Astrocytes sustain long-term productive HIV-1 infection without establishment of reactivable viral latency

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Abstract
The “shock and kill” HIV-1 cure strategy proposes eradication of stable cellular reservoirs by clinical treatment with latency-reversing agents (LRAs). Although resting CD4+ T cells latently infected with HIV-1 constitute the main reservoir that is targeted by these approaches, their consequences on other reservoirs such as the central nervous system are still unknown and should be taken into consideration. We performed experiments aimed at defining the possible role of astrocytes in HIV-1 persistence in the brain and the effect of LRA treatments on this viral sanctuary. We first demonstrate that the diminished HIV-1 production in a proliferating astrocyte culture is due to a reduced proliferative capacity of virus-infected cells compared with uninfected astrocytes. In contrast, infection of non-proliferating astrocytes led to a robust HIV-1 infection that was sustained for over 60 days. To identify astrocytes latently infected with HIV-1, we designed a new dual-color reporter virus called NL4.3 eGFP-IRES-Crimson that is fully infectious and encodes for all viral proteins. Although we detected a small fraction of astrocytes carrying silent HIV-1 proviruses, we did not observe any reactivation using various LRAs and even strong inducers such as tumor necrosis factor, thus suggesting that these proviruses were either not transcriptionally competent or in a state of deep latency. Our findings imply that astrocytes might not constitute a latent reservoir perse but that relentless virus production by this brain cell population could contribute to the neurological disorders seen in HIV-1-infected persons subjected to combination antiretroviral therapy.

Keywords
astrocytes, central nervous system, HIV-1 reservoirs, latency-reversing agents

1 | INTRODUCTION

One of the major complications of human immunodeficiency virus type-1 (HIV-1) infection in the post-antiretroviral therapy era is the development of a broad variety of neurological dysfunctions such as motor disturbances, cognitive impairments and behavioral changes, better known as HIV-1-associated neurological disorders (HAND). It has been estimated that HAND, which is associated with a worse overall prognosis, could affect up to 50% of HIV-1-infected patients on combined antiretroviral therapy (cART) (Valcour, Paul, Chiao, Wendelken, & Miller, 2011). The most severe form of central nervous system (CNS) damages is called HIV-1-associated dementia (HAD), whereas a milder affliction is referred to as minor cognitive motor disorder (MCMD). Although the incidence of HAD has dropped significantly since the introduction of cART, there is an increase in the prevalence of the less severe MCMD (Harezlak et al., 2011; Saylor et al., 2016). The longer life span of HIV-1-infected persons may provide an explanation for this situation. However, it is also believed that the persistence of HIV-1 replication at low copies in the CNS might induce a progressive neurodegeneration.

Lately, much enthusiasm and interest have arisen about developing a cure for HIV-1 infection through various eradication strategies (Datta et al., 2016; Rasmussen, Tolstrup, & Sogaard, 2016). Most of these cure therapies are aimed at reactivating virus production in latently infected resting memory CD4+ T cells by using latency-reversing agents (LRAs). However, the potential consequence of these LRAs on other reservoirs such as the CNS is still unknown. Indeed, the brain is considered as both a sanctuary site (due to a limited drug penetration)
and a reservoir (pool of long-lived/nonproductively infected cells) for HIV-1. The potential risks for the CNS that are associated with proposed eradication strategies include increased brain infection (due to infectious virus produced by latently-infected cells), virally-mediated damages (via production of neurotoxic viral proteins) and immune-related injuries (such as lymphocyte-mediated encephalitis) (Churchill, Cowley, Wesselingh, Gorry, & Gray, 2015; Nath and Clements, 2011). Hence, a better understanding of the mechanisms of HIV-1 latency/reactivation in the CNS is needed.

HIV-1 enters the CNS early after systemic infection, most likely by infiltration through the blood-brain barrier (BBB) of cells carrying infectious virus particles (e.g., monocytes) (Gras and Kaul, 2010). The perivascular macrophages and microglia are thought to be the major HIV-1-producing cells in the CNS (Gartner, Markovits, Markovitz, Betts, & Popovic, 1986; Koenig et al., 1986), whereas astrocytes could constitute a long-lived reservoir (Kramer-Hammerle, Rothenaigner, Wolff, Bell, & Brack-Werner, 2005). Astrocytes are the most abundant cell type in the CNS and have a crucial role in brain homeostasis (Sofroniew and Vinters, 2010), with very diverse functions such as glutamate transport, synapse remodeling, control of the cerebrovasculature and immune surveillance (Anderson and Swanson, 2000; Dong and Benveniste, 2001; Gordon, Mulligan, & Macvicar, 2007; Kim, Nabekura, & Koizumi, 2017). HIV-1 infection in astrocytes has been a controversial topic for many years. Early post-mortem brain tissue examinations by immunohistochemistry from patients with HAD have revealed the presence of HIV-1 proteins in astrocytes. More recent studies using highly sensitive PCR in single astrocytes have shown that up to 20% of astrocytes could carry viral DNA, the extent of virus infection being correlated with the severity of the neuropathology (Churchill et al., 2009; Trillo-Pazos et al., 2003). Moreover, HIV-1 was detected in pure populations of astrocytes from brain tissues of asymptomatic individuals, therefore suggesting that this cell type contributes very early to the reservoir in the CNS (Thompson, Cherry, Bell, & Mclean, 2011). Although detection of viral DNA might be a consequence of the phagocytosis of HIV-1-infected dead cells by astrocytes rather than a true infection, virus-infected astrocytes are also detected in the simian immunodeficiency virus (SIV) rhesus macaque model (Overholser et al., 2003). Whether HIV-1 causes abortive or productive infection of astrocytes is still a matter of debate (Li, Henderson, & Nath, 2016). Although late HIV-1 markers such as Gag and Envelope (Env) structural proteins were sometimes detected in infected astrocytes, most in vivo studies demonstrate that only early gene virus products such as Nef are detected (Anderson et al., 2003; Ranki et al., 1995; Saito et al., 1994), thus leading to the concept of a restricted infection with limited or no virus production (Ranki et al., 1995; Saito et al., 1994). Even more confusing and contradicting data have emerged from in vitro studies since several investigators claimed that astrocytes and astrocytic cell lines can be either productively (Chauhan, Mehla, Vijayakumar, & Handy, 2014), nonproductively infected (Sabri et al., 1999), or totally resistant to HIV-1 entry (Boutet et al., 2001). Some other experiments suggested a block in the virus life cycle either at the level of transcription or a defect in Rev function (Neumann et al., 1995; Vincendeau et al., 2010). It has been proposed that the main restriction is at the level of virus entry since productive infection can be established when glial cell lines or primary human fetal astrocytes (HFA) are transfected with HIV-1 proviral vectors (Bencheikh, Bentsman, Sarkissian, Canki, & Volsky, 1999) or infected with VSV-G-pseudotyped HIV-1 particles (to bypass the normal process of entry via CD4 and the appropriate co-receptor) (Canki et al., 2001). The entry blockade is seemingly caused by the absence of CD4 expression in astrocytes and infection could be restored following transfection with a CD4 expression vector. Recent studies have suggested two possible modes of HIV-1 entry in astrocytes. First, endocytosis leads to a very inefficient infection mode due to degradation of most HIV-1 particles in the endosomal apparatus (Chauhan et al., 2014; Hao and Lyman, 1999). Second, a more efficient mode of entry relies on cell-to-cell contacts through cocultivation with virus-infected lymphocytes, which involves a CD4-independent fusion of immature/budding virions via gp120-CXCR4 interactions (Li et al., 2015, 2016; Luo and He, 2015).

The concept of HIV-1 latency in astrocytes is also unclear and controversial. It has been shown that HIV-1 can achieve a state of dormancy in astrocytes and can be reactivated by a treatment with tumor necrosis factor (TNF) or interleukin (IL)-1β to produce infectious virus particles (Atwood, Tornatore, Meyers, & Major, 1993; Atwood et al., 1994; Tornatore, Meyers, Atwood, Conant, & Major, 1994; Tornatore, Nath, Amemiya, & Major, 1991). However, most of these data were obtained following transfection of HIV-1 DNA in established cell lines, which may not adequately represent in vivo conditions where proviral DNA is integrated into the host genome. The effect of LRAs to purge HIV-1 from CD4+ T cells has been recently tested in astrocytes, again with conflicting results (Chauhan, 2014; Narasipura, Kim, & Al-Harthi, 2014).

In the present study, we initially assessed the ability of astrocytes to constitute a latent reservoir for HIV-1 and next monitored the effect of various LRAs. Using primary HFA and different infection methods, which mimic as closely as possible the in vivo situation, we report that human astrocytes can sustain a persistent productive HIV-1 infection, possibly for their entire lifespan. Moreover, we engineered a new dual-color HIV-1 molecular clone, which expresses all the viral genes, permitting the detection of latently and productively infected astrocytes. This new tool allowed us to demonstrate that a very small proportion of infected astrocytes display a ‘latent’ phenotype that cannot be reactivated by any of the LRAs tested.

### 2 MATERIALS AND METHODS

#### 2.1 Study approval

This study was approved by the Bioethics Committee at the Centre Hospitalier Universitaire de Québec-Université Laval, Pavilion CHUL. Human peripheral blood mononuclear cells (PBMCs) were obtained from anonymous healthy volunteers who were specifically solicited for the donation of these samples. Written informed consent was obtained from each participant before inclusion in this study. Human fetal brain tissues from 15- to 22-weeks fetuses were obtained from adult female patients undergoing elective abortion at the Clinique de Planification des Naissances du Pavilion CHUL from the Centre Hospitalier Universitaire de Québec-Université Laval with the written informed consent of the patient.
2.2 | Reagents

The 5-Aza-dc (2′-deoxy-5-azacytidine), BIX-01294 (2-[hexahydro-4-methyl-1H,1,4-diazepin-1-yl]-6,7-dimethoxy-N-[1-(phenylmethyl)-4-piperidinyl]-4-quinoxalinamine trihydrochloride hydrate), suberoylanilide hydroxamic acid (SAHA), N,N′-hexamethylene bisacetamide (HMBA), tetraethylthiuram disulfide (dissulfiram), valproic acid, bryostatin-1 and DMSO were all purchased from Sigma–Aldrich (Oakville, ON). The Toll-like receptor (TLR) ligands Pam3Csk4 and polyinosinic:polycytidylic acid (poly(I:C)) were purchased from InvivoGen. Raltegravir and JQ1 were obtained from Cayman chemical (Ann Arbor, MI). The following reagent was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 Nef Polyclonal from Dr. Ronald Swanstrom (Catalog #2949).

2.3 | Isolation and purification of HFA

Fetal brain tissues from 15- to 22-weeks aborted fetuses were obtained from Novogenix Laboratories (Los Angeles, CA) and our institution. HFA were isolated as previously described by Walsh et al. (2014). In brief, blood vessels and meninges were removed from the fetal brain tissues. Thereafter, the tissues were minced, treated for 30 min with 0.2 mg mL\(^{-1}\) DNase I (Roche, Nutley, NJ) and 0.25% trypsin (Life Technologies, Invitrogen, Carlsbad, CA) before being passed through a 70-μm cell strainer. The flow-through was plated in T75 tissue culture flasks for adherent cells (Sarstedt, Numbrecht, Germany) at a concentration of 2×10⁶ cells per flask in MEM supplemented with 10% fetal bovine serum (FBS), 100 U mL\(^{-1}\) penicillin, 100 μg mL\(^{-1}\) streptomycin, 0.3 mg mL\(^{-1}\) L-glutamine, 1 mM sodium pyruvate, MEM nonessential amino acids, 0.5 μg mL\(^{-1}\) amphotericin B (all from Life Technologies) and 0.1% dextrose (Sigma–Aldrich, St. Louis, MO). HFA were grown in a humidified incubator maintained at 37°C under a 5% CO₂ atmosphere and left untouched for 2 weeks before being passaged once a week. After 2 passages, the cell culture consisted of ≥97% astrocytes identified as positive for both glial fibrillary acidic protein (GFAP) and the astrocyte specific transmembrane glycoprotein GLUTamate A5partate Transporter (GLAST, EAT1) (Jungblut et al., 2012) as assessed by flow cytometry (Supporting Information Figure 1).

2.4 | Cell lines and primary human CD4\(^{+}\) T cells

Human embryonic kidney 293T (HEK293T) cells were kindly provided by Dr. Warner C. Greene (The J. Gladstone Institutes, San Francisco, CA) and were in Dulbecco’s Modified Eagle Medium (DMEM) (Fisher Scientific, Ottawa, ON) supplemented with 10% FBS (Fisher Scientific, Ottawa, ON). The reporter LuSV cell line, kindly provided by Janice E. Clements (Johns Hopkins University School of Medicine, Baltimore, MD), was derived from the CEMx174 parental cell line (B-cell/T-cell hybrid) and carries the luciferase reporter gene under the control of the SV40mac239 long terminal repeat (LTR) (Roos, Maughan, Liao, Hildreth, & Clements, 2000). The following reagent was obtained from the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: A3.01 from Dr. Thomas Folks (Buttke and Folks, 1992). To obtain purified primary human CD4\(^{+}\) T cells, PBMCs from healthy donors were obtained initially by Ficoll Hypaque gradient centrifugation and CD4\(^{+}\) T cells were isolated by immunomagnetic negative selection as described in the manufacturer’s protocol (StemCell Technologies, Vancouver, BC) and cultured at a concentration of 2×10⁶ cells mL\(^{-1}\) in RPMI 1640 culture medium (Fisher Scientific) supplemented with 10% FBS.

2.5 | Molecular virus vectors and preparation of virus stocks

The NL4.3 eGFP-ires-Nef Bal env vector and its Env-deficient derivative molecular construct (called NL4.3 eGFP-ires-Nef env) have been described previously (Imbeaut, Lodge, Ouellet, & Tremblay, 2009). The double-labeled Red-Green-HIV-1 (RGH) vector was a kind gift from Dr. I. Sadowski (University of British Columbia, Vancouver, BC) (Dahabieh, Ooms, Simon, & Sadowski, 2013). Expression vectors encoding for the wild-type (CD4-dependent) and mutated (CD4-independent) (called m7NDK) NDK Env were provided by Drs. B. Spire (Institut Cochin de Génétique Moléculaire, Paris, France) and U. Hazan (Institut Cochin, Paris, France), respectively (Dumonceaux et al., 1998; Spire et al., 1989). The infectious molecular clone of HIV-1 89.6 (dual-tropic) was a kind gift from Dr. R. Collman (University of Pennsylvania School of Medicine, Philadelphia, PA) (Collman et al., 1992). VSV-G- and m7NDK-pseudotyped HIV-1 particles were obtained by cotransfection of the NL4.3 eGFP-ires-Nef env vector with pCHMV-G and pJD34, respectively. Virus stocks were produced by calcium-phosphate transient transfection of HEK293T cells as described previously (Cantin, Fortin, Lamontagne, & Tremblay, 1997). Cell-free supernatants were ultracentrifugated to eliminate free p24, resuspended in endotoxin-free phosphate-buffered saline (PBS) (Sigma–Aldrich) and quantified by an in-house double-antibody sandwich ELISA specific for the major core p24 protein (Bounou, Leclerc, & Tremblay, 2002).

2.6 | Construction of a novel HIV-1-based dual-reporter virus vector

The NLENG1-IRES vector, which is a replication-competent X4-using virus construct into which the reporter gene eGFP was inserted in front of an IRES sequence and the Nef gene (kindly provided by Dr. David N. Levy, New York University College of Dentistry) (Levy, Aldrovandi, Kutsch, & Shaw, 2004), was used to engineer the NL4.3 eGFP-IRES-Nef Bal env molecular construct as reported previously (Imbeaut et al., 2009). In brief, the wild-type X4 Env from NLENG1-IRES was replaced with a corresponding fragment of the Bal R5 Env from NL4.3 Bal env (Dornadula, Zhang, Shetty, & Pomerantz, 1999). Next, the infectious molecular clone of HIV-1 89.6 (dual-tropic) was a kind gift from Dr. R. Collman (University of Pennsylvania School of Medicine, Philadelphia, PA) (Collman et al., 1992). VSV-G- and m7NDK-pseudotyped HIV-1 particles were obtained by cotransfection of the NL4.3 eGFP-IRES-Nef env vector with pCHMV-G and pJD34, respectively. Virus stocks were produced by calcium-phosphate transient transfection of HEK293T cells as described previously (Cantin, Fortin, Lamontagne, & Tremblay, 1997). Cell-free supernatants were ultracentrifugated to eliminate free p24, resuspended in endotoxin-free phosphate-buffered saline (PBS) (Sigma–Aldrich) and quantified by an in-house double-antibody sandwich ELISA specific for the major core p24 protein (Bounou, Leclerc, & Tremblay, 2002).
**2.8 | Quantification of HIV-1 proviral DNA copies**

Virus stocks were treated with DNase I (40 μg mL⁻¹) (Roche, Bâle, Switzerland) in the presence of MgCl₂ (10 mM) and EGTA (2 mM) for 45 min at room temperature before HIV-1 infection of HFA. Genomic DNA was extracted from infected cells using Nucleospin® tissue DNA purification kit (Macherey-Nagel, Duren, Germany). Finally, integrated proviral DNA copies were quantified using a combined Alu-HIV-1 PCR and real-time quantitative PCR assay as described previously (Suzuki et al., 2003; Thibault, Fromentin, Tardif, & Tremblay, 2009). To ensure quantification precision, all HIV-1 real-time PCR amplified samples were normalized using the β-globin housekeeping gene. In brief, from every diluted DNA samples, β-globin was quantified using 1 μM of sense (TGGTCTATTTCCTACCCCT) and antisense (TGCCAAAGTGGCCCT-TTGA) specific primers and 0.3 μM of the TaqMan probe 5’-β-globin-VIC-TCTGTCCACTCTGATGCTG-NFQ-MGB 3’. Control experiments consisted of HFA infection in the presence of the reverse transcriptase inhibitor efavirenz (50 nM) or the integrase inhibitor raltegravir (2 μM). These two drug treatments abolished detection of integrated HIV-1 DNA (data not shown), thus confirming the validity of this test to quantify HIV-1 provirus in virus-infected HFA.

**2.9 | Western blotting**

Briefly, cells were washed with cold PBS and resuspended in lysis buffer (50 mM HEPES pH 7.4, 125 mM NaCl, 0.2% NP40, 1 mM DTT and 0.1 mM PMSF). Protein content in cell extracts was determined by a BCA protein assay (Thermo Scientific, Rockford, IL) and equal amounts of proteins (10 μg) were submitted to 12% SDS-PAGE and transferred to Immobilon PVDF membranes (Millipore, Bedford, MA). Immunoblotting was performed using the appropriate antibodies. Rabbit polyclonal anti-Nef (Shugars et al., 1993) (1:1,000) and monoclonal anti-HIV-1 Gag (clone 183-H12-5C, 1 μg mL⁻¹) were detected using a horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibody, and visualized using the Western Lightning Plus-ECL kit (Perkin Elmer, Waltham, MA). The following reagent was obtained from the NIH AIDS
Reagent Program, Division of AIDS, NIAID, NIH: Anti-HIV-1 p24 hybridoma (183-H12-5C).

2.10 | Treatments with LRAs

HFA were treated with VSV-G-pseudotyped NL4.3 eGFP-IRES-Crimson viruses (5 ng p24/10⁵ cells) or m7NDK-pseudotyped NL4.3 eGFP-IRES-Nef env' viruses (50 ng p24/10⁵ cells) and then cultured in DMEM supplemented with 1% FBS for 18–21 days. Thereafter, cells were treated with the following LRAs at the indicated concentrations: JQ-1 (0.5 μM), SAHA (1 μM), HMBA (1 mM), Disulfiram (1 μM), Bix-01294 (1 μM), bryostatin-1 (25 nM) and TNF (100 ng mL⁻¹) for 24 or 72 hr. Cells were finally analyzed by flow cytometry and cell-free p24 contents were measured by ELISA.

2.11 | Flow cytometry

For characterization of HFA cultures, cells were detached using EDTA (5 mM) in the presence of 0.5% BSA, and then stained (surface) with APC-conjugated anti-GLAST (Miltenyi Biotec) and intracellularly with Alexa Fluor 488-conjugated anti-GFAP antibodies (eBiosciences) using BD cytofix/cytoperm kit according to manufacturer’s instructions (BD Biosciences, Mississauga, ON). HFA infected with HIV-1 were detached by a trypsin treatment and fixed with 4% paraformaldehyde for 20 min at room temperature. For intracellular staining, cells were permeabilized and stained with the anti-p24 mAb (clone KC57-RD1, Beckman Coulter). After acquisition on a FACS Canto A flow cytometer (BD Biosciences), data were analyzed with the FCS Express 4 software (De Novo Software, Los Angeles, CA).

2.12 | Immunofluorescence staining and microscopy analyses

HFA were cultured either on μ-Slide eight-well ibiTreat (ibidi, Martinsried, Germany) or glass coverslides. Cells were fixed in 4% paraformaldehyde for 30 min at 4°C. After fixation, cells were permeabilized with 0.2% Triton X-100 and incubated for 30 min with a buffer containing PBS (pH 7.4), 1% bovine serum albumin (BSA) (Fitzgerald Industries International), 20% normal goat serum (Jackson Immunoresearch, West Grove, PA) and 10% human AB serum (Corning) to block nonspecific binding sites. Subsequently, cells were incubated with an anti-p24 mAb (clone 183-H12-5C, 1 μg mL⁻¹) in PBS-1% BSA for 1 hr at room temperature. After extensive washes with PBS, cells were incubated for 45 min at room temperature with Alexa Fluor 546-conjugated goat anti-mouse IgG (1:500; Thermo Fisher Scientific) and 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) (1:1000; Sigma-Aldrich). In some experiments, cells were also stained with an eFluor®660-conjugated anti-GFAP mAb (1:300; eBiosciences). Stained cells were washed extensively before addition of ibidi mounting medium (ibidi, Madison, WI). Visual observations with a 20× objective under a fully automated inverted Leica DMi6000 B microscope (Leica Microsystems, Mississauga, ON, Canada) were then performed. Image acquisition and analysis were done using Volocity Software Version 5.4.0 (PerkinElmer, Waltham, MA).

2.13 | Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6. Ratio paired t test and One-way analysis of variance (ANOVA) with corrections for multiple comparisons (Dunnett) were performed to define the statistical significance of our data. All statistical analyses were performed on raw data. The arcsin transformation was applied to data expressed in percentages. A threshold p value of ≤ 0.05 (*) was considered statistically significant.

3 | RESULTS

Some studies have suggested that HIV-1-infected astrocytes gradually enter in a latent state in which virus transcription is shut down. Most of these experiments were performed in transformed glial cell lines that were transfected with HIV-1 molecular constructs. However, these observations are contradicted by reports showing a sustained productive infection with HIV-1 (Canik et al., 2001; Carroll-Anzinger and Al-Harthi, 2006; Chauhan et al., 2014). We set out to clarify this subject using infectious HIV-1 particles and HFA, which can proliferate up to 10 passages under in vitro conditions. HFA were initially infected with two HIV-1-based reporter viruses, namely RGH and NL4.3 eGFP-IRES-Nef env'. RGH (Red-Green-HIV-1) is a double-labeled, single cycle HIV-1 vector that allows for detection of infected cells independently of long terminal repeat (LTR) activity (Dahabieh et al., 2013), whereas NL4.3 eGFP-IRES-Nef env' is an Env-deficient version of the eGFP-encoding NLENG1-IRES molecular clone (Levy et al., 2004). Progeny viruses produced by these two vectors were pseudotyped with the VSV-G Env to circumvent the established low susceptibility of human astrocytes to HIV-1 infection due to the absence of CD4 surface expression. A gradual decrease in the number of cells productively infected with HIV-1 (as measured by GFP expression) was seen over time when using the two reporter viruses (Figure 1, panels a and b). However, this time-dependent diminution in the percentage of GFP-positive cells was not paralleled by a concomitant increase in the number of mCherry single positive cells (a measure of the frequency of latently infected cells) (Figure 1a). On the contrary, this population was also gradually lost, albeit much slowly that the GFP-expressing cells. This observation suggests that HFA productively infected with HIV-1 did not enter into a latent state but were rather killed or overgrown by uninfected cells. A decline in the p24 levels was also observed at each cell passage, therefore suggesting a gradual loss of cells productively infected with HIV-1 (Figure 1, panels c and d). Next, we evaluated the amount of integrated HIV-1 DNA by a quantitative polymerase chain reaction (qPCR) method. We found a decrease of integrated HIV-1 DNA that paralleled the loss of GFP expression (Figure 1, panels e and f). Cell survival was not affected by HIV-1 infection (data not shown), thus implying that the observed decline in productively infected cells was associated with a reduced cell proliferation. To validate this hypothesis, HFA infected with VSV-G-pseudotyped RGH or NL4.3 eGFP-IRES-Nef env' reporter viruses were stained with violet proliferation dye (VPD), which is a reagent that can be used to monitor individual cell divisions. The
mean fluorescence intensity (MFI) was measured at two distinct time points in mock-infected (used as negative controls), bystanders (uninfected cells in an infected population), latently infected (single mCherry-positive) and productively infected cells (GFP-positive). Results showed that the VPD dilution factor was much higher in mock-infected and bystanders compared with both latently and productively infected cells (Figure 1g). These results indicate that infection with VSV-G-pseudotyped HIV-1 reporter viruses inhibits proliferation of HFA. Interestingly, inhibition of cell proliferation was slightly more important in HFA infected with NL4.3 eGFP-IRES-Nef env− viruses compared with cells inoculated with the Nef-deficient RGH reporter virus (i.e., 2.89- vs. 2.08-fold reduction in VPD dilution).

In the human adult brain, astrocytes are thought to proliferate only in response to trauma and inflammation and do not normally undergo proliferation in vitro. It was not possible to study HIV-1 infection in human adult astrocytes for ethical and practical reasons. Thus, we used HFA from late passages that do not display any proliferative capacity (Yong, Kim, & Pleasure, 1988) to mimic the nonproliferating human

**FIGURE 1** HIV-1 inhibits proliferation of HFA. Proliferating HFA were infected with VSV-G-pseudotyped RGH (a, c, and e) and NL4.3 eGFP-IRES-Nef env− viruses (b, d, and f). (a) The percentage of cells expressing mCherry, GFP, or both was evaluated at the indicated days postinfection by flow cytometry analysis. (b) Virus gene expression was assessed by monitoring the number of GFP-positive cells at the listed days postinfection. (c,d) Virus production was evaluated at the indicated days postinfection by measuring the extracellular cell-free p24 content. Arrows indicate cell passage at a 1:5 dilution factor. (e,f) The percentage of positive cells and integrated HIV-1 DNA copies were measured at the indicated times post-infection and plotted on the same graph. (g) HFA were infected with VSV-G-pseudotyped RGH and NL4.3 eGFP-IRES-Nef env− (named NL4.3-GFP) viruses. Forty-eight hours postinfection, HFA were stained with the Violet Proliferation Dye (VPD) to estimate cell proliferation. Results are presented as the ratio of the MFI for the VPD at day 0 and 3. Fold changes between uninfected bystanders and virus-infected cells are indicated at the top of the column bars [Color figure can be viewed at wileyonlinelibrary.com]
adult astrocytes. When late-passage HFA were infected with RGH or NL4.3 eGFP-IRES-Nef env− pseudotypes, a very stable virus-encoded GFP expression was detected up to 62 days postinfection (Figure 2). No cytopathic effect was observed in virus-infected cells during this period (data not shown). These observations confirm that human astrocytes can sustain a productive HIV-1 infection for an extended time period without any virus-induced cytopathic effect.

The high proportion of so-called latently infected cells observed upon infection with the RGH reporter virus was very surprising. Indeed, the RGH molecular clone is prone to recombination events, hence HFA expressing mCherry but not GFP could likely carry defective proviruses. Also, RGH virus stocks display very low infectivity and this molecular clone does not encode for the regulatory Nef protein. To circumvent these limitations, we designed a replication-competent dual-color reporter virus called NL4.3 eGFP-IRES-Crimson that encodes for all viral proteins and is less susceptible to recombination events (unpublished observations). In brief, we used the NL4.3 eGFP-IRES-Nef Bal env vector into which we inserted the E2-Crimson sequence, which codes for a bright far-red fluorescent protein, under the control of the CMV promoter at the 3′-end of the Nef sequence. To allow for an efficient reverse transcription, the polypurine track (PPT) domain within the Nef sequence was destroyed and inserted in front of the 3′-LTR (Figure 3a). Additional mutagenesis was performed in the Nef sequence that is part of the U3 region to avoid possible recombination events. Importantly, the Nef gene was preserved and the protein was expressed normally in infected target cells as monitored by western blot analysis (Figure 3b). Following infection of HFA with VSV-G-pseudotyped NL4.3 eGFP-IRES-Crimson viruses, we found that the majority of infected cells expressed both GFP and Crimson and a very small proportion expressed only Crimson (Figure 3c). Intracellular staining with the anti-p24 KC57 monoclonal antibody (mAb) showed that the GFP-positive cells also expressed Gag, thus confirming that they are productively infected with HIV-1, whereas the majority of the single mCherry-positive cells were not expressing Gag (i.e., around 90%) (Figure 3d). To better reproduce the brain environment where astrocytes are not subjected to high concentrations of serum, HFA were cultured either in medium supplemented with a much lower concentration of FBS (i.e., 1% instead of 10%) or in serum-free culture medium designed specifically for neural cell culture (i.e., chemically defined N-2 Supplement). A modest decrease in the percentage of productively infected cells was seen in the population cultured in 1% serum, most likely due to a very slow proliferation of HFA. However, the proportion of HIV-1-infected HFA remained very stable over the course of the experiment for cells maintained in N-2 Supplement medium (Figure 3, panels e and f). In addition, the MFI for both GFP- and Crimson-expressing cells did not show any diminution over time, therefore suggesting that HFA could sustain long-term viral gene expression. On the contrary, an increase in MFI intensity was observed that is possibly due to an intracellular accumulation of these stable proteins. In the NL4.3 eGFP-IRES-Crimson molecular virus construct, the GFP gene is expressed from the same mRNA as the viral Nef protein and is thus produced during the early phases of viral gene expression. To assess whether the sustained and early virus-encoded reporter gene expression observed in HFA is paralleled by expression of structural proteins and virus egress, we monitored progeny virus production by measuring the extracellular p24 content. A sustained virus production was observed over 4 weeks (Figure 3, panels e and f). In serum-free N-2 Supplement medium, virus production was reduced 2- to 3-fold but remained stable over time.

The use of HIV-1-based VSV-G pseudotypes in order to circumvent the absence of CD4 surface expression in astrocytes may not adequately mimic the in vivo situation where such cells are infected at a very low rate by a CD4-independent entry pathway and/or through a physical contact with virus-infected cells. The VSV-G Env allows a massive entry of viral particles via a low pH-dependent endocytic pathway and was also shown to suppress requirement for Nef for efficient infection (Aiken, 1997). In addition the VSV-G Env, in contrast to the HIV-1 Env, is not capable of mediating latent virus infection of resting CD4+ T cells (Yu, Wang, Yoder, Spear, & Wu, 2009). Consequently, we used a mutated form of the X4-tropic HIV-1 NDK Env called m7NDK, which permits to achieve infection through a CD4-independent and CXCR4-dependent pathway. This Env glycoprotein is derived from a spontaneously emerged clone of the HIV-1 NDK strain after long-term culture in CEM cells and the CD4-independent phenotype was mapped to 7 mutations in the C2 and C3 regions of the V3 loop (Dumonceaux et al., 1998, 1999). When the m7NDK Env was used to pseudotype NL4.3...
FIGURE 3  Infection of HFA with a new dual-color HIV-1 reporter virus. (a) Schematic representation of the NL4.3 eGFP-IRES-Crimson virus construct. (b) HFA were infected with VSV-G-pseudotyped NL4.3 eGFP-IRES-Nef env” (called GFP-IRES-Nef) or NL4.3 eGFP-IRES-Crimson viruses (called GFP-IRES-Crimson). Cell lysates obtained at 72-hr postinfection were subjected to western blot analysis for the detection of viral Nef and Gag proteins. (c) HFA were infected with VSV-G-pseudotyped NL4.3 eGFP-IRES-Crimson viruses (2.5 or 12.5 ng p24) and analyzed by flow cytometry at 6-days postinfection. (d) HFA were infected with VSV-G-pseudotyped NL4.3 eGFP-IRES-Crimson viruses (50 ng of p24). At 18-days postinfection, cells were stained intracellularly with the anti-p24 KC57-RD1 mAb and analyzed by flow cytometry. The Crimson“GFP” and Crimson“GFP” populations were both analyzed for their p24 content. (e,f) HFA were infected with VSV-G-pseudotyped NL4.3 eGFP-IRES-Crimson viruses (5 ng of p24) and then cultured in DMEM culture medium supplemented either with 1% FBS (e) or serum-free N-2 supplement (f). Expression of both virus-encoded reporter proteins as well as virus production (as revealed by measuring the cell-free p24 content) were evaluated at the indicated days postinfection. The proportion of cells expressing Crimson, GFP or both is shown on the left panels, whereas the MFI is depicted in the center panels. Virus production is displayed on the right panels. Results for one representative experiment out of three performed with different donors are shown [Color figure can be viewed at wileyonlinelibrary.com]
eGFP-IRES-Nef env’ viral particles, we detected a low but reproducible number of virus-infected HFA (Figure 4, panels a and b). Importantly, a sustained GFP expression and virus production were observed over time. To determine the proportion of HIV-1-infected HFA responsible for the observed virus production, HFA were stained with an anti-p24 mAb and visualized by fluorescence microscopy. Results showed that almost all GFP-positive cells were also expressing p24 even at 28 days postinfection, in culture media either devoid of serum or containing a very low level of serum (Figure 4, panels c and d). These data were confirmed by flow cytometry using a very high multiplicity of infection (Figure 4e). A very low infection rate (<0.01%) was observed using the complete, infectious HIV-1 NDK molecular clone, which does not possess the seven mutations conferring the CD4-independent entry phenotype. A persistent viral production was also observed over time with HIV-1 NDK (Figure 5).

Recent data from in vitro studies have suggested that astrocytes could be infected following cell-to-cell contacts with HIV-1-infected cells (Li et al., 2015; Luo and He, 2015). It has been proposed that the
mechanism by which astrocytes get infected in vivo is most likely through physical contacts with virus-infected lymphocytes and/or microglia/macrophages. In an attempt to decipher whether this infection mode would lead to a productive or non-productive infection of HFA, primary human CD4$^+$ T cells were first infected with replication-competent NDK (X4-tropic), 89.6 (dual-tropic), NLENG1-IRES (X4-tropic) and NL4.3 eGFP-IRES-Nef Bal env viruses (R5-tropic) for 72 hr, and were next cocultured with HFA either in absence or presence of the integrase inhibitor raltegravir. A decrease in p24 production was seen over time as remaining infected CD4$^+$ T cells died to become undetectable after 21 days (Figure 6a). However, when LuSIV indicator cells (Roos et al., 2000) (which produce luciferase upon HIV-1 infection) were cocultured with these HFA, a high reporter gene activity was observed suggesting that HFA could transmit infection to other more susceptible cell types even when no virus production can be detected (Figure 6, panels b and c). This infection mode was totally abrogated when raltegravir was present during the T cell-HFA coculture, or if HFA were fixed with PFA before initiation of the coculture, thus indicating that HFA must be effectively infected to transmit HIV-1. Also, a higher infection was observed when CD4$^+$ T cells were left in contact with HFA for several days (Figure 6c). A coculture with CD4$^+$ T cells infected with NL4.3 eGFP-IRES-Nef Bal env viruses did not lead to any detectable infection in HFA or indicator cells (i.e., MOLT-4 CCR5 that were used in replacement to LuSIV which are not susceptible to R5-tropic viruses), thus indicating that HFA are not susceptible to R5-tropic HIV-1. Lastly, no virus infection in HFA was observed when using cell-free viruses, as a coculture step with LuSIV did not produce luciferase activity (Figure 6, panels b and c). Altogether these results indicate that HFA can be infected by X4 or dual-tropic, CD4-dependent HIV-1 strains through cell-to-cell contacts with virus-infected cells such as CD4$^+$ T cells, and that virus transmission can be achieved from such cells toward other susceptible target cells even though viral production is under the detection limit. To confirm this interesting observation and distinguish between a very low productive infection and a latent (non-productive) infection that would be reactivated upon coculture with LuSIV cells, we used fluorescence microscopy to detect HIV-1 production in HFA. We used the highly permissive human T-lymphoblastoid cell line A3.01 (Folks et al., 1985), which was infected with the X4-tropic NLENG1-IRES virus, before coculture with HFA. Although very few virus-infected cells could be detected, we observed that almost all GFP-expressing HFA were also positive for the viral capsid protein p24 (Figure 6d). Staining of the glial fibrillary acidic protein (GFAP), which is a marker to distinguish astrocytes from other glial cells, confirmed that cells expressing GFP and p24 were indeed astrocytes. In summary, our findings suggest that astrocytes can be infected by X4 and dual-tropic HIV-1 strains and sustain a long-term productive HIV-1 infection.

Infection of HFA with our dual-color NL4.3 eGFP-IRES-Crimson reporter virus pseudotyped with VSV-G led to a majority of cells productively infected with HIV-1 (i.e., double-positive for GFP and p24) and a small percentage of Crimson-positive and GFP-negative cells (Figure 3). The vast majority of these cells (90%) were also p24-negative (as monitored by intracellular staining), which might suggest a latent infection. Therefore, our next series of investigations were aimed
FIGURE 6  HFA are productively infected upon coculture with HIV-1-infected CD4\(^+\) T cells. (a) Primary human CD4\(^+\) T cells from two different donors were infected with replication-competent NDK, 89.6, NLENG1-IRES and NL4.3 eGFP-IRES-Nef Bal viruses for 72 hr, thoroughly washed and cocultured with HFA in the absence or presence of raltegravir (2 \(\mu M\)) for 28 days with weekly medium change. Virus production was evaluated at the indicated days postcoculture (dpcc) by measuring the extracellular cell-free p24 content. (b) HFA from panel A were next cocultured with LuSIV indicator cells for 14 days before measuring luciferase activity. Controls consisted of cell-free supernatants from virus-infected CD4\(^+\) T cells (called cell-free) (a,b). (c) Primary human CD4\(^+\) T cells were first infected with the HIV-1 isolate 89.6 and next cocultured with HFA either left untreated, fixed with paraformaldehyde, or treated for 18 hr with raltegravir (Ral). Next, CD4\(^+\) T cells were washed away or maintained in contact with HFA (no wash). Finally, cells were cultured for 21 days before addition of LuSIV indicator cells and luciferase activity was assessed after 4 days. (d) A3.01 T lymphoid cells were infected with X4-tropic NLENG1-IRES viruses for 10 days and next cocultured with HFA for 7 days, either in DMEM supplemented with 10% FBS or in serum-free X-VIV-OTM20 medium. Cells were fixed and stained with DAPI (blue staining) and mAbs directed against GFAP (white staining), GFP (green staining) and intracellular p24 (red staining). Cells were finally visualized by confocal microscopy (original magnification: \(\times 200\)). The percentage of cells positive for each marker is indicated in the table [Color figure can be viewed at wileyonlinelibrary.com]
at defining whether these cells were indeed latently infected with HIV-1 by monitoring the effect of several LRAs known to induce virus gene expression in CD4 T cells. We measured the ratio of latently infected (Crimson\textsuperscript{−}/GFP\textsuperscript{+}) to productively infected HFA (Crimson\textsuperscript{+}/GFP\textsuperscript{+}) and the MFI for both fluorescence markers. Data depicted in Figure 7a demonstrate that none of the tested LRAs used either alone or in combination was able to induce a diminution of the latent/productive ratio (indicative of virus reactivation). GFP fluorescence intensity was not...
significantly affected by the tested compounds (Figure 7b). However, the bromodomain protein inhibitor JQ1 induced a modest and not statistically significant increase in expression of the Crimson reporter gene with no effect on HIV-1 gene expression (Figure 7c). An increase in virus production was seen when bryostatin was used in combination either with JQ1 or SAHA (Figure 7d). This augmentation was variable among donors but did reach statistical significance. Because this phenomenon was not observed with GFP expression (which parallels the expression of Nef), we hypothesized that it could reveal a form of latency where only non-structural proteins such as Nef would be expressed. To test this hypothesis, we assessed both intracellular Gag and GFP expression in infected cells by flow cytometry. We found no significant change in the percentage of GFP-positive cells that also express the p24 protein following treatment with these LRA compounds (i.e., bryostatin/JQ1 or bryostatin/SAHA) (Figure 7e), therefore suggesting that the increase in p24 production was not due to reactivation of latently infected cells but to a higher virus gene expression in productively infected cells. Indeed, an increase in the MFI for KC57 staining was observed for some donors and LRAs (used either alone or in combination) (Figure 7f).

A similar drug treatment for a shorter time period (i.e., 24 hr) had also no significant effect on HIV-1 gene expression (data not shown). Additional studies using the DNA methyltransferase inhibitor 5-aza-dC, two histone deacetylase (HDAC) inhibitors (i.e., valproic acid and romidepsin), TLR2 and TLR3 ligands (i.e., Pam3Csk4 and poly(I:C), respectively) or coculture with CD4+ T cells did not reveal any effect of these treatments on either viral production or the percentage of latently infected cells (data not shown). It can be concluded that none of the tested LRAs were able to reactivate these so-called latently infected cells (based on Crimson expression), which might suggest that these cells either harbor defective proviruses or are in a state of deep latency involving a complete blockade of HIV-1 transcription that cannot be reactivated (Darcis, Van Driessche, & Van Lint, 2017). Additional studies are needed to solve this issue.

Lastly, to define whether our observations were influenced by the use of VSV-G-pseudotyped viruses or a transcriptional interference caused by the CMV promoter present in the NL4.3 eGFP-IRES-Crimson vector, we assessed the outcome of these LRAs on cells infected with NL4.3 eGFP-IRES-Nef particles pseudotyped with the m7NDK Env that were cultured in absence or presence of serum. We found no significant LRA-mediated effect neither on the percentage of GFP- and p24-positive cells nor the MFI for GFP (Figure 8, panels a and b). Again, an increase in the MFI for the p24 staining was observed with some LRAs (e.g., JQ1), thus suggesting that some LRAs might increase HIV-1 gene expression in cells that are productively infected rather than reactivate latently infected cells.

4 | DISCUSSION

Although latently infected resting CD4+ T cells constitute the main reservoir that is targeted by the current “shock and kill” approaches to eradicate HIV-1 from infected individuals, the presence of other reservoirs such as the CNS and the effect of these strategies on them should be taken into account. The persistence of neurological disorders despite effective cART further warrants a better understanding of the mechanisms involved in viral persistence in the CNS. In the current work we have attempted to shed light on the role of astrocytes in HIV-1 persistence in the brain.

We demonstrate first that astrocytes infected with VSV-G-pseudotyped HIV-1 particles display a reduced proliferative capacity compared with uninfected cells (Figure 1). This finding is in agreement with other studies showing a modulation of astrocyte proliferation by HIV-1 (Cosenza-Nashat, Si, Zhao, & Lee, 2006). This might be related with the previously reported down-modulation of several genes involved in the control of the cell cycle (Kim, Li, Bentsman, Brooks, & Volsky, 2004). Although the exact mechanism responsible for this inhibition is still unclear, it has been proposed that it might involve various viral proteins such as Tat, Nef and Vpr (Cosenza-Nashat et al., 2006; Kohleisen et al., 1999; Zhou and He, 2004). In our hands, both Nef-deficient (RGH) and Nef-competent HIV-1 particles induced an inhibition of cell proliferation, although this inhibition was slightly lower for RGH, thus suggesting that this process is independent of Nef. When using the RGH molecular clone, cells expressing mCherry but not GFP showed an impairment of astrocyte proliferation similar to their productively infected counterparts, which indicates that expression of HIV-1 structural proteins might not be necessary for this effect to occur. The physiological relevance of this observation is still unclear since astrocytes in the adult brain are normally quiescent and proliferate only following traumatic, ischemic, and inflammatory insults (Pelny and Nilsson, 2005). Nevertheless, our findings might help to explain the gradual disappearance of virus production over time reported by some studies (Luo and He, 2015; Tornatore et al., 1991).

Interestingly, infection of nonproliferating late-passage HFA led to a productive HIV-1 infection that was sustained for over 60 days (Figure 2). Comparable observations were made when HFA were cultured in serum-free medium that do not promote astrocyte proliferation (Figure 3). A similar persistent viral production was observed in other studies (Chauhan, 2014, 2015). However, the use of HIV-1-based viruses pseudotyped with the VSV-G Env to bypass the natural entry blockade in astrocytes may not adequately represent HIV-1 neuroinvasion in vivo. VSV-G that mediates entry through endocytosis is known also to eliminate the requirement for Nef (Aiken, 1997). Some investigators reported a non-productive infection using non-pseudotyped HIV-1 isolates (Messam and Major, 2000; Sabri et al., 1999). Yet, an important limitation of these studies is that they only measured cell-free p24 production and did not include controls with antiretroviral drugs. Hence, the reported transient HIV-1 production could be due to a slow release of the initial viral inoculum, which can be observed for over 10 days (Chauhan et al., 2014; Clarke et al., 2006). To confirm the existence of a persistent productive infection with HIV-1 particles that possess all the viral genes including Nef and gp120, we infected HFA with GFP-encoding viruses pseudotyped with the CD4-independent NDK Env (i.e., m7NDK) or cocultured together astrocytes and primary human CD4+ T cells infected with fully replicative viruses. We detected GFP- and/or Gag-producing cells up to 1 month following infection. In our
hands, HIV-1 infection was very limited, strictly CXCR4-dependent, totally suppressed by antiretroviral agents and bypassed the CD4-gp120 interaction either through the use of a mutated form of gp120 that can probably interact directly with CXCR4 or via cell-to-cell transmission by a fusion process initiated by immature viral particles in a CXCR4-dependent, CD4-independent manner as demonstrated previously (Li et al., 2015). Other studies reported infection of astrocytes by R5-tropic viruses that could be transferred to T cells (Eugenin and Berman, 2007; Narasipura et al., 2014). The discrepancy with our data might be explained by the use of different viral strains or a different cell model such as progenitor-derived astrocytes that may express the CCR5 co-receptor. Recently, Russel et al. demonstrated that astrocytes were not infected by cell-free viruses or following a contact with virus-infected macrophages, but could engulf debris from HIV-1-infected macrophage (Russell et al., 2017). Their hypothetical model is in line with our observations that HFA are not infected by R5-tropic viruses.

FIGURE 8 LRAs do not reactivate latent virus in HFA upon infection with m7NDK pseudotypes. HFA were infected with m7NDK-pseudotyped NL4.3 eGFP-IRES-Nef env viruses (50 ng of p24) and maintained for 18 days in DMEM culture medium supplemented either with 1% FBS (left panels) or serum-free N-2 supplement (right panels). Cells were then treated with the indicated LRAs for 48 hr. Finally, cells were fixed and stained for intracellular p24 with the KC57 mAb before analysis by flow cytometry. The percentage of GFP- and p24 positive cells is depicted in panel (a) whereas the MFI for GFP and KC57 staining are shown in panels (b,c), respectively [Color figure can be viewed at wileyonlinelibrary.com]
However, our data clearly demonstrate that HFA can be infected by X4-tropic viruses through their contact with virus-infected primary human CD4\(^+\) T cells. It has been proposed that HIV-1 infection in astrocytes may be restricted when the infection is very low, a process that can be overcome when the entry barrier is bypassed by VSV-G pseudotyping (Li et al., 2016). We recapitulated such a very low infection process by using either m7NDK pseudotypes or a coculture step with virus-infected CD4\(^+\) T cells. A sustained productive virus infection was seen in such instances, therefore suggesting the absence of restriction factor(s) after virus entry in astrocytes.

The biological consequences of such persistent viral production in astrocytes are numerous. For example, astrocytes are long-lived cells with a very low turnover rate and can thus ultimately act as a stable HIV-1 reservoir. In addition, virus gene expression is not suppressed by the current ART. Hence viral products may persist for an extended period of time without any new round of infection. A continuous secretion of viral products such as Nef, Tat, or Vpr, which have all been shown to induce neurotoxicity (Chang et al., 2011; Fan and He, 2016; Fields et al., 2015; James, Nonnemacher, Wigdahl, & Krebs, 2016; Ranki et al., 1995; Sami Saribas et al., 2017), could contribute to the pathogenesis of HAND. In addition, astrocytes expressing HIV-1-derived peptides at their surface could be targeted by the immune system, which could promote brain inflammation and immune activation, two processes leading to neurodegeneration and increased risks of lymphocyte-mediated encephalitis. The BBB integrity could also be compromised by very few HIV-1-infected astrocytes through misguided endfeet as well as endothelial apoptosis induced via a gap junction-dependent mechanism (Eugenin, Clements, Zink, & Berman, 2011).

Identification of latently infected cells has been a hurdle in the quest for a cure for HIV-1 infection. Contrary to CD4\(^+\) T cells, in which productively infected cells die rapidly allowing quantification of latently infected cells using a viral outgrowth assay, assessment of latently infected astrocytes within a mixed population of uninfected and productively infected cells requires the use of a reporter protein that is expressed in all cells carrying a provirus. A limited number of dual-color HIV-1 molecular constructs have been designed for such a purpose. We performed our studies with the previously described RGH virus construct (Dahabieh et al., 2013). However, an unusually high amount of eGFP\(^-\)/mCherry\(^+\) cells was detected with this vector, which we believe might be linked with defective proviruses due to recombination, rather than true latent proviruses. We thus engineered a dual-color infectious molecular clone of HIV-1 that encodes for all viral proteins and should be less susceptible to recombination. A much lower percentage of cells expressing only the Crimson reporter protein was obtained with this virus construct. Whether these cells are really carrying latent HIV-1 provirus is still unclear since we were unable to induce viral reactivation using a large panel of LRAs from different classes (e.g., PKC activators, HDAC inhibitors, histone methyltransferase inhibitors and bromodomain inhibitors), including very strong inducers such as TNF and phorbol 12-myristate 13-acetate (PMA) (data not shown for PMA). In addition to the more classical HIV-1 inducers, we also evaluated the reactivating potential of some TLR ligands since they were identified as potential LRAs in CD4\(^+\) T cells (Novis et al., 2013) and very recently in latently infected microglial cell lines (Alvarez-Carbonell et al., 2017). Again, no virus reactivation was observed with TLR2 and TLR3 ligands either used alone or in combination with TNF. Lastly, a contact with live, apoptotic or necrotic CD4\(^+\) T cells did not reverse virus latency. These data are in agreement with previous studies showing no increase in HIV-1 production by LRAs in persistently infected astrocytes (Chauhan, 2015; Li et al., 2016). However, other studies reported some reactivation with bryostatin (Diaz et al., 2015), QJ1 and various HDAC inhibitors (Gray et al., 2016; Narasipura et al., 2014). Differences in experimental methodologies may account for the discrepant findings. For example, Narasipura et al. used primary human progenitor-derived astrocytes and astrocytoma cell lines, and in addition they report a productive infection with the R5-tropic HIV-1 BaL virus, whereas others and we found that HFA are totally resistant to infection with R5-tropic viruses (Li et al., 2015; Russell et al., 2017). They also used LTR-driven luciferase reporter virus constructs, which may not adequately represent infection with a complete molecular clone of HIV-1. In addition, the high concentration of SAHA used in this study (i.e., 10 \(\mu\)M) was toxic when we used it with HFA (data not shown). Gray et al. reported that some LRAs promote HIV-1 transcription in HFA (Gray et al., 2016). However, they only monitored HIV-1-specific RNA levels, which do not necessarily reflect protein expression. In fact, it was recently shown that HIV-1 RNA production in CD4\(^+\) T cells is not always accompanied with p24 production (Passaes et al., 2017). The very modest increase in p24 production reported by Diaz et al. (Diaz et al., 2015) is in our view not significant and may very well be caused by a delayed release of the initial viral inoculum, which can occur up to 10-days postinfection (Chauhan et al., 2014). Lastly, these studies did not assess HIV-1 expression at the single-cell level. Hence, a global increase in RNA expression or p24 production may very well be caused by a stimulation of cells already productively infected rather than the reactivation of latently infected cells. Indeed, we did observe a modest increase in p24 production following treatment with some combinations of LRAs. However, this increase was not caused by reactivation of latently infected cells (Figures 7e and 8a). Our data were obtained exclusively in primary HFA that were inoculated with HIV-1 reporter viruses containing all viral genes, and we monitored simultaneously GFP and p24 expression. Hence, we believe that our conclusions may be more relevant with regards to the in vivo situation. In addition, we tested different culture media that are more representative of the brain environment (absence or reduced levels of serum), with similar observations albeit with a reduced p24 production.

Taken together our observations suggest that, although HIV-1 infection of astrocytes with cell-free virus is very inefficient due to a block in virus entry, these cells can nonetheless support viral production for a very long-time period once they are infected by CD4-independent viral strains or by a coculture step with HIV-1-infected CD4\(^+\) T cells. Moreover, HFA do not appear to support a latent HIV-1 infection that can be reactivated upon treatment with LRAs. One should exert caution when drawing conclusions from these data as we have used HFA that are likely to differ phenotypically from adult astrocytes. It would then be of great interest to repeat these experiments.
with adult astrocytes, as well as with mixed cultures that could more adequately recapitulate the brain microenvironment. In addition, it would be useful to use HIV-1 clones isolated from the brain of infected patients. Indeed, there is strong evidence for compartmentalization of HIV-1 in the CNS, therefore suggesting that the virus evolves in the brain and may acquire unique properties. Some studies have demonstrated the compartmentalized nature of the HIV-1 promoter region and LTRs from brain-derived viral isolates tend to display a lower basal transcriptional activity (Churchill et al., 2015; Gray et al., 2013), a feature that could promote the establishment of latency. Also, analyses of brain-derived Env sequences suggest a high compartmentalization and selection for a lower CD4 dependence (Vazquez-Santiago and Rivera-Amill, 2015). The reduced contribution of CD4 in the virus life cycle appears to correlate with the extent of astrocyte infection in a simian-human immunodeficiency virus model of encephalitis (Zhuang et al., 2014).

Our data raise the question of the existence of latency in astrocytes. Although the Crimson+/GFP− cells may harbor defective proviruses, it can also be proposed that such cells carry silent proviruses in which latency might be due to mechanisms that could be different from the ones seen in CD4+ T cells. They could also be similar to the replication-competent noninduced proviruses described in CD4+ T cells from treated patients, which inability to be reactivated cannot be explained by repressive chromatin modification or transcriptional interference (Ho et al., 2013). Further analyses will be necessary to solve this issue, including fluorescent in situ hybridization targeting ribonucleic acid molecules (RNA FISH) to assess if these cells express viral RNA and testing new compounds exhibiting distinct mechanisms of action. For example, benzotriazoles were recently identified as potential LRAs through inactivation of STAT5 SUMOylation (Bosque et al., 2017). Nevertheless, whether or not latently infected astrocytes are present in the brain of HIV-1-infected patients, our data suggest that eradication of HIV-1 from the brain via the “shock and kill” approach might be challenging. In fact, the unique feature of HIV-1 infection in the CNS such as the presence of long-lived cells that support HIV-1 production without significant cytopathic effects and low accessibility to antiretrovirals and the immune system, hint towards an alternate strategy of locking out proviruses in a deep latency state. The recent interest in the development of therapeutic agents aimed at repressing HIV-1 transcription has already yielded several promising research avenues (Darcis et al., 2017; Mousseau, Mediouni, & Valente, 2015). This “lock” strategy that would lead to a functional rather than sterilizing cure would have the advantage of being safer for the CNS.

ACKNOWLEDGMENT

The authors thank Caroline Côté for her technical support. They acknowledge the special contribution of the Bioimaging platform of the Infectious Diseases Research Centre, which was funded by an equipment and infrastructure grant from the Canada Foundation for Innovation, and are also grateful for the service provided by the clinical research team at the infectious disease unit. This study was supported by the Canadian Institutes of Health Research grant MOP-133696. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication. M.J.T. holds the CIHR-Canada Research Chair in Human Immuno-Retrovirology (Tier 1 level).

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*How to cite this article:* Barat C, Proust A, Deshiere A, Leboeuf M, Drouin J, Tremblay MJ. Astrocytes sustain long-term productive HIV-1 infection without establishment of reactivable viral latency. *Glia*. 2018;00:1–19.  https://doi.org/10.1002/glia.23310