used to address the true cellular targets of HIV in the CNS and the role of surrounding CNS cells in supporting infection and the development of neural injury.[109, 124, 125] Furthermore, as iPSC-derived CNS cells and cerebral organoids can be maintained in culture for long periods of time (months to years), it will be interesting to investigate whether these cells revert to a latent state postinfection making them suitable models to study HIV persistence and latency in the CNS within a CNS-like environment.[109, 126] To conclude, several promising in vitro model systems are available to study HIV persistence and latency. However, it should be kept in mind that HIV CNS in vitro models can only partially recapitulate HIV persistence and latency in vivo and are a simplification of the complexity of the human brain. Therefore, caution should be used when translating findings to the in vivo scenario.

HIV transcription and latency in the CNS

Regulation of (general) HIV transcription and latency

Multiple mechanisms are known to regulate transcriptional silencing of integrated HIV, which have been extensively reviewed,[127, 128] (Figure 2A). During latency, repressors of TFs and repressive epigenetic marks around the HIV integration site and long terminal repeat (LTR) inhibit transcription. The negative elongation factor (NELF) and DRB-sensitivity inducing factor (DSIF) cause pausing of the RNA polymerase II (RNAPII) on the LTR. In addition, latent cells are characterized by low levels of Tat. If levels of Tat are low, only low-level basal transcription of viral genes occurs and RNAPII is interrupted after the synthesis of short transcripts (±80 base pairs) that include the transactivation response element (TAR).[127, 128] However, for productive infection these factors inhibiting HIV transcription need to be removed. Upon activation of the latent cells, Tat levels will increase.

Tat binds to the TAR element and recruits the P-TEFb complex (positive transcription elongation factor) to the LTR. The RNAPII carboxyterminal domain and DSIF are phosphorylated, causing dissociation of NELF, which enables the efficient transcription of the HIV genome. Moreover, Tat recruits several factors that are needed for the inhibition of the repressive (epigenetic) transcription mechanisms and efficient elongation of HIV transcription.[127, 128]

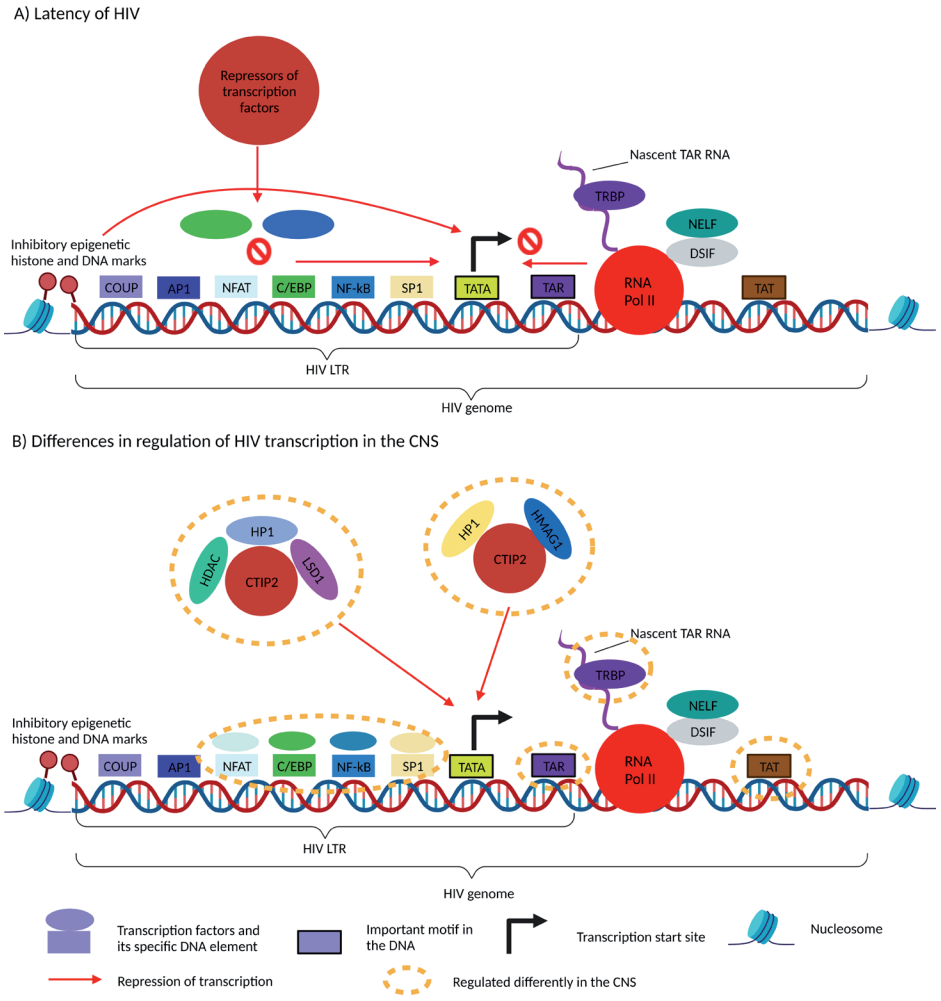


Figure 2. HIV latency regulation in periphery and its differences within the CNS. (A) Latency of HIV: The presence of transcription repression factors and inhibitory epigenetic around the HIV integration site and LTR prevent the transcription of HIV. Tat is not transcribed, which is needed for full-length HIV transcription. **(B) Differences in regulation of HIV transcription in the CNS:** Within the CNS multiple factors within transcription regulation are differently regulated compared with the general situation in the periphery. Factors of which it is reported that they are altered in the CNS are outlined with a dashed orange line. Polymorphisms in the LTR and the Tat gene cause an altered binding of transcription factors and a different function of Tat. Moreover, within brain target cells, unique isoforms and levels of C/EBP, SP1, SP3, COUP, and TRBP alter their transcriptional activity. At last, within brain target cells, increased levels of transcription repression factors are observed, which repress transcription by blocking TF binding sites or establishing epigenetic modifications. *Created with Biorender.com. HDAC, histone deacetylase; CTIP2, COUP-TF interacting protein; HIC1, hypermethylated in Cancer 1; LSD1, lysine specific demethylase; HP1, heterochromatin protein 1; HMAG1, high mobility group AT-hook 1; AP1, activator protein 1; C/EBP, CCAAT enhancer binding protein; COUP, chicken ovalbumin upstream promotor; SP1, specificity protein 1; LTR, long terminal repeat; RNA Pol II, RNA Polymerase II; TF, transcription factor; Tat, transactivator of transcription; TAR, transactivation response element; TRPB, TAR binding proteins; NELF, Negative Elongation Factor; DSIF, DRB-sensitivity inducing factor*

HIV transcription in the CNS

Polymorphisms in HIV LTR of CNS strains isolated from autopsy tissue of virally unsuppressed PWH are reported to have considerable consequences on the latency and transcription of HIV as shown during in vitro infection in astrocyte and T cell lines.[17, 24, 129] Generally, based on sequence analyses these LTR polymorphisms were expected to result in an altered binding of multiple TFs such as CCAAT/enhancer binding protein (C/EBP),[130, 131] NFAT,[132] NF-kB,[132] or SV40-promoter specific factor (SP).[24, 132] One study reported that these CNS-derived LTR sequences resulted in reduced basal transcription in T cells and astrocytes compared with HIV populations present in the periphery.[129] Binding of these TFs to the LTR is essential for initiation of LTR-dependent transcription and synthesis of Tat protein. Viral sequences obtained from the CNS of PWH without suppressed ART indicate also sequence variations in the Tat gene.[133-135] Overall, these sequences did not change the ability of Tat to transactivate the HIV LTR in glial, astrocyte, monocyte, and T cell lines, although this was the case in material obtained from some individuals,[133] (Figure 2B) and (Table 1).

TABLE 1. Differences of transcription regulation within the CNS compared with the periphery. Polymorphisms within the LTR of CNS viral populations, unique (expression of) transcription factors and a different epigenetic regulation within CNS cells might have an influence on the regulation of transcription and the induction of latency within the CNS. *LTR, long terminal repeat*

Change of transcription mechanism in the CNS	What effect?	Reference
CCAAT/enhancer binding protein (C/EBP)	Altered binding of C/EBP to LTR	(130,131)
NFAT	Altered binding of NFAT to LTR	(132)
NF-kB	Altered binding of NF-κβ to LTR	(132)
SV40-promoter specific factor (SP)	Altered binding of SP to LTR	(24,132)
Transactivator protein (Tat) gene	No change on reactivation	(133,134)
Unique transcription factors		
Unique interactions of C/EBP, COUP, SP1 and SP3	Inhibition of HIV transcription in glial cells	(139,140)
Dexamethasone activates the glucocorticoid receptor	Repression of HIV transcription in microglia	(121)
Nerve Growth Factor IB-like nuclear receptor Nurr1 (NR4A2)	Silencing of HIV in microglia	(141)
Distinct levels of isoforms of SP	Restricted HIV transcription in astrocytes	(17)
Low levels of TAR RNA binding proteins (TRBP)	Restricted HIV transcription in astrocytes	(142)
Epigenetic regulation		
Increased levels of COUP-TF interacting protein (CTIP2), histone deacetlyase (HDAC) and heterochromatin protein 1 (HP1)	Differently regulated transcription in microglia	(94)

Additionally, it has been shown that cultured primary microglia and astrocytes produce unique TFs with a distinct working mechanism, compared with other cells.[17, 136, 137]

First, HIV transcription in the CNS is majorly dependent on C/EBP TFs in the cells of the myeloid cell-line lineages.[138] Second, in in vitro glial cell-lines transcription inhibitors C/EBPγ, a truncated form of C/EBP, chicken ovalbumin upstream promotor TF (COUP), SP1 and SP3 show unique interactions with the LTR and can inhibit HIV transcription.[139, 140] Moreover, dexamethasone activates the glucocorticoid receptor and represses HIV transcription in in vitro immortalized microglia.[121] Similarly, the Nerve Growth Factor IB-like nuclear receptor Nurr1(NR4A2) led to silencing of HIV by binding to the HIV LTR.[141] Astrocyte cell lines express distinct levels of isoforms of SP resulting in restricted transcription[17] and express low levels of TAR RNA binding proteins (TRBP),[142] important for Tat-dependent HIV transcription. It has been suggested that in astrocyte and glial cell lines HIV transcription occurs in the absence of TAR[143, 144] and is more dependent on Tat binding to NF-kB,[145] (Figure 2B) and (Table 1).

Transcription is also regulated via epigenetic regulation in multiple brain cell types, such as microglia and astrocytes.[128, 146] COUP-TF interacting protein (CTIP2), also known as BCL11b, is an important regulator in the epigenetics of HIV transcription and increased levels of CTIP2 are seen in microglial cells and contribute to differently regulated transcription.[64, 94] An extensive explanation of the role of CTIP2 within HIV latency has been previously reviewed.[147] At first, CTIP2 serves as a platform to anchor several protein complexes having different functions, which together disfavor viral reactivation.[147, 148] One complex of lysine specific demethylase (LSD1), histone deacetylases (HDACs) and heterochromatin protein 1 (HP1) induces the formation of heterochromatin.[149, 150] Another complex with hypermethylated in cancer 1 (HIC1) and high mobility group AT-hook 1 (HMAG1) with CTIP2 represses HIV transcription via the inhibition of TEFb,[150, 151] (Figure 2B). Interestingly, in infected cells obtained from postmortem brain tissues of PWH with and without ART therapy, an increase in CTIP2, HDAC, and HP1 levels was observed,[94] (Table 1). Second, CTIP2 regulates multiple cellular genes important in HIV transcription via TF p21 and thus indirectly inhibits transcription.[147] However, in microglia cell lines it is seen that this is counteracted via HIV viral protein R.[152]

Altogether, these studies suggest that there might be a differentially regulated transcriptional activity and latency induction for HIV CNS viral populations as compared with those seen in peripheral blood. This can be caused by polymorphisms, especially in the LTR and its transcription bindings sites, and the expression of proteins, such as TFs, Tat and epigenetic modifiers. These differences might be the consequence of the selection pressure and different microenvironment in the CNS, leading to the compartmentalization of the CNS. These differences in transcription mechanisms may also contribute to differences in the establishment and maintenance of viral reservoirs observed in CNS and periphery (Figure 2).

Notably, these findings were obtained from studies performed mainly on in vitro cell lines and a few on cultured primary cells. As discussed before in Section **2.5**, the translation of the results in these model systems should be done with caution, regarding their differences with the human in vivo situation. Moreover, most of the viruses isolated from human brain tissue to study these transcription mechanisms were derived from PWH without suppressive therapy, which might be not identical or comparable to the regulation of latency and transcription in PWH on successful therapy.

Shock and kill eradication strategy

Theoretical approach

The persistence of latent reservoirs is in general the major obstacle to HIV cure. The immune systems fails to detect the presence of transcriptional silent latently infected cells, limiting recognition for elimination by immune-mediated clearance or direct viral cell-lysis by viral production. Theoretically, reactivation of HIV with LRAs by targeting the latency mechanisms ("shock") will lead to the synthesis of HIV RNA and viral protein production. Subsequently, these reactivated cells are ultimately recognized and killed ("kill") by the host immune defense mechanisms or viral cytolysis.[21, 153] This potential cure strategy is known as "shock and kill" and is performed in combination with ART.[21]

Classes of LRAs

LRAs can be classified into different classes, based on their mechanism of action, shown in (Figure **3**). First, epigenetic modifiers reverse the repressing epigenetic marks around the integrated provirus, which influence the transcription of HIV. The most studied ones are the histone methyltransferase (HMT) inhibitors and the HDAC inhibitors (HDACis). These agents reverse the repressing epigenetic acetyl and methyl marks in the integrated HIV-genome, its surrounding genome, and the associated histone tails in nucleosomes.[21, 153] The BRG1/BRM-associated factor (BAF) inhibitors modulate the histone position of the nucleosome of the integrated HIV DNA and facilitate thereby the transcription of the HIV genome. Second, the intracellular signaling modulators include drugs that regulate the protein kinases in signaling pathways modulating the TFs binding to the LTR such as protein kinase C (PKC) agonists and compounds within the PI3K/Akt pathway or JAK/STAT pathway.[21, 153] Also second mitochondria-derived activator of caspases (SMAC) mimetics can be used, which inhibit the degradation of NF-kB inducing kinase (NIK), allowing for the accumulation of NF-kB.[154] Another class of LRAs are the cytokine or immune receptor agonists, which stimulate the immune cell by using ILs or cytokines, TCR, checkpoint inhibitors, or the TLR agonists. After transcription initiation, transcription elongation factors can be used to promote the activity of Tat, important for the elongation of the transcription.[153, 155] Important examples are the bromodomain and extra terminal domain (BET) inhibitors, which antagonize the inhibitor

of P-TEFb and consequently activate the recruitment of P-TEFb to the LTR.[21, 153] Finally, a class of unclassified LRAs includes previously used drugs, which were found to reactivate HIV. However, their working mechanism for reactivating HIV is still unknown. Antioxidants and phosphatases are examples of these drugs that induced HIV latency reversal.[153] Examples of drugs of each class are listed in (Table **2**) and in other reviews.[153, 156, 157]

Many compounds in these classes have been studied in vitro, but only a few of them are studied in clinical trials. In most of these studies an increase in HIV transcription was observed, but without a reduction in HIV DNA.[155] Combinations of LRAs with different working mechanism may function in a synergistic manner to reactivate HIV and consequently increase the transcription of HIV.[157] Nevertheless, because of a decreased susceptibility of HIV-infected cells to apoptosis, direct inhibition of the immune cells by LRAs, immune escape mutations, issues with penetration of LRAs within tissues, decreased potency of some LRAs, and immune dysfunction in PWH, it is unlikely that reactivation alone is enough to completely eradicate latently infected cells.[21] Therefore, stimulation of immune-based elimination and apoptotic pathways combined with LRAs will be needed to eradicate HIV-infected cells.[158]

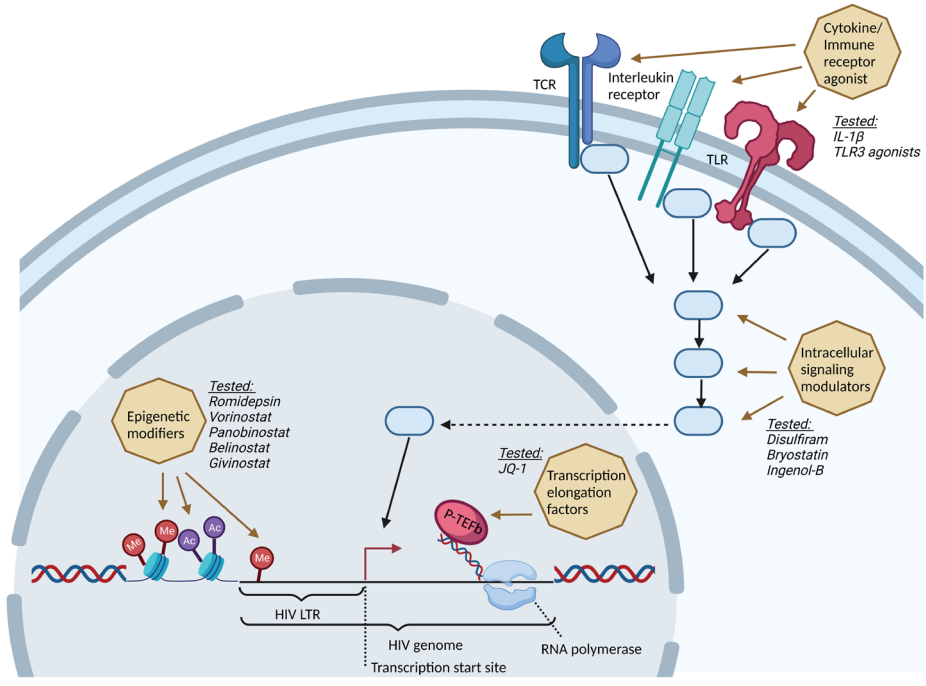


Figure 3. The different classes of LRAs and compounds that are tested for their efficacy in culture models CNS cells or CNS quasispecies. Latency reversal agents (LRAs) can be divided within 5 classes, based on their working mechanisms. Within each of these classes, subclasses are defined. Within the figure, the LRAs that have been tested on CNS culture models or CNS viral quasispecies are indicated. *Created with Biorender.com.*

TABLE 2. Overview of the different classes of LRAs and examples of drugs. Latency reversal agents (LRAs) are classified within 5 different classes and multiple subclasses. Of each of these classes examples of drugs are listed.[153, 156, 157]

(Sub)class of LRA	Examples of drugs
Epigenetic modifiers	
Histone methyltransferases (HMT) inhibitors	Chaetocin, AZ505, BIX01294
Histone deacetylase inhibitors (HDACis)	Vorinostat (SAHA), panobinostat, romidepsin, chidamide, valproic acid, belinostat, givinostat
BRG1/BRM-associated factor (BAF) inhibitors	Pyrimethamine
Intracellular signaling modulators	
Protein kinase C (PKC) agonists	Bryostatin, Ingenol-B, prostatin
Modulators in the PI3K/Akt pathway	Disulfiram
Modulators in the JAK/STAT pathway	Benzotriazole
Second mitochondria-derived activator of caspases (SMAC) mimetics	SBI-0637142, CAPE
Cytokine or immune receptor agonists	
ILs and cytokines	IL-1β, IL-2, IL-7, IL-15 agonists
TCR activators	Maraviroc
Checkpoint inhibitors	Anti-CTLA4, anti-PD1
TLR agonists	TLR2,3,7,8, 9 (MGN1703) agonists
Transcription elongation factors	
Bromodomain and extraterminal domain (BET) inhibitors	JQ1, MMQO
Unclassified	
Antioxidants	Auranofin
Phosphatases	SMAPP1

Shock and kill in the CNS

The evidence for HIV persistence and potential latency in CNS-specific cells and resident $CD4^+$ T cells emphasizes that also the CNS reservoir needs to be considered in HIV curative strategies. One of the most studied approaches is the "shock and kill" strategy (Section **3**). In the coming paragraphs, issues regarding the effectivity and safety of "shock and kill" within the context of the CNS will be discussed.

Penetration of LRA compounds in the CNS

Penetration of LRAs in the CNS could be a limiting factor for the success of the "shock and kill" strategy. Churchill *et al.*[17] reviewed many of the currently known LRAs and showed that CNS penetration varied from poor as in the case of romidepsin toward very good as seen with vorinostat and disulfiram. Although most LRAs show relatively good CNS penetration, multiple approaches are under development, such as nanoparticle delivery, to improve the passage of drugs/compounds across the BBB.[20]

Latency reversal in CNS cells

HIV transcription and latency can be differentially regulated in the CNS as compared with that in peripheral $CD4^+$ T cells (Section **2.6.2**). Although there is no direct proof of latency in the CNS, there are strong indications of latency as discussed in Section **2.3**. It is likely that these differences have an impact on the responsiveness and efficacy of the LRAs.[24]

Several in vitro studies have been performed with different LRAs that showed variable effects in the previously discussed culture model systems (Section **2.5**) of microglia (Figure 3) and (Table 3).[22, 115, 122, 159, 160] Contradictory data were shown regarding the induction of viral transcription of romidepsin, panobinostat, vorinostat, bryostatin, and the BET inhibitor JQ1 in monocyte-derived macrophages,[22, 115] (Table 3). A study testing HIV reactivation of HDACis (belinostat, givinostat, panobinostat, romidepsin, and vorinostat) on in vitro-infected monocyte-derived macrophages showed that these HDACis reduced the amount of integrated HIV DNA, but without measurable reactivation because of the formation of autophagosomes.[160] This might explain the differences in measurable HIV reactivation between the studies,[160] but also donor variability has been suggested to cause these differences.[115] Bryostatin/ingenol-B combined with JQ1 reactivated HIV in microglial cell lines.[159] Finally, it is reported that treatment with TLR3 agonists reactivated HIV transcription in immortalized primary microglia,[122] (Table 3).

Although microglia are suggested to be the main reservoir of HIV in the brain (Section **2.4**), multiple studies have tested the effects of LRAs on primary astrocytes or astrocyte cell lines (Figure 3) and (Table 3). Some LRAs did induce viral transcription in primary astrocytes, such as romidepsin, panobinostat, and disulfiram,[22] whereas others did not such as IL-1β.[159] However, in general, conflicting data were reported regarding the reactivation effects of LRAs in the astrocytes, for example for bryostatin and vorinostat.[22, 98, 99, 146, 161]

Altogether, this indicates LRAs might have promising effects in the microglial or macrophage cells with respect to latency reversal and that conflicting data are seen in astrocytes, (Table 3). The discrepancy between microglia and astrocytes may be attributed to the fact that astrocytes have limited productive infection in vivo, *ex vivo*, and in vitro[98, 101, 102, 161] and consequently, have limited ability to increase their viral expression after reactivation. Moreover, it is important to notice that many of these studies are performed on primary cells or even cell lines, which do not present the true HIV latency situation and are a simplification of the complex CNS reservoir, as discussed in Section **2.5**. Last, some of these studies only report differences in viral transcription, whereas this is not directly result in an increase in viral production.

TABLE 3. Overview of the effects of LRAs within culture models of different brain cells. The listed latency reversal agents (LRAs) are reported to have varying effects within astrocytes or (monocyte-derived) macrophages or microglia. PKC, protein kinase C

LRA	Cell type	What effect?	Reference
Microglial models			
Romidepsin, panobinostat & JQ1	Monocyte-derived macrophages	Viral transcription	(22)
Vorinostat	Monocyte derived macrophages	Little viral transcription	(22)
Bryostatin & borinostat	Monocyte derived macrophages	HIV reactivation	(115)
Panobinostat	Monocyte derived macrophages	No HIV reactivation	(115)
Belinostat, givinostat, panobinostat, romidepsin and vorinostat	Monocyte derived macrophages	Reduced amount of HIV DNA	(160)
Combination therapy bryostatin & Ingenol-B	Microglial cell-lines	Reactivation of infected cells	(159)
TLR3 agonists	Immortalized primary microglia	Viral reactivation	(121)
Astrocyte models			
Disulfiram, romidepsin, panobinostat	Astrocytes	Viral transcription	(22)
IL-1β	Astrocytes	No impact on viral transcription	(161)
Vorinostat	Astrocytes	Conflicting results on viral transcription	(22,146,161)
Bryostatin	Astrocytes	Conflicting results on viral transcription	(98,99)

Latency reversal of CNS viral populations

Some of the differences in HIV transcription and persistence in the CNS might be explained by compartmentalization of HIV and differences in LTR and Tat sequences, as discussed before in Section **2.6.2**. A study on CNS-derived LTRs obtained from unsuppressed PWH, showed that viral reactivation by LRAs romidepsin, panobinostat, and JQ1 in combination with Tat was lower for the brain-derived viruses compared with the lymphoid-derived viruses in human fetal astrocytic cell lines (SVG),[24] (Figure 3). However, to the best of our knowledge, this is the only study in which these effects are investigated. Therefore, it is unknown whether these effects also occur for other LRAs and for viral strains obtained from suppressed individuals. This emphasizes the urgent need to screen the current LRAs for their efficacy toward CNS-derived viruses from both ART-suppressed and unsuppressed PWH.

The impact of the "shock" in the CNS

It is important to keep in mind that in vitro and *ex vivo* experiments do not completely reflect the direct and indirect effects of LRAs within the complex human brain and its neurocognitive performance. LRAs themselves and the resulting viral reactivation might cause damage to the CNS. Therefore, it is needed to monitor potential toxic effects, preferably in 3D human cerebral organoid models, animal studies or clinical trials.

Impact of viral reactivation in the CNS

To the best of our knowledge, only one in vivo study has been performed that investigated the potential of LRAs to reactivate HIV in the CNS.[23] This study showed that the administration of Ingenol-B and vorinostat in ART-suppressed SIV-infected macaques lead to a 10-fold higher viral load in CSF as compared with plasma in one macaque. Postmortem in situ hybridization showed viral transcripts in myeloid cells ($CD68^+$ cells) within the occipital cortex,[23] suggesting that perivascular macrophages or microglia represent an HIV reservoir that can be reactivated via the usage of these LRAs. However, in the latency reversed SIV-infected macaque, also increased levels of markers for neuronal degradation, inflammation, and SIV encephalitis were found in brain tissue and CSF compared with the macaque without latency reversal. This implies that although these LRAs might result in successful viral reactivation, the production of viral RNA and inflammatory markers of this HIV-cure strategy might also result in neuronal degradation and consequently HAND, as discussed previously in Section **2.2**.

In line with this observation, ART interruption in PWH lead to increases in HIV RNA in CSF and plasma and increased levels of NFL in the CSF,[162] which is associated with neuronal degradation.[46, 162] Interestingly, it has been shown that neuronal damage enhances HIV expression in latently infected microglia,[163] suggesting a positive feedback loop after the first reactivation and its induced neuronal damage. Together, this indicates that reactivation of the CNS reservoir may impact neuronal degradation.

Moreover, it is important to be aware of the limited potential of CNS cells, predominantly neurons, to replenish as compared with peripheral cells.[164] Although the number of HIV-infected cells in the CNS is low, there could still be an effect of HIV-induced bystander apoptosis.[165] Therefore, LRA-induced activation of HIV-infected cells might lead to even more killing of surrounding (neuronal) cells with possible detrimental consequences on an individual's brain functioning. Altogether, viral reactivation induced by LRAs may result in a reduction of neuronal cells, which might lead to a decline in neurocognitive performance and the development of HAND.[166]

Neurotoxicity of LRAs

Besides the effects of viral reactivation in the CNS and its impact on neuronal functioning, LRAs might have direct neurotoxic effects as well. One important issue of latency reversal agents is that they may not only activate cells that harbor latent HIV, but instead cause activation of other resting cells.[157, 167] The first LRAs induced global (T cell) activation as bystander effect, leading to severe toxicity. Therefore, most contemporary LRAs are designed to more specifically activate HIV, for instance by preferential methylation of HIV DNA as discussed in section **3.2**.[167] Nevertheless, current LRAs are not exclusively reactivating HIV-infected cells resulting in immune activation and toxicity in bystander cells, as discussed for $CD4^+$ T cells.[157, 167-169] It is suggestive that activation of noninfected cells in the CNS may lead to toxicity in these bystander cells as well. Moreover, it has been shown that the treatment of primary astrocytes with multiple combinations of LRAs resulted in secretion of several inflammation markers[170] and accumulation of amyloid beta,[171] which may have detrimental consequences such as neuronal injury. At last, it has been shown that the LRAs prostratin and bryostatin damage the integrity of the BBB, allowing immune cells to cross this barrier and thus enhance neuroinflammation.[172]

Neurotoxic effects of LRAs are reported in clinical trials as well. For example, HDACis and also disulfiram are associated with neurotoxic symptom.[173, 174] In a recent clinical trial investigating the combination of vorinostat and high dose disulfiram, the first 2 participants enrolled developed neurotoxicity grade 3, leading to cessation of the study.[174] In contrast, other clinical latency reversal studies reported that administration of the standard licensed doses of romidepsin and panobinostat was not associated with CNS side effects, as assessed by analysis of expression of CSF biomarkers and by performing cognitive tests.[175, 176] However, panobinostat could not be detected in the CSF, indicating that there was potentially no penetration of this LRA in the CNS.[175] Increased neurotoxicity as observed in some of these clinical trials might also be related to the usage of LRA combinations as opposed to LRA monotherapy. Together these results strongly suggest integration of CNS-specific monitoring in clinical studies to gain more insight in the efficacy of latency reversal and the consequences of LRA treatment and HIV reactivation in the CNS. In future, it may be a good idea to combine the "shock and kill" strategy with anti-inflammatory compounds, although these might reduce subsequent immune-mediated clearance.[147, 177]

The "kill" in the CNS

The last important step of the "shock and kill" strategy is killing of the HIV-expressing cells, either via virus-mediated cytotoxicity or immune-mediated clearance. For both of these mechanisms, critical differences are to be expected in the CNS as compared with killing of $CD4^+$ T cells in the periphery.

Virus-mediated cell killing

It is reported that myeloid cells, including the perivascular macrophages and the microglial cells, are more resistant to cytopathic effects and apoptosis.[147, 148] The mechanisms behind the reduced cytopathic effects in myeloid cells are still largely unknown. However, recently it has been shown that CTL-mediated killing of infected macrophages compared with $CD4^+$ T cells required longer cell–cell contact and that a higher concentration of secreted IFN-γ by CTLs was needed for cellular killing.[178]

Moreover, studies have shown that HIV infection makes infected macrophages and microglia more resistant to apoptosis.[179] Gene expression in monocytes from PWH shows apoptosis-resistant expression patterns,[180] and Bim, a proapoptotic negative regulator of CTIP2, is up-regulated in latently infected macrophages in vitro.[117] These would allow microglia and perivascular macrophages to harbor latent HIV for months or years.[64, 147] The influence of HIV on apoptosis in astrocytes is currently unknown, although resistance of HIV-infected astrocytes to apoptosis has been described.[181]

Current in vitro LRA studies using primary CNS cells did not show a clear direct effect on reducing the number of infected cells despite viral reactivation.[22, 98, 99, 115, 122, 146, 159, 161]

However, as discussed before in section **3.2**, it is unlikely that administering LRAs alone will eliminate these cells and therefore LRAs need to be combined with compounds that stimulate cell killing. To our knowledge, there are no studies on the effect of this strategy in the CNS of PWH on ART.

Immune-mediated clearance

The most prominent immune cells in the CNS are the resident perivascular macrophages and microglia, but other peripheral immune subsets such as macrophages, dendritic cells, and T cells are also present.[182] The number of HIV-specific $CD4^+$ and $CD8^+$ T cells is limited in the CNS as compared with the periphery but they can be detected in the CSF of PWH.[183] This indicates that immune-mediated clearance of HIV can occur in the CNS and is suggested to be one of the mechanisms that may be involved in killing reactivated cells following latency reversal.

As discussed in section **2.2**, the induction of HIV inflammatory responses in the brain, results in microglia activation, an up-regulation of cytokines and chemokines, and the influx of cells from the periphery, including monocytes and lymphocytes. A process called viral encephalitis.[184] This can lead to neuronal injury and degradation. To avoid these inflammatory processes, immune responses are regulated strictly in the CNS.[185] As a result, immune-mediated clearance is limited in the CNS, which might also limit the "kill" part of this eradication strategy. Together, the increased resistance of HIV-infected

CNS cells to apoptosis and the reduced ability of immune-mediated killing suggests that the "kill" after latency reversal in the CNS will be suboptimal or even very limited. If viral production/reactivation and immune activation by LRAs is induced, but the removal of the activated cells is limited, this could have detrimental consequences on neuronal injury and performance. Therefore, it is questionable whether inducing structural mechanisms of immune activation in the CNS via LRAs with a possible risk on neuronal degradation is beneficial and/or preferable while treating (suppressed) PWH.

Evaluation of LRA effects in CNS

Most techniques to evaluate the effects and safety of HIV eradication strategies in PWH are developed for the peripheral $CD4^+$ T-cell reservoir and are hard to use for the CNS reservoir.[186] Current assays to study the size of the HIV reservoir are the quantitative viral outgrowth assay, Tat/Rev Induced Limiting Dilution Assay, and the intact proviral DNA assay.[187, 188] These assays are generally performed on blood-derived samples, which do not reflect the CNS reservoir. Some HIV-induced markers of CNS injury can be found in the blood.[189] Ideally, the impact of LRAs on the viral reservoir in the CNS is investigated on human brain tissue on time points before and after an intervention. However, it is clear that these analyses cannot be performed in the setting of human clinical trials.[190] Therefore, longitudinal analyses will depend on the analyses of viral RNA and biomarkers for neuroinflammation and neuronal injury in CSF,[9, 46, 55] which are of interest for the evaluation of the effects and safety of HIV eradication strategies. However, this option is also restricted because of the high invasiveness of every puncture.[191]

Other less invasive options to study the effects and safety of HIV eradication strategies on the CNS in vivo are imaging techniques. Although, MRI does not allow us to detect changes at the cellular level, such as neuronal death, as discussed before. [192] More informative techniques to monitor HIV infection in the CNS are nuclear imaging approaches such as single photon emission computed tomography and PET scans. These techniques are used to observe immune activation, inflammation and neuronal injury related to HIV infection via radioactive tracers, as reviewedearlier.[193] For example PET imaging of CSF1R and TSPO were shown to track neuroinflammation in microglia[63, 194] and tracers are available to monitor synaptic density.[195] These techniques are interesting to utilize in an HIV-context.[63] However, to directly target HIV-infected cells in the CNS, there is an urgent need of HIV-specific traces, which are able to penetrate the CNS.[193] Metabolic imaging using magnetic resonance spectroscopy is another promising imaging technique measuring chemical changes in (neuro)metabolites, which can be used to monitor neuroinflammation and its related neuronal injury. Unfortunately, just a limited number of brain regions can be studied and comorbidities have a great confounding effect.[196] Altogether, these imaging techniques are of great promise to monitor the effects of eradication strategies and its safety in the CNS, although it is hard to monitor the direct viral reactivation of HIV. The usage and further

development of these novel imaging techniques is of great importance to evaluate the effects and safety of HIV eradication strategies in the CNS.

Concluding remarks

Eradication of the latent and persistent CNS reservoir is important because continued viral production during ART can contribute to the development of HAND. Cessation of ART can lead to systemic viral recrudescence contributing to HAND. HIV RNA in the CNS has been associated with severe neurologic manifestations. The CNS is shielded from the periphery by the BBB, which results in a unique microenvironment with CNS-specific cells that may be infected by HIV such as microglia, perivascular macrophages, and astrocytes. Localized HIV replication in these cells results in the generation and selection of compartmentalized CNS- specific viral quasispecies. Moreover, in these cells, HIV transcriptional activity and latency induction may be different, due to cellular characteristics and polymorphisms in the LTR of these CNS viral populations. Most HIV cure research efforts to date have focused on the "shock and kill" strategy. Unfortunately, the evaluation of HIV cure strategies in the CNS, including the "shock and kill" strategy is challenging, and several limitations regarding the success and safety of reactivation of the viral reservoir with LRAs in the CNS exist.

It is therefore important to get more insight in the mechanisms associated with HIV latency in the CNS and the potential impact of latency reversal on neuropathogenesis. Improved delivery of LRAs is being explored such as the development of nanoparticles that can specifically target latently infected HIV cells in the CNS. Moreover, the differences in the viral LTR sequences could imply that CNS-specific LRAs may be needed to specifically reactivate brain-specific viral populations. The use of combinations of LRAs with apoptotic inducers may also improve the efficacy and safety of the "shock and kill" strategy, by sensitizing cells for apoptosis shortly after HIV is reactivated and thereby limiting continued viral production and bystander cell death. Ideally, these strategies will lead to a higher efficacy and specificity of the drug compounds toward HIV-infected cells, in order to increase eradication of HIV-infected cells and limit the eradication of noninfected brain cells.

Furthermore, better in vitro models such as human cerebral organoid model systems or in vivo animal studies are needed to investigate mechanisms of HIV persistence in the CNS including latency, latency reversal and its impact on cell activation, viral production, immune activation, and safety. Robust HIV reactivation via LRAs likely leads to encephalitis, neuronal damage, or neuronal loss due to the relative aspecific LRA stimulation, induced inflammation, viral toxicity causing bystander cell death. Considering the limited potential to replenish these neurons, the "shock and kill" strategy might have detrimental consequences on brain function. Therefore, it is of great importance to monitor HIV RNA

in CSF and specific CSF parameters during clinical trials that may help us to assess the impact and safety of the "shock and kill" strategy. Moreover, further development of imaging techniques can provide a less invasive method to evaluate the effects and safety of the LRAs in treated individuals. As an alternative, at least CNS parameters in the blood need to be monitored. Anti-inflammatory compounds could be combined with the "shock and kill" strategy to diminish neuroinflammation seen in PWH. However, it is questionable whether the elimination of infected and reactivated cells will still occur if inflammation is inhibited.

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CHAPTER 7

Summary and Discussion

Antiretroviral therapy (ART) is by far the most significant breakthrough in HIV research to date, enabling millions of people to live long lives by suppressing HIV replication to undetectable levels [1]. While there have been major differences in ART through the years, none of the current drugs are able to target HIV-infected cells, thereby allowing HIV reservoirs to persist. The persistence of HIV reservoirs has proven to be the biggest obstacle to HIV cure. One of the proposed anatomical reservoirs for HIV is the central nervous system (CNS). For the CNS to qualify as a reservoir for HIV, (i) it must contain cells with a long lifespan or which undergo (homeostatic) proliferation that harbor integrated proviral DNA, (ii) are capable of establishing and retaining the virus in a latent state, (iii) and which can then be reactivated to produce new replication-competent viral particles and reseed infection [2–4]. In this thesis, we provide evidence to support the CNS as a viral reservoir for HIV, which include the identification of genetic and phenotypic differences between CSF and plasma variants (**chapter 3**), and the detection of intact proviral DNA in the CNS (**chapter 4).** We also characterized productive HIV infection in 2D and 3D human microglial culture models that can be used to further HIV research on the CNS **(chapter 2, 5)** and ultimately discussed the impact of HIV cure strategies on the CNS reservoir **(chapter 6)**.

HIV infection of the CNS

The central nervous system is a unique anatomical compartment that is enclosed by the blood-brain barrier (BBB) and surrounded by cerebrospinal fluid (CSF). The BBB controls the selective communication and transport of cells and molecules between the periphery and the CNS. For HIV to remain dormant for years despite treatment, it must first productively enter and integrate into the host genome of a cell. HIV utilizes two major receptors to enter a target cell, the CD4 receptor, and the CCR5 coreceptor. In regards to the CNS, it is well-known that both receptors are expressed on infiltrating and CNS resident CD4+ T cells, microglia, and perivascular macrophages, making them the most likely target cells for HIV in the CNS (**chapter 2**) [5].

HIV has been detected in the CSF as early as 8 days post estimated infection [6]. Initial evidence for the CNS as a separate viral compartment came from the detection of discordant viral load between the CSF and plasma, suggestive of selective viral entry and replication in the CNS [4,6]. In untreated infection, HIV-1 RNA in CSF is generally lower than that in plasma, but increases with the progression of disease, resulting in a reduction of the median viral load difference from 2.36 log10 (copies/mL) during acute infection [7] to one log10 or below during chronic infection [8]. Within our study population (**chapter 3**), consisting of ART-naïve or off-treatment individuals with high-level CSF and plasma viremia, we also found CSF HIV-1 RNA levels to be significantly lower than plasma with a median viral difference of 0.89 log10. In contrast, in 3 individuals (1 with and 2 without neurological complications), we observed higher RNA levels in CSF than in plasma suggesting greater transmigration of HIV into the CNS and/or an increase in HIV production and/or replication within the CNS.

The most widely accepted hypothesis for the entry of HIV into the CNS is the "Trojan horse theory", which proposes that the virus enters mainly through the transmigration of infected CD14+/CD16+ monocytes, and CD4+ T cells during routine immune surveillance [9]. Once within the CNS, monocytes may differentiate into perivascular macrophages and, together with CD4+ T cells, release infectious virus that subsequently infects resident CNS cells [10,11]. Circulating viral particles and infected cells then activate other CNS cells and collectively participate in the production and secretion of viral proteins, neurotoxins, and pro-inflammatory compounds. The ensuing chronic neuroinflammatory environment and neuronal damage are believed to be responsible for many of the neurocognitive deficits observed in HIV-associated neurological disorders (HAND) [12–15]. Successful virologic control with ART has shifted the incidence of HAND from severe, HIV-associated dementia (HAD), to the milder forms, asymptomatic neurocognitive impairment (ANI) and mild neurocognitive disorder (MND), affecting 20-50% of the HIV-infected population today despite suppressive ART [16–18].

Post-mortem studies on archival human brain tissues from the pre-ART era detected HIV DNA in CD68+ microglia/macrophages [19,20], astrocytes [20,21] and neurons [19,22,23] of AIDS patients with encephalitis or dementia. However, modern-day virally suppressed patients on ART were found to harbor HIV DNA mostly in microglia and perivascular macrophages [24,25], with one recent study reporting HIV DNA in astrocytes [26]. In line with these reports, we found HIV DNA in both microglia (CD11b+) and non-microglial (CD11b-) cells, isolated from a virally suppressed individual on long-term ART (**chapter 4**). While we can't rule out possible cross contamination of some microglial cells in the CD11b negative fraction, our findings support that non-microglial cells such as CNS resident CD4+ T cells, also harbor HIV DNA during treatment although the exact cell composition of this microglia-depleted cellular fraction remains to be determined. However, the mere detection of proviral DNA, without any additional information regarding the integrity of the genome or integration site, is not enough to qualify a cell as a cellular HIV reservoir. This will be further discussed in the next paragraph.

The persistence of HIV DNA in perivascular macrophages, microglia, and astrocytes has been corroborated by the detection of low-copy viral RNA in the CNS in individuals with undetectable RNA in CSF and plasma [24,26]. Perivascular macrophages and microglia have also been readily proven to support productive HIV infection through the CD4 and the CCR5 receptor (chapter 2, 5), but the productive infection of astrocytes remains controversial and highly debated. Astrocytes lack the expression of CD4 and cannot support HIV entry through the classic CD4-mediated mechanism. Several studies have advocated for the productive infection of astrocytes without the CD4 receptor including (i) receptor-mediated endocytosis; (ii) direct cell-to-cell contact with infected CD4+ T cells and/or (iii), direct binding of immature viral particles, with exposed CXCR4-binding sites,

to the CXCR4 coreceptor which induces membrane fusion [27,28]. Productive infection through endocytosis, however, requires escape from lysosomal degradation, which is not normally seen in astrocyte cultures without prior treatment with lysosomotropic agents such as chloroquine, bafilomycin, or Tat-HA2 peptide [27,29]. To date, these CD4-independent mechanisms proposed for productive HIV infection of astrocytes remain theories based on *in vitro* studies with human fetal astrocytes and are yet to be confirmed *in vivo*.

Furthermore, while CD4+ T cells, microglia, and perivascular macrophages all utilize the well-established CD4-mediated mechanism for cell entry, one important point of distinction is the low surface expression of the CD4 receptor on myeloid cells compared to T cells. It is therefore believed that for HIV to replicate within these cells it must adapt to the low CD4 surface expression [30]. Initial evidence supporting this hypothesis came from phenotypic studies characterizing the entry phenotype of viral isolates obtained from the periphery and CNS of viremic individuals in advanced HIV disease with HAD. CSF viral isolates were more efficient at infecting monocyte-derived macrophages than the plasma viral isolates, suggestive of an evolutionary path towards target cells with low CD4 density [31–38]. Similar studies quickly followed, after the introduction of the Hek293-affinofile cell line, in which the surface density of CD4 can be experimentally manipulated to resemble the low CD4 surface density on macrophages or the high surface density on CD4+ T cells [39,40]. In a cohort of ART-naïve subjects, CNS compartmentalization was found in up to 20% of paired CSF and blood samples obtained over the first two years of primary infection [41]. Viral tropism analysis in Affinofile cells revealed that all compartmentalized variants required high levels of CD4 surface expression, mostly found on CD4+ T cells and classified as R5 T-tropic. This R5 T-tropic population consisted of identical or nearly identical variants representative of clonal expansion within local CNS CD4+ T cells [Figure 1]. As the disease progresses towards chronic infection and advanced disease, compartmentalized CSF populations are readily found in subjects diagnosed with HIV-1-associated dementia (HAD) that can efficiently enter cells with high (R5 T-tropic) and low CD4 surface levels [31]. R5 Tropic viruses capable of using both high and low CD4 surface expression are classified as R5 M-tropic. Both the R5 T-tropic and R5 M-tropic viruses were more genetically diverse indicative of persistent viral replication, beyond a single clonal amplification event, within local CNS cells with high (CD4+ T cells) and low levels of CD4 surface expression (microglia and macrophages) [31,42–44]. R5 M-tropic variants were also rarely observed in the blood and peripheral tissues [31,32,38,44], further supporting the prevalence of local CNS viral replication.

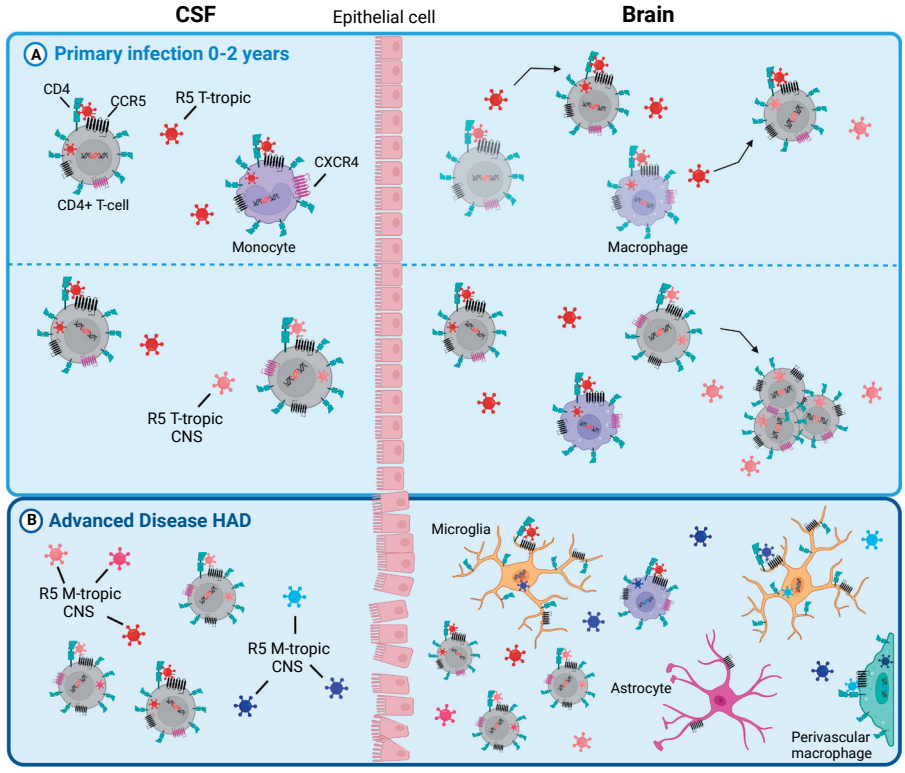

Figure 1: Progression of viral tropism in the CSF and brain during disease progression. HIV bound to the CD4 and CCR5 receptor represent the virus that infects the cell, and the virus inside the cell represent the virus that the cell produces. M-tropic variants are depicted in blue and T-tropic variants are depicted in red/pink. (A) During primary infection, R5 T cell-tropic virus (red) from the periphery transmigrate into the CSF within CD4+ T cells and monocytes/macrophages or as cell-free virion. R5 T-tropic virus within the CSF is then believed to infect infiltrating CD4+ T cells with minimal to no local replication in the brain (transparent). (B) As the disease progresses towards chronic infection, compartmentalized CNS R5 T-tropic virus (pink) are found in the CSF, representing local CNS viral replication in resident CD4+ T cells. Compartmentalized R5 T-tropic viral populations consisted of identical or nearly identical variants suggestive of clonal expansion within CD4+ T cells with high CD4 surface levels. (C) In the advanced disease state (correlated with HIV-associated dementia), genetically complex compartmentalized CNS R5 T-tropic (shades of red/pink) and R5 M-tropic virus (shades of blue) are found in the CSF. This is indicative of persistent replication, beyond a single clonal amplification event, within high CD4 (CD4+ T cells) and low-CD4 (microglia, macrophages) expressing cells. Created with BioRender.com

In line with the aforementioned studies, **in chapter 3**, we also investigated the genetic and phenotypic characteristics between CSF- and plasma-derived HIV variants in a cohort of HIV-infected individuals, not on treatment, in the chronic stage of infection. As mentioned, these individuals were viremic in both CSF and plasma with significantly lower RNA levels in CSF than in plasma, providing the first evidence for a unique viral population in the CSF. Compartmentalization analyses revealed a genetically distinct CSF viral population

in two subjects, subjects 13 and 19, of which only subject 13 had mild neurological complications. Next, we phenotypically characterized the CSF and plasma-derived variants of compartmentalized subject 19 and two equilibrated subjects, subjects 8 and 27, in human CD4+ T cells and primary microglia. To our knowledge, this is the first study to examine the entry phenotype of patient-derived CSF and plasma-derived viruses in both CD4+ T cells and primary microglia. To account for the inherent variability in CD4 expression between donors, we utilized the lab strains BaL and YU-2, R5 M-tropic, and JRCSF, R5 T-tropic, as controls. Most CSF and plasma-derived viruses were not able to mediate infection of microglial cells and required high levels of CD4, found on the CD4+ T cells, for cell entry. We also observed no significant difference between the compartmentalized CSF-derived clones from subject 19 and the equilibrated CSF-derived clones from subjects 8 and 27, in terms of their ability to utilize low CD4 surface expression for cell entry. There was, however, one CSF clone in each subject that exhibited an intermediate enhancement, defined as ≥50% of YU-2 infection, for entry into microglia. This intermediate entry phenotype was also observed for one plasma clone in subject 27, which suggests that a subset of the viral population within plasma originated from the CNS or from low-CD4 expressing cells such as tissue macrophages. Other than CSF and plasma [40–42], viruses with an intermediate entry phenotype, determined by low CD4 Affinofile cells and/or monocyte-derived macrophages, have also been detected in peripheral tissues, such as the colon, lungs, and lymph nodes [38]. Unfortunately, without longitudinal data samples, it is unclear whether these viruses represent evolutionary intermediates on a multi-step path to macrophage tropism. Several envelope substitutions have been reported to be associated with macrophage tropism, such as N283 in the CD4bs [36], a conserved amino acid in the V1 loop [45], and the loss of an N-linked glycosylation site at 386 [35,46]. However, none of these genetic mutations were found to be conserved across different studies. Furthermore, Brese et al [38], using single-molecular real-time (SMRT) sequencing, found that, in addition to the genetic evolution between CSF and plasma viruses, isolated viral replication and evolution also occurs between different brain regions (frontal, occipital, and parietal lobe).

In summary, the prevalence of HAND and the persistence of HIV DNA in CNS cells despite ART, combined with genetic and phenotypic CNS compartmentalization, provide strong evidence for the CNS as an anatomical reservoir for HIV.

Measuring the HIV reservoir

As stated before, HIV DNA has been found in microglia, perivascular macrophages, and astrocytes. However, for this DNA to be of clinical relevance and considered part of the HIV reservoir, it must be intact, encode for a replication-competent virus, and integrated into the host genome in a region that is permissive for transcription. Due to the high error rate of reverse transcription, most integrated proviruses are defective containing premature

stop codons, deletions, mutations, or other defects that prevent the production of new infectious virus [47].

The large population of defective proviruses and the high heterogeneity of the reservoir have made it extremely challenging to get an accurate measurement of the intact reservoir. Two of the most prominent assays for the measurement of the HIV reservoir are the quantitative viral outgrowth assay (QVOA), based on the assessment of viral production following *ex vivo* stimulation, and the intact proviral DNA assay (IPDA), capable of distinguishing intact from defective proviruses using specific primer/probe sets. Each assay has well-described limitations causing an underestimation (QVOA) or overestimation (IPDA) of the true reservoir size. The IPDA, however, has several advantages over the QVOA, such as its high throughput capability and cost-effectiveness, and requires only a relatively low number of cells [48,49]. These characteristics make the IPDA a favorable method for the measurement of the CNS reservoir since human brain tissue and CNS cells are difficult to obtain in large quantities.

Prior to ART, circulating CD4+ T cells with intact provirus represent the majority of the proviral population (mean 63%) in the periphery [50], however, these cells gradually decline during treatment at a faster decay rate than the cells containing defective DNA [51,52]. In HIV-infected individuals on long-term suppressive ART, CD4+ T cells with intact provirus represented only ≤10% of the total proviruses [51,52]. This discrepancy in decay rate between the intact and defective proviral DNA following ART initiation indicates that the replication-competent (intact) HIV reservoir is under a distinct immune clearance mechanism and potentially a higher susceptibility to cell death due to the toxicity of viral products such RNA transcripts and viral proteins. This also raises the question of whether this mechanism applies to all cell types harboring intact proviral DNA, or whether it is specific to CD4+ T cells in the blood.

In **chapter 4**, we characterized for the first time, the genomic integrity of proviral DNA in CNS cells isolated from a virally suppressed individual on long-term ART. Using the IPDA, we detected intact proviral DNA in the CD11b+ microglia population of three different brain regions (frontal lobe, occipital lobe, and subventricular zone). In line with the proviral DNA composition in circulating CD4+ T cells in treated individuals, intact proviruses represented only 13% of the total viral population and were greatly outnumbered by defective proviruses (~6.5 fold). The prevalence of intact provirus in the microglia population was about 2.8-fold higher than in the CD11b- microglia-depleted population. Modeling the longitudinal dynamics of the intact proviral reservoir in the CNS is ethically impossible, but the higher prevalence of defective rather than intact proviral DNA suggests that the intact DNA decays at a faster rate, similar to peripheral CD4+ T cells.

Furthermore, as the IPDA does not measure replication competence, it remains to be studied how much of the intact proviral reservoir in the CNS is inducible and can fuel rebound viremia following *in vivo* activation or stimulation. In **chapter 4**, we generated CNS-derived luciferase reporter viruses, utilizing the full-length Env gene amplified from the microglia fraction, and showed that the Env gene derived from microglia can efficiently replicate within CD4+ T cells, hereby supporting the potential of CNS-derived viruses to reseed viremia in the periphery.

CNS culture models to study neuroHIV

To officially designate the CNS as an HIV reservoir, proof of replication competence is required. The CNS resident cells need to be able to support viral replication while the intact proviral DNA needs to encode for a replication-competent virus capable of productively infecting local CNS cells. Due to the inaccessibility and scarcity of human brain tissue, however, this is an extremely challenging undertaking primarily due to the ethical and practical restrictions. Non-human primates (NHP) share similar anatomical and physiological features to humans and have been well-established as a human surrogate for the investigation of HIV-1 pathogenesis [53].

Historically, early models of neuroAIDS research typically utilized different viral clones or swarms, that target both CD4+ T cells and monocyte/macrophages, causing AIDS-like immunosuppression and SIV encephalitis (SIVE) in about 30% of Rhesus macaques and higher percentages of Pigtailed macaques within 2-3 years [54–57]. Since then, several NHP models have been developed that reliably develop AIDS with a high incidence of SIVE within 3 to 6 months after infection. Rapid AIDS progression is induced through inoculation of Rhesus or pigtailed macaques with neurovirulent or macrophage-tropic viruses combined with the suppression or depletion of either CD4+ or CD8+ T cells using immunosuppressive viruses or monoclonal antibodies [54,55,57]. SIV infection, albeit accelerated, was found to mirror many of the key pathophysiological features of HIV infection in humans [54–57]. It is nonetheless noteworthy that NHP have significant genetic and physiological differences from humans and thus caution should be taken when translating results to the *in vivo* scenario in humans.

This being said, some of the most prominent CNS findings were obtained using the rapid neuroAIDS SIV models, including proof of a macrophage functional latent reservoir. Using a macrophage-specific quantitative viral outgrowth assay (MΦ-QVOA), CD11b+ brain macrophages (microglia and perivascular macrophages) were found to harbor replication-competent SIV in macaques suppressed on ART for 4 months up to 1.5 years [58–60]. In addition, viruses induced in the MΦ-QVOA were able to infect and propagate normally in healthy activated CD4+ T cells [58–60]. These studies hereby provide solid evidence that CD11b+ brain macrophages, in macaques, satisfy all the criteria of an HIV reservoir.

Yet, while the use of NHP provides many practical benefits over human studies including experimentally controlled infection and ART regimen, sampling of brain tissue, and the possibility to perform longitudinal analyzes, it is expensive and increasingly restricted by serious ethical concerns for the primates [53,55]. Current legislation and public opinion progressively push for an end to the use of animals for scientific research, especially NHP. As one of the partners in the national Non-animal Testing Innovation Transition (TPI) initiative, ZonMw started a program in 2021 entitled "More Knowledge with Fewer Animals" that provides funding for research into the development and implementation of animal-free models (**chapter 2 and 5**).

In **chapter 4**, we utilized the well-characterized primary CD4+ T-cell culture model to prove that full-length Env genes found in human microglial cells are replication-competent and can refuel viremia in the periphery (CD4+ T cells). However, we also need human CNS culture models that resemble the human CNS *in vivo* to further research on HIV infection in the CNS.

In **chapter 2**, we interrogated a variety of human microglial culture models on their resemblance to microglia *in vivo*, on a morphologic, transcriptomic, and functional level, and their ability to support productive HIV infection. Except for the microglial cell lines, each model was able to recapitulate aspects of primary microglia morphology and function however none of the models were able to fully recapitulate the transcriptomic signature of uncultured primary microglia. Several research groups have somewhat rectified this transcriptomic deficiency by adapting the cytokine cocktail in the culture medium, co-culture with astrocytes and/or neurons, or transplantation in mice, however complete correction remains an ongoing undertaking. Based on their transcriptome, cerebral organoids and iPSC-derived microglia have the closest resemblance to cultured primary microglia. In terms of HIV replication, we found the SV40 and HMC3 cell lines to be resistant to HIV infection and observed a significant difference in infection pattern between the commonly used microglial models, primary microglia (pMG) and monocyte-derived microglia (MDMi), and the cerebral organoid model. pMG and MDMi were highly susceptible to HIV replication with continuous virus production and viral spread, and thus not reflective of the limited infection and viral spread observed among microglial cells *in vivo*. HIV infection of organoid-derived microglia and cerebral organoids, in contrast, reached peak infection in the first week of infection with limited viral spread within the organoids. This observation somewhat aligns with a recent study by Alvarez-carbonell et al [61], that observed co-culture with neurons to have an initial inhibitory effect on microglia infection, suggesting that the surrounding CNS cells play a role in the infection of microglia *in vivo* either through direct cell-to-cell contact or indirect communication via the release of cytokines and/or chemokines. Overall, based on our findings we found cerebral organoids

to have the closest resemblance to (cultured) primary microglia and most representative of microglia HIV infection *in vivo*.

In the cerebral organoid model, consisting of a variety of CNS cells including microglia, astrocytes, and neurons, we found that productive HIV infection occurred exclusively within microglial cells via the CCR5 receptor (**chapter 5**). A similar finding was reported by Dos reis et al., following the incorporation of HIV-infected microglia into their human brain organoid (hBORG) model [62]. Furthermore, despite the detection of HIV DNA and msRNA in astrocytes *in vivo* [26], neither organoid model found astrocytes to be susceptible to productive HIV infection. A very recent paper by Woodburn et al [63], also reported that, contrary to primary microglia and monocyte-derived macrophages, astrocytes were completely refractory to HIV infection using both M-tropic and T cell-tropic HIV-1 Env proteins. Albeit, the absence of HIV infection in astrocytes in our organoid model may be explained by two notable limitations of our organoid model, namely the "age" of the astrocytes and the absence of CD4+ T cells. Immature astrocytes might be less susceptible to HIV infection than fully mature astrocytes in older organoids, whereas CD4+ T cells are proposed to be essential for CD4-independent infection of astrocytes *in vitro*.

Overall, the cerebral organoid model holds great promise as a human CNS-like *in vitro* culture model for the study of HIV neuropathogenesis, including the establishment, maintenance, and reactivation of HIV latency in the CNS. Notably, as with any *in vitro* model, it comes with a set of limitations. Some of the biggest challenges we came across while setting up the cerebral organoid model for HIV research were: (i) the high variability between organoids from the same batch and across iPSC lines, (ii) the lack of control over mesoderm induction and the subsequent low frequency of microglia within the organoids (iii) and the inability to completely remove unbound virus from the Matrigel. Since the publication of our organoid differentiation protocol in 2018 [64], there have been considerable improvements in the protocols used for the generation of cerebral organoids such as the replacement of Matrigel with polymer scaffolds and adapting the differentiation cocktail to decrease heterogeneity and improve reproducibility. One protocol that piqued our interest was published by Xu et al. and entails the co-culture of iPSC-derived primitive macrophage progenitors and primitive neural progenitor cells at the onset of 3D organoid formation to generate microglia-containing brain organoids and hereby gain control of the frequency of microglia within the organoids [65]. Furthermore, as the cerebral organoid technology field continues to advance to better recapitulate the human CNS *in vitro*, it also gives rise to various ethical concerns [66]. Particularly, one can wonder whether a human cerebral organoid could develop some degree of consciousness and whether, under certain conditions, it could acquire its own moral status with the related rights [67].

Lastly, cerebral organoids have been proposed as an alternative model for animal research. Currently, the development of cerebral organoids utilizes several animal-derived components, such as sera and Matrigel. Furthermore, cerebral organoids lack interorgan communication, do not contain a blood-brain barrier, and lack peripheral immune cells all of which are essential for the accurate representation of CNS HIV infection *in vivo*. As a result, cerebral organoids cannot completely replace the use of animals in HIV research but can be used as a pre-screening tool to help reduce the number of animals used.

Targeting the HIV reservoir

The main objective of cure interventions is to enable HIV-infected individuals to discontinue treatment without the consequence of rebound viremia and the ensuing opportunistic infections. The achievement of a sterilizing HIV cure in the "Berlin patient", and the recent "London patient", as well as the "Dusseldorf patient", have given new hope that a cure for HIV is possible [68–71]. These patients underwent an allogeneic stem cell transplant from a homozygous CCR5Δ32 donor, whose cells are resistant to R5-tropic HIV variants due to the 32-base pair deletion in the CCR5 receptor. There have been several attempts to replicate the success of the "Berlin patient", which included the "Boston patients" who received an allogeneic stem cell transplant from homozygous wildtype CCR5+/+ donors. Both patients remained HIV negative up until 129- and 226-days after ART interruption, when the virus rebounded in blood and CSF, resulting in HIV-associated meningitis in one patient [72].

On a positive note, the achievement of a cure following the transplantation of CCR5Δ32 stem cells has prompted research into gene editing to develop HIV-1 resistant cells. This included the use of a variety of nuclease-mediated gene editing tools, such as transcription activator-like effector nucleases (TALEN), zinc-fingers (ZNF), and clustered regularly interspaced short palindromic repeats (CRISPR) to manipulate the CCR5 and CXCR4 receptor *in vitro*, however, due to the high-cost and time-consuming process of ZNF and TALEN, CRISPR/Cas9 has become the preferred method [73]. The CRISPR/Cas9 system involves the use of a custom-designed guide RNA and the Cas9 nuclease to excise a specific DNA sequence from a cell, such as CCR5, or HIV DNA, to generate cells that are devoid of these sequences [74,75]. The CRISPR/Cas9 system can also be used to reactivate latently infected cells or suppress HIV expression by fusing an activator or repressor to a defective Cas9 (dCas9) protein [76]. Using the CRISPR system, latent HIV could be successfully eradicated from microglia [77], perivascular macrophages [77], and astrocytes [78] *in vitro*, suggesting that this method could be an effective strategy for targeting the CNS. However, it remains to be established whether CRISPR can effectively cross the BBB and have the same impact on these CNS cells *in vivo*.

In addition to gene therapy, there are two pharmacological approaches to HIV cure namely, the "Shock and Kill" and the "Block and Lock" strategy. The Shock and Kill strategy,

extensively discussed in **chapter 6**, is the most explored strategy for HIV cure and entails the reactivation of the latent reservoir with potent latency-reversing agents (LRA), followed by cell killing either directly due to the cytopathic effect of the virus or by cell-mediated immune responses [79]. The success of this method in the eradication of the CNS reservoir is highly dependent on the ability of the LRAs to cross the BBB and efficiently activate all the latently infected CNS cells without causing overt neuroinflammation and neurotoxicity. Equally important is the penetration of immune cells and/or neutralizing antibodies into the CNS and their efficacy in killing the activated reservoir [80].

The "Block and Lock" strategy, in contrast, aims to permanently lock infected cells into a deep latent state and prevent HIV gene transcription using latency-promoting agents (LPAs) [79]. The most popular LPA is Didehydro-cortistatin A (dCA) and was reported to efficiently cross the BBB and significantly reduce HIV RNA levels in the brain of the bone marrow-liver-thymus (BLT) mouse model of HIV latency and persistence. In addition, dCA also decreased the uptake of HIV-1 transactivator of transcription (Tat) in microglia-like and astrocyte cell lines [81,82]. Considering Tat's pro-inflammatory and cytotoxic properties, inhibition of Tat activity in the CNS may alleviate Tat-mediated neurotoxicity and neuroinflammation [83].

Final thoughts and Concluding remarks

In this thesis, we advocate and provide supporting evidence for the CNS as a viral reservoir for HIV. In particular, microglia were identified as a major CNS target cell susceptible to HIV replication, CSF-derived viral clones were detected with an intermediate enhancement for entry into microglia, and in the CNS CD11b positive cells from a suppressed individual, we found intact proviral DNA whose envelope gene was capable of replication.

Thus, the major question is "**Do we, in conjunction with current literature, now have sufficient evidence to permanently designate the CNS as an HIV reservoir?** "Simply put, no. We have shown that CNS cells harbor intact HIV proviral DNA; however, if we strictly adhere to the criteria and requirements of an HIV reservoir, we still need definitive evidence that latently infected CNS cells can produce infectious virus upon reactivation and reseed peripheral viremia after ART is discontinued. To date, this criterion can only be addressed by performing a QVOA on the reservoir of interest. While performing the QVOA on human brain tissue is technically feasible, it involves a considerable number of practical challenges which include the difficulty of obtaining large amounts of brain tissue from well-characterized virally suppressed HIV-infected individuals and finding appropriate stimuli and target cells that can efficiently activate CNS cells and maintain the (macrophage-tropic) characteristics of the CNS viral population through sequential rounds of culture. Therefore, apart from the ethical issues, such a study would still take several years to complete. In the meantime, as cure strategies and HIV-1 intervention trials are quickly progressing (clinicaltrials.gov), we contend that the CNS should be regarded as an HIV reservoir, despite its unproven replication competence, and incorporated into current and new curative strategies.

Subsequently, to incorporate the CNS in cure interventions, we need a comprehensive understanding of the molecular mechanisms responsible for the establishment and maintenance of HIV latency in the infected CNS cells. In this thesis, we propose that microglia are the predominant cellular HIV reservoir in the CNS and characterized several human microglial culture models. The question is: "**How do we move forward with these models to advance neuroHIV research?**". In chapters 2 and 5, cerebral organoids are highlighted as the most representative CNS culture model for the recapitulation of HIV infection in the CNS *in vivo*. However, the cerebral organoid field is still in its infancy and requires additional optimization to increase reproducibility, as well as the consistent induction of microglial cells. The latter is especially vital for HIV research, as we and others have shown microglia to be the only cells susceptible to HIV infection within cerebral organoids. Thus, until we have a protocol that can reproducibly generate microglia-containing organoids, this model is not yet suitable for wide-scale HIV research.

An alternative model would be iPSC-derived tri-culture models of microglia, astrocytes, and neurons, in which the prevalence rate of each cell type can be readily manipulated. This model could be used to further investigate the inhibitory role of surrounding CNS cells on HIV infection of microglia, observed in the cerebral organoids. Furthermore, one of the main research questions for the CNS reservoir is to determine if and how HIV latency in the CNS cells differs from the CD4+ T cells. Using the iPSC-derived tri-culture model or primary microglia we can assess the integration site of HIV, identify epigenetic modulators of transcription that can be used for LRAs and LPAs, and also elucidate the efficacy and toxicity of current LRAs, LPAs, and the CRISPR/Cas9 system. In addition, the iPSC-derived tri-culture model can also be used to study HIV-induced neuronal damage and neuroinflammation responsible for the development of HAND. Lastly, primary microglia and monocyte-derived microglia can be used to assess the replication-competence and macrophage tropism of HIV variants isolated from the CSF and brain tissue. Primary microglia and monocyte-derived microglia can also be used in the QVOA as target cells for the propagation of the viruses, following *ex vivo* stimulation.

The final question is: **which cure strategy should we use for the CNS reservoir and how should we monitor this reservoir in cure inventions?** The main goal of targeting the CNS reservoir will be to select a carefully tailored combination of two or more strategies that can eradicate, or permanently block, the replication-competent viral reservoir in the CNS while preventing permanent neurological damage.

Currently, the most popular approach is the shock and kill, however, implementing this approach without having proper knowledge of the CNS reservoir and the efficacy of killing, carries a great deal of risk. In contrast, the block and lock strategy or the use of CRISPR to excise proviral DNA or block gene transcription is much more neurologically safe, in terms of bypassing cell activation and HIV production. Although it remains to be studied how much of the reservoir needs to be blocked or eradicated to prevent rebound viremia, as none of these methods are likely to be capable of targeting all latently infected cells. A case in point is the Boston patients, who despite having an undetectable viral load in their blood and peripheral tissues, faced HIV reactivation and viral rebound suggesting that HIV cure most likely requires complete eradication or blocking of *de novo* infections. Furthermore, while the defective reservoir is not considered part of the HIV reservoir due to its replication incompetence, defective proviruses have been repeatedly reported to express HIV RNA transcripts and proteins that have not only been found to play a role in the development of neuropathogenesis but are also hypothesized to function as a decoy by distracting immune cells from targeting the intact reservoir. Taking this into consideration, the prevalence of a transcriptionally active defective reservoir, after the eradication of the intact reservoir, could still cause serious neurological complications among the HIV-infected population.

Unfortunately, determining the degree of depletion or eradication of the CNS reservoir in living subjects is not possible due to the ethical restriction of pre-mortem brain biopsies. Consequently, the examination of CSF will remain the best resource for assessing HIV in the brain. CNS HIV monitoring should include serial sampling of paired CSF and plasma, before, during, and after a cure intervention [84]. Several CSF markers have been reported that can be used to monitor CNS immune activation, inflammation, and neuronal injury including neopterin [85], neurofilament light chain [86], YKL-40 [87,88], and Trem2 [89]. Measurement of these CSF markers during the intervention would give insight into the state of microglia activation, neuroinflammation, and possible neuronal damage. However, since lumbar puncture is an invasive procedure and uncomfortable for the patient, more research is needed on the development of blood biomarkers to monitor HIV activity in the CNS [90]. In HIV cure-directed clinical trials using analytical treatment interruptions (ATIs), CSF viral rebound should be phylogenetically characterized together with plasma rebound viruses to investigate CNS compartmentalization. Finally, after ART resumption, CSF viremia and inflammation must be monitored to ensure a return to baseline values and a clinical neurological examination or neuropsychological testing should be conducted to determine whether any neurological impairment occurred [84].

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APPENDICES

Appendices

NEDERLANDSE SAMENVATTING

Sinds de introductie van antiretrovirale therapie (ART) is een infectie met het humaan immunodeficiëntie virus (hiv) in de meeste gevallen een chronische aandoening, met een normale levensverwachting. Behandeling van hiv is erop gericht om de hoeveelheid virus in het lichaam (virale load) te onderdrukken tot ondetecteerbare waarden door het vermenigvuldigen (replicatie) van het virus te voorkomen. Echter, ondanks langdurige behandeling, is ART niet in staat om hiv te genezen. Dit komt omdat het virus zich, direct na infectie, nestelt in het DNA van onze afweercellen waarin het "slapend" aanwezig blijft, het zogenaamd latent viraal reservoir. ART verhindert de infectie van nieuwe cellen, maar kan deze reservoircellen niet verwijderen. Hierdoor dient ART levenslang te worden voortgezet om te voorkomen dat het reservoir "wakker" wordt (reactiveert) en in afwezigheid van ART weer nieuw infectieus virus produceert (virale rebound). De genezing van hiv berust dus op het vermogen om deze geïnfecteerde reservoircellen te verwijderen of de productie van nieuw infectieus virus permanent te blokkeren.

Het is bekend dat hiv zich ook in de hersenen nestelt. Maar hoe, waar precies en wat het volledige gevolg is, is nog onduidelijk. De hersenen vormen samen met het ruggenmerg het centrale zenuwstelsel (CZS), een uniek anatomisch compartiment dat beschermt wordt door de bloed-hersenbarrière (BBB) en omgeven is door hersenvocht (CSF). De persistentie van hiv in het CZS draagt bij tot de ontwikkeling van een spectrum van cognitieve stoornissen die gezamenlijk hiv-geassocieerde neurocognitieve stoornis (HAND) wordt genoemd. Ondanks langdurige behandeling treedt bij 20-50% van de hiv-geïnfecteerd populatie nog steeds HAND op, waarvan de meeste een milde vorm hebben.

In dit proefschrift leveren we bewijs ter ondersteuning van het CZS als een viraal reservoir voor hiv. Hiervoor hebben we gebruik gemaakt van verschillende kweekmodellen en technieken om hiv-infectie in het CZS te onderzoeken. Eerst beschrijven we in hoofdstuk 2, een overzicht van kweekmodellen die gebruikt kunnen worden om hiv-infectie in het CZS en met name in de microglia cellen te onderzoeken. In de daaropvolgende hoofdstukken leveren we bewijs ter ondersteuning van het CZS als viraal reservoir, waaronder de verschillen tussen de virus populatie in hersenvocht en bloed (hoofdstuk 3), detectie van intact hiv-DNA in hersenweefsel gedurende langdurige ART (hoofdstuk 4), en het ontrafelen van de target cellen van hiv in de hersenen (hoofdstuk 5). Als laatst geven we in hoofdstuk 6 een overzicht van de huidige literatuur over hiv-genezing strategieën en impact op het CZS viraal reservoir.

Hiv en de CZS

Bij het binnendringen van het lichaam gaat het virus op zoek naar target cellen die een CD4 receptor en een hulp receptor, CCR5 of CXCR4, aan de buitenkant van de cel tot

expressie brengen. Het virus gebruikt deze twee receptoren als een soort antenne om aan de cel te binden en vervolgens te infecteren. In het bloed infecteert hiv voornamelijk CD4 positieve T-lymphocyten, kortweg CD4-cellen. Door de CD4-cellen te gebruiken als een soort kopieermachine, kan hiv zichzelf vermenigvuldigen en vervolgens een groot aantal nieuwe virussen verspreiden door het lichaam waaronder naar het centrale zenuwstelsel.

Kort na de primaire infectie, dringt hiv de hersenen binnen. In **hoofdstuk 3** hebben we de hoeveelheid virus (virale load) in bloed en hersenvocht van mensen met hiv die niet op behandeling zijn geanalyseerd en vonden dat in de meeste individuen (84%) de virale load in het hersenvocht significant lager is dan in het bloed. Hiernaast vonden we 3 individuen die meer virus in het hersenvocht hadden dan in het bloed. Deze bevindingen duiden erop dat de toegang van hiv naar de hersenen selectief is en dat waarschijnlijk ook hiv-productie en/of replicatie in de hersenen plaatsvindt.

Het is bekend dat twee celtypes in de hersenen, namelijk microglia en perivasculaire macrofagen, de CD4 receptor en de hulp receptor CCR5 tot expressie brengen. Hierdoor zijn deze cellen, naast de vanuit het bloed infiltrerende CD4-cellen de meest waarschijnlijke target cellen in het CZS die door hiv geïnfecteerd kunnen worden. In post-mortem (na de dood) hersenonderzoek van hiv-positieve individuen wordt hiv-DNA ook het vaakst aangetroffen in deze microglia en perivasculaire macrofagen. Deze cellen hebben een lange halfwaardetijd van ongeveer 3 maanden (macrofagen) tot 4.2 jaar (microglia). Microglia zijn, in tegenstelling tot macrofagen, een zelfvoorzienende populatie en hebben hiernaast ook speciale mechanismen om het virus in slaap, oftewel latent, te houden. Hierdoor zou hiv voor een lange tijd in de hersenen kunnen overleven. Naast microglia en perivasculaire macrofagen worden astrocyten ook verdacht vatbaar te zijn voor hiv-infectie hoewel dit in het hiv onderzoeksveld controversieel is, omdat astrocyten geen CD4 receptor tot expressie brengen.

Helaas is het zelden mogelijk om humaan hersenweefsel direct te bestuderen, voornamelijk omdat het moeilijk is om vers humaan hersenweefsel te krijgen. Hierdoor gingen we in **hoofdstuk 2** op zoek naar een humaan microglia kweek model dat geschikt is voor hiv-onderzoek. We hebben onderzocht hoe goed de verschillende modellen in staat zijn om de echte microglia zoals verkregen uit hersenweefsel na te bootsen en of ze een productieve hiv-infectie kunnen ondersteunen. Onder productieve hiv-infectie verstaan we het vermogen van een cel om geïnfecteerd te raken en vervolgens nieuwe infectieuze virussen te produceren. Tot voor kort, werd microglia-onderzoek doorgaans uitgevoerd op microglia cellijnen, monocyten-afgeleide microglia (MDMi) en geïsoleerde microglia uit humaan hersenweefsel, de zogenaamde primaire microglia (pMG). Recente technologische vooruitgang in stamcelonderzoek heeft de functionele modellering van de humane

hersenen mogelijk gemaakt door het omzetten van stamcellen tot een verscheidenheid van hersencellen, waaronder microglia (iPSC-MG) en 3D cerebrale organoïden.

Wij vonden dat hoewel elk microglia kweekmodel, behalve de microglia cellijnen, verschillende aspecten van de celstructuur en de functie van microglia kan repliceren, geen enkel kweekmodel in staat is om de echte microglia in de hersenen volledig na te bootsen. Desondanks vonden we dat primaire microglia, monocyten-afgeleide microglia en cerebrale organoïden productieve hiv-infectie ondersteunen met behulp van de CD4 receptor en de CCR5 hulpreceptor die op alle 3 kweekmodellen tot expressie komt. In tegenstelling tot primaire microglia en monocyten-afgeleiden microglia, was de infectie in cerebrale organoïden beperkt met weinig virale spreiding en is hierdoor het meest representatief voor hiv-infectie in de hersenen. Al met al, zijn primaire microglia, monocyten-afgeleide microglia en cerebrale organoïden, op basis van onze bevindingen, allemaal geschikte microglia kweekmodellen voor hiv-onderzoek hoewel cerebrale organoïden en ook de infectie hiervan de meeste gelijkenissen vertonen met de echte infectie van microglia in de hersenen.

Hiv infectie in de CZS

Na het bepalen van het beste kweekmodel hebben we in **hoofdstuk 5** de cerebrale organoïden gebruikt om te onderzoeken welke hersencellen, naast microglia, mogelijk vatbaar zijn voor productieve hiv-infectie. 3D cerebrale organoïden worden vaak mini-breintjes genoemd, wat suggereert dat het om kleine versies van de complexe menselijke hersenen gaat. In werkelijkheid, zijn de humane hersenen te complex om volledig na te bootsen in een laboratorium. Echter de cerebrale organoïden zijn kleine 3D hersenstructuren (2 tot 5mm groot) die een grote verscheidenheid van hersencellen waaronder microglia, astrocyten en neuronen laten zien, die zich structureel organiseren zoals in de ontwikkelende hersenen tijdens de zwangerschap. Door deze cerebrale organoïden te infecteren met een hiv-virus met een fluorescentie label, konden we vaststellen welke cellen geïnfecteerd raakten door de fluorescentie onder de microscoop te visualiseren. Het enige celtype dat productief door hiv geïnfecteerd kon worden, waren de microglia. Hiernaast waren er ook verschillende overeenkomsten tussen hiv-infectie in de cerebrale organoïden en hiv-infectie in de hersenen, waaronder beperkte infectie, weinig virale spreiding en de typische grote microglia complexe celstructuren die vaak gezien wordt in het hersenweefsel van hiv-positieve individuen met hiv-geassocieerde dementia en encefalitis.

Hoewel we nu overtuigend bewijs hebben dat microglia het hoofddoelwit van hiv in de hersenen is, is er één kenmerkend verschil tussen de distrubutie van de CD4 en de CCR5 receptor op microglia en CD4-cellen in bloed. Vergeleken met de CD4 positieve T cellen, is de dichtheid van de CD4 en CCR5 receptoren aan de buitenkant van microglia veel

lager. Hierdoor wordt het verondersteld dat, tijdens chronische hiv-infectie, de virussen afkomstig van het bloed zich genetisch aanpassen aan de lage oppervlaktedichtheid van de hiv receptoren op microglia om ze vervolgens effectief te kunnen infecteren. In **hoofdstuk 3** hebben we in een groep van hiv-positieve individuen die niet op behandeling stonden, onderzocht of er genetische verschillen zijn tussen virussen in het bloed en virussen in het hersenvocht. Hiernaast hebben we gekeken hoe goed deze virussen CD4-cellen en microglia infecteren. Uit onze studiepopulatie bleek dat er over het algemeen weinig genetische verschillen zijn tussen de viruspopulaties in hersenvocht en bloed in de meeste individuen, maar bij twee individuen vonden we dat de viruspopulatie in het hersenvocht aanzienlijk verschilde van de viruspopulatie in het bloed. Dit noemen we compartmentalizatie. De detectie van CZS compartmentalizatie in deze twee individuen duidt erop dat er hoogstwaarschijnlijk niet alleen hiv-infectie maar ook replicatie plaatsvond in hersencellen, dat leidde tot de productie van deze CZS-specifieke virussen. Daarnaast, hoewel alle virussen de CD4-cellen konden infecteren, waren de meeste virussen niet in staat om microglia te infecteren. Dit suggereert dat deze CZS-specifieke virussen waarschijnlijk geproduceerd zijn door T cellen in het CZS. Echter waren er wel enkele virussen die het redelijk goed deden in microglia. Het is nog de vraag of deze virussen tussenproducten zijn op weg naar de transformatie tot virussen die effectief microglia kunnen infecteren.

Het CZS als een hiv-reservoir

Kortom, de bovengenoemde studies leveren samen bewijs voor hiv-infectie en replicatie in het CZS, maar om vast te stellen dat het CZS een hiv-reservoir is hebben we hard bewijs nodig van geïnfecteerde reservoircellen in de hersenen die na reactivatie in staat zijn om nieuwe virussen te produceren die ook weer nieuwe cellen kunnen infecteren. Om kopieën van zichzelf te kunnen maken moet eerst het RNA van hiv ingebouwd worden in het DNA van de cel. Hiervoor gebruikt hiv twee virale eiwitten (enzymen) namelijk reverse-transcriptase, die het viraal RNA omzet in viraal DNA, en integrase die ervoor zorgt dat het virale DNA ingebouwd wordt in het DNA van de cel. Reverse transcriptase maakt constant fouten tijdens het omzetten van RNA in DNA, waardoor het meeste hiv-DNA in CD4-cellen defect is en geen nieuw infectieus virus kan produceren.

Voorgaande post-mortem studies hebben stukjes hiv-DNA aangetoond in de hersenen, voornamelijk in microglia en macrofagen, maar om beschouwd te worden als een onderdeel van het hiv-reservoir moet dit hiv-DNA intact zijn. In **hoofdstuk 4** kijken wij naar de integriteit van hiv-DNA in hersencellen geïsoleerd uit een individu met hiv op langdurige behandeling (post-mortem). Hiervoor hebben we een nieuwe techniek toegepast, genaamd IPDA (intact proviral DNA assay), waarmee we een onderscheid kunnen maken tussen intact en defect hiv-DNA. Zoals verwacht was alleen een klein deel van het totale hiv-DNA intact (13%), maar de prevalentie van intact DNA was ongeveer 2.8-voud hoger

in microglia dan in de resterende hersencellen. Dit levert verder bewijs op dat microglia de meest waarschijnlijke reservoircel voor hiv in het CZS is, maar wijst erop dat ook andere celtypes intact hiv-DNA zouden kunnen hebben en mogelijk kunnen fungeren als een reservoir. Het is nog de vraag of deze reservoircellen met intact hiv-DNA ook in staat zijn om na reactivatie nieuw infectieus virus te produceren die vervolgens bijdraagt aan virale rebound. Om hier inzicht in te krijgen hebben we een belangrijk onderdeel van het hiv-DNA verder onderzocht. Dit is het zogenaamde virale envelop eiwit, dat verantwoordelijk is voor het binden van het virus aan de hiv receptoren op de cel. We vonden dat het envelop stuk van de virussen, geïsoleerd uit de bovengenoemde microglia hersencellen, intact is en effectief konden binden aan de CD4 en CCR5 receptor van CD4-cellen. Na binding, kon het virus de cel binnendringen, zichzelf inbouwen in het DNA van de CD4-cel en vervolgens nieuwe infectieus virus aanmaken. Dit preliminaire experiment ondersteunt het potentieel van microglia-specifieke virussen om naast virale rebound in het CZS ook bij te dragen aan de virale rebound in het bloed door infectie en replicatie in (perifere) CD4-cellen.

Hiv genezing en het CZS reservoir

Hiv is helaas nog niet globaal te genezen. Wereldwijd zijn er 4 mensen genezen van hiv na het ondergaan van een stamceltransplantatie. Helaas kan stamceltransplantatie niet op grote schaal gebruikt worden als genezing voor hiv omdat de kans op overlijden bij een stamceltransplantatie erg groot is. Het hoofddoel van hiv-genezing strategieën is om hiv-positieve individuen in staat te stellen hun hiv-therapie te staken zonder de gevolgen van virale rebound en de daaropvolgende opportunistische infecties die uiteindelijk leiden tot AIDS.

Naast stamceltransplantatie worden er verschillende manieren onderzocht om het slapende virus te verwijderen uit het lichaam en/of permanent te onderdrukken. Twee van de meest veelbelovende hiv-genezing strategieën zijn de “Block and Lock” en de “Shock and Kill” strategie. De block and lock strategie heeft tot doel om geïnfecteerde reservoircellen permanent in een diepe slaap te vergrendelen (lock) om reactivatie en nieuwe virus productie te voorkomen (block). De shock and kill strategie, daarentegen, is de meest onderzochte strategie voor hiv-genezing en berust op het reactiveren van de geïnfecteerde reservoircellen (shock), zodat ze herkent en opgeruimd kunnen worden door het immuunsysteem (kill).

In **hoofdstuk 6** hebben we uitvoerig de shock and kill strategie besproken met betrekking tot eradicatie van het CZS reservoir. Het succes van deze strategie bij de eradicatie van het CZS reservoir is sterk afhankelijk van het vermogen om de reservoircellen in de hersenen efficiënt te activeren zonder gevaarlijke inflammatie en toxiciteit in de hersenen te veroorzaken. Even belangrijk is het binnendringen van immuuncellen vanuit het bloed in het CZS om effectief de geactiveerde reservoircellen op te ruimen. Tot nu toe

is uit onderzoek gebleken dat het mogelijk is om een deel van de slapende virussen in het reservoir te reactiveren, maar helaas is het nog niet gelukt om het virus hierna uit te schakelen en het viraal reservoir te verkleinen. Momenteel wordt er onderzoek gedaan om te kijken hoe we de reactivatie van de reservoircellen kunnen optimaliseren en het immuunsysteem versterken om het virus beter op te ruimen.

Conclusie

In dit proefschrift, leveren we sterke aanwijzingen dat het CZS kan functioneren als een viraal reservoir voor hiv. Wij hebben de microglia geïdentificeerd als het hoofddoelwit van hiv in de hersenen, CZS-specifieke virussen gevonden die aanzienlijk verschilde van de virussen in het bloed en in het bijzonder voor het eerst intact hiv-DNA aangetoond in post-mortem verkregen hersencellen van iemand met hiv die langdurig antiretrovirale behandeling had ondergaan.

Op basis van voorgaande studies zoals beschreven in de wetenschappelijke literatuur, in combinatie met onze eigen bevindingen, zijn we overtuigd dat microglia de voornaamste reservoircel is voor hiv in het CZS en ongetwijfeld een belangrijke rol speelt in het bereiken van hiv genezing. Om CZS te betrekken bij strategieën voor genezing van hiv, hebben wij een uitgebreid begrip nodig van de moleculaire mechanismen die verantwoordelijk zijn voor het vormen en behouden van hiv in de slaapstand in deze cellen. Hiervoor kunnen we de verschillende microglia kweekmodellen gebruiken, waaronder primaire microglia, monocyten-afgeleide microglia en 3D cerebral organoïden, benoemd in hoofdstuk 2. Tenslotte bevelen wij aan om CZS-monitoring toe te voegen aan lopende en toekomstige klinische studies gericht op de genezing van hiv om mogelijke schadelijke neveneffecten op het CZS te beperken en tegelijkertijd meer inzicht te krijgen in het CZS viraal reservoir.

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LIST OF PUBLICATIONS

Publications related to this thesis

1. **Gumbs SBH**, Berdenis van Berlekom A, Kübler R, Schipper PJ, Gharu L, Boks MP, Ormel PR, Wensing AMJ, de Witte LD, Nijhuis M. Characterization of HIV-1 Infection in Microglia-Containing Human Cerebral Organoids. Viruses. 2022 Apr 16;14(4):829.

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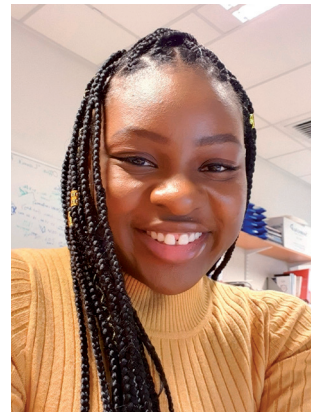
CURRICULUM VITAE



Stephanie Gumbs was born on the 26th of November 1992 in Curaçao. She attended secondary education (VWO) at Maria Immaculata Lyceum in Curaçao, where she obtained her diploma in 2010. Following her graduation, she moved to the Netherlands where she continued her education at Maastricht University and received her Bachelor's in Biomedical Sciences in 2013. She then went on to receive a Master's degree in Biomedical Sciences, majoring in Infection and Immunity, from the University of Amsterdam in 2015. In her master's, she interned at the Amsterdam University Medical Center in the department of Experimental Vaccinology under the supervision of Tom van den Kerkhof and Rogier Sanders. Her research project focused on optimizing the functionality and capability of HIV trimeric envelope glycoproteins to be used as immunogens to elicit broadly neutralizing antibody responses. Her second internship was under the supervision of Sake van Wageningen at the Division of Molecular Carcinogenesis in the Netherlands Cancer Institute where she worked on elucidating the molecular mechanism of the synthetic lethality between MEK-inhibition and knockout of IRE-1α in colorectal cancer cells.

In September 2016, she started as a PhD candidate in the department of Translational Virology at The University Medical Center Utrecht under the supervision of Monique Nijhuis, Annemarie Wensing and Lot de Witte. The aim of her PhD project was to investigate whether the central nervous system, with a particular focus on microglial cells, could function as an HIV reservoir. The results of this work have been presented at multiple (inter)national conferences, published in peer-reviewed international scientific journals, and are collectively described in this thesis.

Following her PhD defense, Stephanie will continue her career as a Postdoctoral Research Fellow in the group of Paul Spearman at the Cincinnati Children's Hospital Medical Center in Cincinnati, Ohio.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