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HIV-1 infection renders brain vascular pericytes susceptible to the extracellular glutamate

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Abstract

Reduced pericytes coverage of endothelium in brain is one of the structural changes leading to breach of the Blood Brain Barrier during HIV infection. We previously showed in central memory T (T_{CM}) cells that HIV latency increases cellular susceptibility to DNA damage. In this study we investigated susceptibility of primary brain pericytes infected with HIV-1 to DNA damage in response to glutamate and TNF α , both known to induce neuronal death during chronic inflammatory conditions. To infect pericytes, we used a single-cycle HIV-1 pseudotyped with VSV-G envelope glycoprotein and maintained the cultures until latency was established. Our data indicate that pericytes silence HIV-1 expression at similar rate compared to primary T_{CM} cells. TNF α and IL-1 β caused partial reactivation of the virus suggesting that progression of disease and neuroinflammation might facilitate virus reactivation from latency. Significant increases in the level of γ H2AX, which reflect DNA damage, were observed in infected cultures exposed to TNF α and glutamate at day 2 post-infection. Glutamate, an excitatory neurologic stimuli, also caused increases in the γ H2AX level in latently infected pericytes, whereas PARP and DNA-PK inhibitors caused reductions in cell population suggesting that HIV-1 latency affects repairs of single and double strand DNA breaks. For comparison, we also analyzed latently infected astrocytes and determined that DNA damage response in astrocytes is less affected by HIV-1. In conclusion, our results indicate that productive infection and HIV-1 latency in pericytes interferes with DNA damage response, rendering them vulnerable to the agents that are characteristic of chronic neuroinflammatory disease conditions.

Keywords

Pericytes; astrocytes; central memory T cells; HIV latency; glutamate; DNA damage response

INTRODUCTION

Pericytes have a crucial role in the formation and maintenance of the Blood Brain Barrier (BBB) integrity, and are also important mediators of neuroinflammation (Cho *et al*, 2017; Jansson *et al*, 2014; Rustenhoven *et al*, 2017). Their significance to BBB function is also

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CONFLICT OF INTEREST

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highlighted by increased density of pericytes covering the central nervous system (CNS) vasculature, when compared to capillaries and venules in other parts of the body (Aguilera and Brekken, 2014; Armulik *et al*, 2010; Bell *et al*, 2010). The pericytes coverage is reduced in diseases characterized by BBB impairment, such as Alzheimer's disease (AD), multiple sclerosis (MS), amyotrophic lateral sclerosis (ALS), and also HIV-associated neurocognitive disorders (HAND) (Aguilera and Brekken, 2014; Persidsky *et al*, 2016; Sengillo *et al*, 2013; Winkler *et al*, 2013). Chronic neuroinflammation associated with these diseases causes pericyte dysfunction characterized by changes in their phenotype leading to detachment from endothelial cells, reduced expression of factors promoting BBB formation and integrity, and increased expression of molecules promoting monocytes infiltration (Jansson *et al*, 2014; Persidsky *et al*, 2016; Rustenhoven *et al*, 2017). All these contribute to increased leakage through the BBB.

HIV-1 invades the CNS early after the initial infection and contributes to development of neuroinflammation. The condition is characterized by chronically increased levels of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF α), and excess extracellular glutamate and nitric oxide (NO) (Brahmachari *et al*, 2006; Holguin *et al*, 2004; Saylor *et al*, 2016). Viral proteins Tat and envelope glycoprotein gp120 have been shown to affect glutamate homeostasis (Haughey *et al*, 2001; Melendez *et al*, 2016; Musante *et al*, 2010; Potter *et al*, 2013; Vesce *et al*, 1997). Tat was also found to induce changes in pericytes resulting in increased cell migration suggesting that the loss of pericyte coverage in the BBB during HAND is due to their detachment from endothelium (Niu *et al*, 2014).

The major cells in the CNS supporting HIV-1 replication are microglia and macrophages, but the virus also has an ability to infect pericytes and astrocytes, and although at a low level, their infection was found to disrupt the integrity of the endothelial barrier (Cho *et al*, 2017; Churchill *et al*, 2006; Eugenin *et al*, 2011; Nakagawa *et al*, 2012; Thompson *et al*, 2011; Tornatore *et al*, 1994; Wiley *et al*, 1986). Permissiveness of pericytes to non-productive HIV-1 infection suggests that the cells might support viral latency. We and others showed that HIV-1 affects DNA damage response (DDR) in cells during virus expression, as well as during latency (Belzile *et al*, 2010; Piekna-Przybylska *et al*, 2017; Roshal *et al*, 2003; Sun *et al*, 2006; Zhu *et al*, 2001; Zimmerman *et al*, 2004). We hypothesize that the loss of pericyte coverage in brains of patients with HAND is also related to an increased death rate of infected pericytes due to higher susceptibility to oxidative stress induced by chronic neuroinflammation, particularly glutamate and TNF α . Chronic excess of extracellular glutamate during neuroinflammation is well known to act as a neurotoxin, which triggers oxidative stress and DNA damage leading to neuronal death (Lau and Tymianski, 2010). TNF α is also a key player in neuronal death during inflammatory disease states (Liu *et al*, 2014; Tweedie *et al*, 2007; Zou and Crews, 2005).

In order to determine susceptibility of HIV-1 infected pericytes to neuroinflammatory conditions, we analyzed the level of DNA damage in response to pro-inflammatory cytokine TNF α and to glutamate soon after infection and during latency. For comparison, we also analyzed infected astrocytes. We show that pericytes, and also astrocytes, can efficiently silence HIV-1 expression at a similar rate as primary central memory T (T_{CM}) cells, and that the viral latency renders pericytes sensitive to the excess of extracellular glutamate. By

exposing latently infected cultures to DDR inhibitors, we determined that HIV latency affected more pericytes than astrocytes, in repairs of single (SSBs) and double strand DNA breaks (DSBs).

RESULTS

Productively infected primary brain pericytes are susceptible to glutamate and TNF α .

To investigate the effect of neuroinflammatory conditions on infected brain pericytes we used primary human brain vascular pericytes and infected them with HIV-1 NL4–3 strain. We used single-cycle virus (DHIV) with defective *env*, which precludes production of infectious viral particles after a single round of infection. We also used HIV-1 NL4–3 engineered to encode green fluorescent protein (GFP) as a transcriptional reporter of virus genes (HIV-GFP). Since permissiveness of pericytes to HIV infection is low, we used VSV-G *env* for pseudotyping HIV to increase efficiency of virus entry into the cell. VSV-G *env* has broad tropism and is used with HIV vectors and other retroviruses to increase population of infected cells in a culture.

For HIV-1 infection, primary brain pericytes were seeded to achieve 80–90% confluency and then exposed to HIV-GFP or DHIV (both at 10 ng/ml of p24) for 24 h. On day 1 post-infection (1 dpi), we observed small population of GFP-positive or p24-positive cells (not shown), but at 2 dpi almost entire population expressed GFP (Fig. 1a), or stained positively for p24 (Fig. 1b). Using Vi-CELL counter and fluorescent microscope we determined that the number of cells in infected population was reduced by about 50% at 2–3 dpi (see Fig. 2a), whereas immunofluorescent staining showed the presence of gamma-H2AX (γ H2AX) foci in nuclei, indicating phosphorylation of histone H2A variant in response to DNA damage (Fig. 1a). Since single-cycle virus cannot produce new viral particles, the reduction in pericytes population could be due to their sensitivity to the expression of viral proteins, and resulted from cell cycle arrest and apoptosis (Badley *et al*, 2000; Cummins and Badley, 2010; de Silva *et al*, 2012; Herbein *et al*, 1998; Piekna-Przybylska and Maggirwar, 2018; Planelles *et al*, 1995; Tachiwana *et al*, 2006).

The γ H2AX protein is a marker of DSBs formation, in response to signaling events triggered by multiple forms of DNA damages, like oxidative DNA damage, formation of covalent DNA adducts, replication stress and formation of SSBs (Guo *et al*, 2010; Khoronenkova and Dianov, 2015; Olive, 2009; Yan *et al*, 2011; Yan *et al*, 2014). Therefore, the effects of extracellular glutamate and TNF α on DDR in pericytes were analyzed by monitoring the level of γ H2AX. At day 1 post-infection, we exposed uninfected and DHIV infected cells to glutamate (250 nM), or TNF α (75 ng/ml); and 24 h later (2 dpi) we analyzed the γ H2AX levels by fluorescent microscope. Since infected cells showed reduction in cell numbers (about 50%), we compared the levels of γ H2AX by calculating average fluorescence intensities of Alexa Fluor 647 used to detect γ H2AX. Data were normalized to basal level of fluorescence calculated for uninfected and untreated pericytes (Fig. 1b). Glutamate and TNF α treatment caused 10–20% increases in fluorescence intensity of the γ H2AX signals for uninfected cells, when compared to untreated cultures. For DHIV infected pericytes, there were 20–30% increases in intensity of the γ H2AX signals, but up to 50% for infected cells exposed to glutamate and TNF α . These increases also correlated with

reduction in the number of cells, especially for cells treated with glutamate for both infected and uninfected pericytes. The results, summarized in Figure 2a, show that in reference to untreated and uninfected pericytes, DHIV infection reduced pericytes population to $52 \pm 2.9\%$. This reduction likely results from HIV-induced cell cycle arrest and apoptotic cells death. In reference to untreated cultures (Fig. 2b), TNF α caused reductions in cell number to $92.3 \pm 2.9\%$ for uninfected pericytes, and to $87.6 \pm 8\%$ for DHIV infected pericytes. This difference was not statistically significant. However, for cells treated with glutamate, there were reductions in the cell number to $74.4 \pm 9.1\%$ and to $58.5 \pm 5.6\%$ for uninfected and DHIV infected cultures, respectively. Thus, although TNF α treatment also induced formation of γ H2AX, it had a smaller effect on the cell number. In conclusion, these results suggest that upon HIV-1 infection brain pericytes become more susceptible to neuroinflammatory mediators.

The rate of HIV-1 latency in primary human brain pericytes is comparable to that in central memory T cells.

To determine whether pericytes support HIV latency, the cultures of infected pericytes were maintained for a period of 11–13 days post-infection and monitored for virus expression. Application of the virus with defective *env* precluded production of infectious viral particles after a single round of infection, while pseudotyping HIV-1 with VSV-G envelope protein allowed infection of entire population. Since virus did not spread in the cell culture, we were able to determine how efficiently pericytes can silence HIV-1 expression once they are infected. The HIV-1 silencing was monitored by flow cytometry and microscopic imaging of the GFP or p24 expression following infections with HIV-GFP and DHIV, respectively. The rate of HIV-1 silencing in pericytes was also compared with that in T_{CM} cells. In this case, the T_{CM} cells were infected with HIV-GFP and DHIV in an analogous manner. As shown in Figure 3a and 3b, cultivation of primary brain pericytes infected with HIV-GFP or DHIV led to a dramatic loss of HIV-1 expression over a period of one week. About 5% of pericytes population were GFP positive at day 1 post-infection. By day 2–3 post-infection over 80% of pericytes turned GFP positive, and this population was reduced by a half by day 5–6 post-infection (2–3 days later). By day 11–12 post-infection, the GFP positive population was only about 2% (not shown). Integration of provirus into the host genome was confirmed by *Alu-gag* polymerase chain reaction (PCR) followed by nested PCR reactions using genomic DNA isolated from cells collected at day 11 post-infection (Fig. 3c). A small population of GFP negative cells at 3 dpi likely represent cells that were GFP-positive at 1–2 dpi that had already silenced virus expression. We observed similar flow cytometry profiles for cells infected with larger quantities of virus. Quick silencing of virus expression indicates that cellular environment in brain pericytes helps to enforce virus latency. This observation is consistent with previous reports indicating that HIV-1 does not propagate well in pericyte cultures (Nakagawa *et al*, 2012).

In addition to TNF α , development of neuroinflammation during HIV infection is associated with increased expression of interleukin 1 beta (IL-1 β), interferon gamma (IFN- γ) and IL-6. Among these, TNF α , IL-1 β and IL-6 were found to have an ability to reactivate latent virus in T cells or macrophages (Chun *et al*, 1998; Devadas *et al*, 2004; Granowitz *et al*, 1995; Oguariri *et al*, 2007; Poli *et al*, 1994; Saleh *et al*, 2011). To test whether latent HIV-1 can be

reactivated in pericytes, on day 10 post-infection the cells were exposed to TNF α (50 ng/ml) together with IL-1 β (50 ng/ml) for 24 h. Flow cytometric analyses showed that TNF α +IL-1 β treatment increased the GFP positive population in latently infected pericytes cultures from about 3% to 11% (Fig. 3d). This suggests that the accumulation of pro-inflammatory milieu in the brain might induce HIV reactivation from latency in pericytes and drive replication of the virus at a low level.

Next, we analyzed the rate of HIV-1 silencing in pericytes by comparing it with the rate observed in T_{CM} cells infected by the same virus. It is noteworthy that the T_{CM} cells constitute the major HIV-1 reservoir known to support viral latency (Soriano-Sarabia *et al*, 2014). We first selected primary naïve CD4⁺ T cells from peripheral blood mononuclear cells (PBMC) and primed them towards differentiation into nonpolarized (NP) cells, which resemble the phenotype of the T_{CM} cells (Fig. 3e) (Bosque and Planelles, 2009; Piekna-Przybylska *et al*, 2017). Infections with HIV-1 were performed when cells were still in an activated stage, and latency was established by progression of activated cells to a quiescent, memory-like stage. As shown in Figure 3f and 3g, by day 3 post-infection about 20% of population were GFP positive, and this population was reduced by half on day 5–6 post-infection (2–3 days), similar to what was observed for infected pericytes. Cells returned to a quiescent stage by day 11–12 post-infection. This observation is also consistent with a rate of HIV silencing in primary memory T cells when latency is established with replication competent virus in the presence of cART (Martins *et al*, 2016), and indicates that HIV-1 expression is silenced in pericytes in similar time frame, and at similar rate as in T_{CM} cells.

HIV-1 latency in brain vascular pericytes affects cellular DDR to glutamate.

To determine the sensitivity of latently infected pericytes to the inflammatory molecules, at day 10 post-infection (DHIV), cells were collected and counted to seed equal number of cells in new dishes. The next day, we treated cells with 250 nM glutamate for 24 h, and analyzed the level of γ H2AX by flow cytometry. No differences in cell numbers were observed for glutamate treated cultures. However, the results summarized in Figure 4, show that infected pericytes exposed to glutamate have greater increases in the γ H2AX levels. Elevated glutamate during chronic neuroinflammation is known to be associated with mitochondrial dysfunction and oxidative stress that generates sufficient reactive oxygen species (ROS) to cause DNA damage (Kritis *et al*, 2015). The damage from ROS is likely to be a mixture of oxidative damage to DNA bases, SSBs and DSBs. Thus, increases in the level of γ H2AX in response to glutamate would indicate that DNA repairs induced by ROS are not processed efficiently in infected cells. Base Excision repair (BER) pathway, which processes oxidative DNA damage, transiently generates abasic sites that can be converted to SSBs and DSBs (Szczepanski *et al*, 2010). Therefore, we also analyzed efficiency of DDR in latently infected pericytes by targeting poly adenosine diphosphate [ADP] ribose polymerases (PARP), DNA-dependent protein kinase (DNA-PK) and protein kinase ataxia-telangiectasia mutated (ATM), the factors that are intimately involved in repairs of SSBs and DSBs.

PARP is a family of enzymes involved in the repair of SSB, and assist in repairs of nicks generated as a result of BER (Ame *et al*, 2004; Schreiber *et al*, 2002). This enzyme is also

involved in repairs of DSBs (Beck *et al*, 2014). DNA-PK is required for the repairs of DSBs through non-homologous end joining (NHEJ) pathway, and maintenance of telomeres (Davis *et al*, 2014; Gilley *et al*, 2001; Williams *et al*, 2009). ATM is well known for its role in activating the DDR due to formation of DSBs (Bakkenist and Kastan, 2003; Ward *et al*, 2001). At day 10 post-infection, equal number of latently infected and uninfected pericytes were seeded on new dishes, and 24h later the cultures were exposed to 3-ABA (6 mM), NU7441 (1 μ M), or KU55933 (1 μ M), which are the inhibitors of PARP, DNA-PK and ATM, respectively. After 30 h of treatment, we analyzed the number of cells and their viability by using Vi-CELL counter or fluorescent microscope. The results, summarized in Figure 5, show that there was no significant difference between cultures of infected and uninfected pericytes treated with ATM inhibitor. However, inhibitors of DNA-PK and PARP reduced population of latent cells for about $19 \pm 4\%$ and $40 \pm 6\%$, respectively, when compared to uninfected pericytes treated with these inhibitors. In conclusion, increased cytotoxic effects of DDR inhibitors for latently infected cells indicate that HIV latency established in brain pericytes may affect cells ability to efficiently respond to DNA damage induced by the excess of extracellular glutamate.

Primary cultures of pericytes and astrocytes are differentially affected by HIV-1 latency.

For comparison, we also analyzed DDR in infected primary brain astrocytes to evaluate increased susceptibility of HIV-1 infected pericytes to neuroinflammatory conditions. Using the same approach described for pericytes, we established infections in astrocytes isolated from cerebral cortex, and observed similar rate of HIV-1 silencing. By day 2–3 post-infection over 90% of astrocytes turned GFP positive, and this population was reduced to about 40% by day 7 post-infection, and to only about 10% by day 10 (Fig. 6a). Cultures of uninfected and latently infected astrocytes were then used to analyze the effects induced by glutamate and inhibitors of PARP, DNA-PK and ATM, using the same conditions as for pericytes.

The results, summarized in Figure 6b, show that about $10 \pm 3\%$ of infected astrocytes stained positively for the presence of γ H2AX, and it increased to $17 \pm 3\%$ following their exposure to 250 nM glutamate. For uninfected cultures, the background level of γ H2AX was $3.5 \pm 1.8\%$, and it increased to $7 \pm 2.5\%$ after glutamate treatment. For cultures exposed to inhibitors, we analyzed the cell numbers using Vi-CELL counter and fluorescent microscope. The results, summarized in Figure 6c, show that population of latently infected astrocytes was less affected by DDR inhibitors, as we observed for latently infected pericytes (Figure 5). When compared to uninfected astrocytes, there was about $7 \pm 4.1\%$ reduction in cells population of latently infected astrocytes in cultures exposed to 1 μ M NU7441, and about $11 \pm 6.1\%$ reduction in cultures exposed to 6 mM 3-ABA.

Taken together, our results indicate that latently infected pericytes are more susceptible to DDR inhibitors, as opposed to latently infected astrocytes, and thus suggest that DDR in pericytes is less efficient after HIV-1 infection. Therefore, reduced ability to perform DNA repairs would predispose pericytes to cytotoxic effects of excessive glutamate implicated in neuronal injury in many disorders of CNS, including HAND (Vazquez-Santiago *et al*, 2014).

DISCUSSION

Inflammation in the CNS is associated with chronic oxidative stress, a key feature of all neurodegenerative diseases, including HAND (Ferrell and Giunta, 2014; Scutari *et al*, 2017). The condition results from progressive loss of neurons due to oxidative stress-mediated DNA damage triggered by excess of extracellular glutamate, nitric oxide (NO) and sustained release of inflammatory mediators, such as TNF α , IL-1 β , and IFN γ (Brabers and Nottet, 2006; Ferrarese *et al*, 2001; Tavazzi *et al*, 2014). In addition to loss of neurons, recent studies show that brain pericyte coverage is diminished in HIV-infected patients with or without combination antiretroviral therapy (cART) (Persidsky *et al*, 2016). Pro-inflammatory cytokines are indicated as major contributors of pericyte depletion during chronic neuroinflammation that results from pericyte detachment and migration from BBB (Jansson *et al*, 2014; Persidsky *et al*, 2016; Rustenhoven *et al*, 2017). In these studies, we investigated whether diminished coverage of pericytes in brain of HIV-infected patients could be also associated with increased susceptibility of infected pericytes to neuroinflammatory conditions.

We recently reported that HIV-1 latency in T_{CM} cells affects cellular DDR, and results in increased susceptibility to DNA damage (Piekna-Przybylska *et al*, 2017). Pericytes are permissive to HIV-1 infection, although the rate of virus replication appears to be even lower than in astrocytes, where HIV-1 replication is also restricted (Nakagawa *et al*, 2012). Nevertheless, low level of HIV-1 replication also suggests that after infection the cells can suppress virus expression and promote viral latency. Since chronic neuroinflammation induces DNA damage in neurons and leads to their death, and HIV-1 has ability to interfere with cellular DDR, we considered that depletion of pericytes in the brain also results from increased susceptibility to neuroinflammation when pericytes are infected. Our data indicate that pericytes, and also astrocytes, can efficiently silence HIV-1 expression, suggesting that these cells support HIV-1 latency in the brain. The ability of TNF α and IL-1 β to reactivate HIV-1 in pericytes also suggests that the progression of HAND disease, characterized by sustained release of pro-inflammatory factors in the CNS, may stimulate virus reactivation by terminating latency. Reductions in cell number observed at day 2–3 post-infection likely result from two factors, one is cell cycle arrest and another is apoptosis. Cell death related to HIV infection is well documented. Most of HIV infected cells undergo apoptotic cell death following infection, and it is associated with pro-apoptotic qualities of viral proteins gp120, Tat, Nef, Vpr, Vpu and HIV protease (Badley *et al*, 2000; Cummins and Badley, 2010; de Silva *et al*, 2012; Herbein *et al*, 1998; Planelles *et al*, 1995; Tachiwana *et al*, 2006). Infected primary CD4⁺ T cells undergo increased rate of apoptosis after HIV infection (Piekna-Przybylska and Maggirwar, 2018). The apoptosis is also induced by HIV reactivation in primary memory T cells and in T cell lines even for latent cells established with defective viruses which cannot produce viral particles (Piekna-Przybylska and Maggirwar, 2018).

Chronic excess of extracellular glutamate acts as a neurotoxin. It is a well-known trigger of calcium influx in neurons leading to their mitochondrial dysfunction, ROS generation, oxidative DNA damage and finally cell death (Kritis *et al*, 2015). Studies suggest that pericytes are also susceptible to ROS-mediated cellular injury. In ischemic stroke, where BBB dysfunction is directly linked to loss of pericytes, it is triggered by calcium influx

mediated by ROS, leading to contraction and death of pericytes (Fernandez-Klett *et al*, 2013; Hall *et al*, 2014; Kamouchi *et al*, 2007). In diabetic nephropathy, loss of retinal pericytes is also linked to oxidative stress and ROS, which are induced by hyperglycemia (Ferland-McCollough *et al*, 2017). Since pericytes appear to be susceptible to intercellular calcium overload and oxidative stress, it suggests that increased extracellular glutamate level during neuroinflammation might contribute to loss of pericytes coverage in BBB, especially when they become infected by HIV. Our data show that HIV-1 latency increased pericytes susceptibility to inhibitors targeting factors involved in DNA repair indicating that virus latency interferes with DDR in pericytes. The level of γ H2AX signaling increased for HIV-1 infected pericytes exposed to glutamate or TNF α at day 2 post-infection, and correlated with increased reduction in cell population. Higher γ H2AX levels in response to glutamate treatment were also observed during latency. Because an increase in γ H2AX signaling in cells reflects an increase in a steady-state level of DNA damage, it suggests that HIV-1 infected pericytes are more susceptible to glutamate-induced DNA damage in the brain.

Studies also indicate that brains of patients with rapidly progressive HAND are also characterized by increased loss of astrocytes (Thompson *et al*, 2001). Our data indicate that astrocytes are less affected by HIV latency, when compared to pericytes, although increases in γ H2AX signaling in latently infected astrocytes in response to glutamate treatment suggest that astrocytes are more susceptible to neuroinflammatory conditions, when they become infected by HIV. Virus interference with cellular DDR would likely affect efficient repairs of glutamate-mediated DNA damages, and therefore influencing the survival rate of infected cells in brain during infection. Analysis of postmortem brain tissue from patients with HIV-Associated Dementia (HAD), which is the most severe form of HAND, showed that an increased rate of astrocyte loss was associated with an increase in number of HIV DNA-positive astrocytes (Thompson *et al*, 2001).

It was determined that HIV infection of pericytes is restricted at the entry, and hence pericytes infection could be limited in the brain, but it is possible that virus can infect pericytes through other mechanisms. For example, astrocytes are also resistant to infection by cell-free HIV-1, but are efficiently infected via cell-to-cell contact (Luo and He, 2015; Nath *et al*, 1995; Schweighardt and Atwood, 2001; Tardieu *et al*, 1992). Regardless of how pericytes get infected, they reside in proximity to tight junctions, a pivotal perivascular niche indicated as a major route of virus entry. This localization suggests that the pericytes together with astrocytes, and endothelial cells, are likely the first cells in the brain to become infected by HIV. Depletion of pericytes and astrocytes in brains of patients with HAND may therefore also be caused by HIV infection and establishment of latency. It would also suggest that although pericytes and astrocytes are mediators of neuroinflammation, their DDR become injured by HIV latency and leads to accumulation of DNA damages triggered by neuroinflammation condition, and increased rate of apoptosis, resulting in their depletion in brain. It would be also important to examine whether HIV latency in pericytes affects formation of functional BBB and maintains its integrity, particularly in response to neuroinflammatory conditions, that are often noted during HIV infection, regardless of cART.

In summary, we demonstrate that the brain vascular pericytes are among the rare brain-derived cells that allow viral latency, which supports the notion that a diverse pool of viral reservoir exists in the CNS of HIV-infected individuals. We also show that the HIV-1 latency renders pericytes vulnerable to the neuroinflammatory conditions that are commonly found in persons with HIV-1. These findings provide an important insight into the establishment of viral reservoirs in the CNS during acute HIV-1 infection and explains the reasons as to why targeting the DNA damage response in latently infected cells would likely support therapeutic approaches to cure HIV/AIDS.

MATERIALS AND METHODS

Ethics statement.

The Research Subjects Review Board at the University of Rochester Medical Center approved studies involving human samples. All the study participants were adults and blood samples were obtained after written informed consent, in accordance with the Declaration of Helsinki.

Cell cultures and Reagents.

Primary human brain vascular pericytes and human astrocytes were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured for additional three passages before used for experiment. The cells were cultured in complete medium provided by the supplier, containing 2% fetal bovine serum (FBS), antibiotics (penicillin and streptomycin) and pericytes or astrocytes growth supplement. Primary central memory T (T_{CM}) cells were generated from naïve $CD4^+$ T cells isolated from peripheral blood mononuclear cells (PBMC) selected from healthy blood donors, as previously described (Bosque and Planelles, 2009; Piekna-Przybylska *et al*, 2017). ATM Kinase Inhibitor (KU55933) and PARP inhibitor I (3-ABA, 3-Aminobenzamide) were obtained from Calbiochem. DNA-PK inhibitor (NU7441) was obtained from Apexbio Technology LLC.

Generation of HIV-1 stocks and infection.

HIV-1-GFP (pNL4-3-deltaE-EGFP) was obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, National Institutes of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). DHIV was obtained from Vicente Planelles (Bosque and Planelles, 2009). Plasmid expressing the vesicular stomatitis virus G protein (VSV-G) under the control of the human cytomegalovirus promoter (pHCMV-g) was obtained from Carrie Dykes (Dykes *et al*, 2010). Human embryonic kidney 293T cells were used for generation of HIV-1 stocks. 293T cells were maintained in Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 µg of streptomycin/ml, and 10% heat-inactivated FBS obtained from HyClone. The cells were transiently transfected with 40 µg of HIV-GFP or DHIV, together with 40 µg of pHCMV-g plasmid DNA by lipofection (SuperFect, Qiagen); supernatants were harvested after 72 h and stored at - 80°C. HIV-1 virus capsid protein (p24) quantitation was performed on virus stocks using an ELISA (R&D systems), according to manufacturer's instructions. VSV-G pseudotyped HIV-1 stocks were used to infect pericytes and astrocytes. Briefly, at 80–90% confluency the cells were exposed to HIV-GFP or DHIV (both at 10. ng/ml of p24) for 24 h.

Then, the medium was removed, the cells were washed and fresh medium was added. To generate latently infected cells, at day 3–4 post-infection the cells were trypsinized and seeded again. When the cultures reached a confluence of 80–90%, they were seeded again at 40–50% confluence. Generation of latently infected primary memory T cells were done as previously described (Bosque and Planelles, 2009; Piekna-Przybylska *et al*, 2017).

Immunocytochemistry.

At endpoint, cells were washed with PBS, then fixed using 4.2% paraformaldehyde solution (BD Cytotfix™ Fixation Buffer) for 15 min, and washed three times with PBS. For intracellular staining, cells were permeabilized with solution containing 0.2% Triton X-100 and 1% BSA. After washing with PBS three times, the cells were blocked for 30 min. at room temperature with blocking buffer BD Pharmingen™ Stain Buffer containing FBS. KC57-FITC antibodies (1:50, Coulter Clone) were used for intracellular staining of p24 antigen for two hours at room temperature. For γ H2AX detection, cells were stained with Alexa Fluor 647 Mouse anti-H2AX (pS139) (1:50, BD Pharmingen) for two hours at room temperature. After staining, the antibody was removed and the wells were washed three times with BD Pharmingen™ Stain Buffer. Nuclei were detected using DAPI or Hoechst stains. For cultures treated with DNA repair inhibitors, cells were collected and counted to seed equal number of cells in new dishes before exposing them to different inhibitors. At endpoint, the cells were washed three times with PBS to remove dead cells, fixed using 4.2% paraformaldehyde solution (BD Cytotfix™ Fixation Buffer) for 10 min and washed three times with PBS. The cells were then stained with DAPI and the density of cells was analyzed by counting nuclei. Olympus IX83 microscope was used for image acquisition from plates at 10x and 20x magnification. Quantitative analysis of positively stained cells and total cells was performed using Olympus Cell Sens Dimension software.

Flow Cytometry.

For flow cytometry analysis the cells were detached enzymatically by standard trypsinization, or mechanically by scraping. For GFP analysis, the cells were washed with PBS and fixed for 15 min. using 4.2% paraformaldehyde solution (BD Cytotfix™ Fixation Buffer). For analysis of intracellular levels of γ H2AX, fixed cells were permeabilized for 5–10 min. using BD Phosflow™ Perm Buffer III, then washed three times with PBS and blocked for 20 min. with BD Pharmingen™ Stain Buffer. To detect γ H2AX, the samples were stained for 1–2 h with specific monoclonal antibodies conjugated to Alexa Fluor® 647 (1:50, BD Pharmingen). Intracellular p24 Gag expression was detected with monoclonal antibody conjugated with FITC (KC57-FITC, Coulter Clone) and analyzed as previously described (Bosque and Planelles, 2009). Sample fluorescence of 10,000 or 20,000 cells was analyzed by using Accuri C6 (BD Biosciences). Cell populations were analyzed using FlowJo software (Version 10.2). Statistical analysis was performed with GraphPad Prism Software. Statistical analysis was done by one-way ANOVA and Tukey's multiple comparisons post-test (significance $P < 0.05$).

Integration analysis.

To detect HIV-1 provirus in host genome we used approach described by Liszewski et al. (Liszewski *et al*, 2009). Briefly, genomic DNA was isolated from cells collected at day 11

post-infection using the DNeasy Tissue Kit (Qiagen). Integration of HIV-1 provirus was determined by *Alu-gag* PCR followed by the nested PCR specific for the 5'-LTR region of HIV-1. For *Alu-gag*, the pair of primers Alu-Forward (GCC TCC CAA AGT GCT GGG ATT ACA G) and gag-Reverse (GCT CTC GCA CCC ATC TCT CTC C; nucleotides (nt): 803–782) was used for amplification (40 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 1 min). 20% of *Alu-gag* PCR amplification reaction was used in the nested PCR. The pair of primers U3 NF-κ3-Forward (CAT CGA GCT TTC TAC AAG GGA; nt: 333–353) and gagUTR-Reverse (CCA GTC GCC GCC CCT CGC CTC TTG CCG TG; nt: 744–716) was used to amplify 411 nt long fragment (product A). The pair of primers U3 Sp1-Forward (CTC AGA TGC TAC ATA TAA GCA GCT; nt: 414–437) and gagUTR-Reverse (CCA GTC GCC GCC CCT CGC CTC TTG CCG TG; nt: 744–716) was used to amplify 330 nt long fragment (product B). The pair of primers R-Forward (CCT GGG AGC TCT CTG GCT AAC T; nt: 482–503) and U5-Reverse (TCC ACA CTG ACT AAA AGG GTC TGA; nt: 622–599) was used to amplify 140 nt long fragment (product C). The cycling conditions for nested PCR reactions were 40 cycles of 95°C for 15 s, 50°C for 15 s and 72°C for 30 s. All PCRs were performed using Platinum *Taq* Polymerase (Thermo Fisher Scientific).

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REFERENCES

- Aguilera KY, Brekken RA (2014) Recruitment and retention: factors that affect pericyte migration. *Cell Mol Life Sci* 71:299–309. doi: 10.1007/s00018-013-1432-z [PubMed: 23912898]
- Ame JC, Spenlehauer C, de Murcia G (2004) The PARP superfamily. *Bioessays* 26:882–93. doi: 10.1002/bies.20085 [PubMed: 15273990]
- Armulik A, Genove G, Mae M, Nisancioglu MH, Wallgard E, Niaudet C, He L, Norlin J, Lindblom P, Strittmatter K, Johansson BR, Betsholtz C (2010) Pericytes regulate the blood-brain barrier. *Nature* 468:557–61. doi: 10.1038/nature09522 [PubMed: 20944627]
- Badley AD, Pilon AA, Landay A, Lynch DH (2000) Mechanisms of HIV-associated lymphocyte apoptosis. *Blood* 96:2951–64. [PubMed: 11049971]
- Bakkenist CJ, Kastan MB (2003) DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 421:499–506. doi: 10.1038/nature01368 [PubMed: 12556884]
- Beck C, Robert I, Reina-San-Martin B, Schreiber V, Dantzer F (2014) Poly(ADP-ribose) polymerases in double-strand break repair: focus on PARP1, PARP2 and PARP3. *Exp Cell Res* 329:18–25. doi: 10.1016/j.yexcr.2014.07.003 [PubMed: 25017100]
- Bell RD, Winkler EA, Sagare AP, Singh I, LaRue B, Deane R, Zlokovic BV (2010) Pericytes control key neurovascular functions and neuronal phenotype in the adult brain and during brain aging. *Neuron* 68:409–27. doi: 10.1016/j.neuron.2010.09.043 [PubMed: 21040844]
- Belzile JP, Abrahamyan LG, Gerard FC, Rougeau N, Cohen EA (2010) Formation of mobile chromatin-associated nuclear foci containing HIV-1 Vpr and VPRBP is critical for the induction of G2 cell cycle arrest. *PLoS Pathog* 6:e1001080. doi: 10.1371/journal.ppat.1001080 [PubMed: 20824083]

- Bosque A, Planelles V (2009) Induction of HIV-1 latency and reactivation in primary memory CD4+ T cells. *Blood* 113:58–65. doi: 10.1182/blood-2008-07-168393 [PubMed: 18849485]
- Brabers NA, Nottet HS (2006) Role of the pro-inflammatory cytokines TNF-alpha and IL-1beta in HIV-associated dementia. *Eur J Clin Invest* 36:447–58. doi: 10.1111/j.1365-2362.2006.01657.x [PubMed: 16796601]
- Brahmachari S, Fung YK, Pahan K (2006) Induction of glial fibrillary acidic protein expression in astrocytes by nitric oxide. *J Neurosci* 26:4930–9. doi: 10.1523/JNEUROSCI.5480-05.2006 [PubMed: 16672668]
- Cho HJ, Kuo AM, Bertrand L, Toborek M (2017) HIV Alters Gap Junction-Mediated Inter cellular Communication in Human Brain Pericytes. *Front Mol Neurosci* 10:410. doi: 10.3389/fnmol.2017.00410 [PubMed: 29311803]
- Chun TW, Engel D, Mizell SB, Ehler LA, Fauci AS (1998) Induction of HIV-1 replication in latently infected CD4+ T cells using a combination of cytokines. *J Exp Med* 188:83–91. [PubMed: 9653086]
- Churchill MJ, Gorry PR, Cowley D, Lal L, Sonza S, Purcell DF, Thompson KA, Gabuzda D, McArthur JC, Pardo CA, Wesselingh SL (2006) Use of laser capture microdissection to detect integrated HIV-1 DNA in macrophages and astrocytes from autopsy brain tissues. *J Neurovirol* 12:146–52. doi: 10.1080/13550280600748946 [PubMed: 16798676]
- Cummins NW, Badley AD (2010) Mechanisms of HIV-associated lymphocyte apoptosis: 2010. *Cell Death Dis* 1:e99. doi: 10.1038/cddis.2010.77 [PubMed: 21368875]
- Davis AJ, Chen BP, Chen DJ (2014) DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. *DNA Repair (Amst)* 17:21–9. doi: 10.1016/j.dnarep.2014.02.020 [PubMed: 24680878]
- de Silva S, Planelles V, Wu L (2012) Differential effects of Vpr on single-cycle and spreading HIV-1 infections in CD4+ T-cells and dendritic cells. *PLoS One* 7:e35385. doi: 10.1371/journal.pone.0035385 [PubMed: 22570689]
- Devadas K, Hardegen NJ, Wahl LM, Hewlett IK, Clouse KA, Yamada KM, Dhawan S (2004) Mechanisms for macrophage-mediated HIV-1 induction. *J Immunol* 173:6735–44. [PubMed: 15557166]
- Dykes C, Wu H, Sims M, Holden-Wiltse J, Demeter LM (2010) Human immunodeficiency virus type 1 protease inhibitor drug-resistant mutants give discordant results when compared in single-cycle and multiple-cycle fitness assays. *J Clin Microbiol* 48:4035–43. doi: 10.1128/JCM.00605-10 [PubMed: 20826651]
- Eugenin EA, Clements JE, Zink MC, Berman JW (2011) Human immunodeficiency virus infection of human astrocytes disrupts blood-brain barrier integrity by a gap junction-dependent mechanism. *J Neurosci* 31:9456–65. doi: 10.1523/JNEUROSCI.1460-11.2011 [PubMed: 21715610]
- Ferland-McCollough D, Slater S, Richard J, Reni C, Mangialardi G (2017) Pericytes, an overlooked player in vascular pathobiology. *Pharmacol Ther* 171:30–42. doi: 10.1016/j.pharmthera.2016.11.008 [PubMed: 27916653]
- Fernandez-Klett F, Potas JR, Hilpert D, Blazej K, Radke J, Huck J, Engel O, Stenzel W, Genove G, Priller J (2013) Early loss of pericytes and perivascular stromal cell-induced scar formation after stroke. *J Cereb Blood Flow Metab* 33:428–39. doi: 10.1038/jcbfm.2012.187 [PubMed: 23250106]
- Ferrarese C, Aliprandi A, Tremolizzo L, Stanzani L, De Micheli A, Dolara A, Frattola L (2001) Increased glutamate in CSF and plasma of patients with HIV dementia. *Neurology* 57:671–5. [PubMed: 11524477]
- Ferrell D, Giunta B (2014) The impact of HIV-1 on neurogenesis: implications for HAND. *Cell Mol Life Sci* 71:4387–92. doi: 10.1007/s00018-014-1702-4 [PubMed: 25134912]
- Gilley D, Tanaka H, Hande MP, Kurimasa A, Li GC, Oshimura M, Chen DJ (2001) DNA-PKcs is critical for telomere capping. *Proc Natl Acad Sci U S A* 98:15084–8. doi: 10.1073/pnas.261574698 [PubMed: 11742099]
- Granowitz EV, Saget BM, Wang MZ, Dinarello CA, Skolnik PR (1995) Interleukin 1 induces HIV-1 expression in chronically infected U1 cells: blockade by interleukin 1 receptor antagonist and tumor necrosis factor binding protein type 1. *Mol Med* 1:667–77. [PubMed: 8529133]
- Guo Z, Kozlov S, Lavin MF, Person MD, Paull TT (2010) ATM activation by oxidative stress. *Science* 330:517–21. doi: 10.1126/science.1192912 [PubMed: 20966255]

- Hall CN, Reynell C, Gesslein B, Hamilton NB, Mishra A, Sutherland BA, O'Farrell FM, Buchan AM, Lauritzen M, Attwell D (2014) Capillary pericytes regulate cerebral blood flow in health and disease. *Nature* 508:55–60. doi: 10.1038/nature13165 [PubMed: 24670647]
- Haughey NJ, Nath A, Mattson MP, Slevin JT, Geiger JD (2001) HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *J Neurochem* 78:457–67. [PubMed: 11483648]
- Herbein G, Van Lint C, Lovett JL, Verdin E (1998) Distinct mechanisms trigger apoptosis in human immunodeficiency virus type 1-infected and in uninfected bystander T lymphocytes. *J Virol* 72:660–70. [PubMed: 9420271]
- Holguin A, O'Connor KA, Biedenkapp J, Campisi J, Wieseler-Frank J, Milligan ED, Hansen MK, Spataro L, Maksimova E, Bravmann C, Martin D, Fleshner M, Maier SF, Watkins LR (2004) HIV-1 gp120 stimulates proinflammatory cytokine-mediated pain facilitation via activation of nitric oxide synthase-I (nNOS). *Pain* 110:517–30. doi: 10.1016/j.pain.2004.02.018 [PubMed: 15288392]
- Jansson D, Rustenhoven J, Feng S, Hurley D, Oldfield RL, Bergin PS, Mee EW, Faull RL, Dragunow M (2014) A role for human brain pericytes in neuroinflammation. *J Neuroinflammation* 11:104. doi: 10.1186/1742-2094-11-104 [PubMed: 24920309]
- Kamouchi M, Kitazono T, Ago T, Wakisaka M, Kuroda J, Nakamura K, Hagiwara N, Ooboshi H, Ibayashi S, Iida M (2007) Hydrogen peroxide-induced Ca²⁺ responses in CNS pericytes. *Neurosci Lett* 416:12–6. doi: 10.1016/j.neulet.2007.01.039 [PubMed: 17350757]
- Khoronenkova SV, Dianov GL (2015) ATM prevents DSB formation by coordinating SSB repair and cell cycle progression. *Proc Natl Acad Sci U S A* 112:3997–4002. doi: 10.1073/pnas.1416031112 [PubMed: 25775545]
- Kritis AA, Stamoula EG, Paniskaki KA, Vavilis TD (2015) Researching glutamate - induced cytotoxicity in different cell lines: a comparative/collective analysis/study. *Front Cell Neurosci* 9:91. doi: 10.3389/fncel.2015.00091 [PubMed: 25852482]
- Lau A, Tymianski M (2010) Glutamate receptors, neurotoxicity and neurodegeneration. *Pflugers Arch* 460:525–42. doi: 10.1007/s00424-010-0809-1 [PubMed: 20229265]
- Liszewski MK, Yu JJ, O'Doherty U (2009) Detecting HIV-1 integration by repetitive-sampling Alu-gag PCR. *Methods* 47:254–60. doi: 10.1016/j.ymeth.2009.01.002 [PubMed: 19195495]
- Liu S, Wang X, Li Y, Xu L, Yu X, Ge L, Li J, Zhu Y, He S (2014) Necroptosis mediates TNF-induced toxicity of hippocampal neurons. *Biomed Res Int* 2014:290182. doi: 10.1155/2014/290182 [PubMed: 25093162]
- Luo X, He JJ (2015) Cell-cell contact viral transfer contributes to HIV infection and persistence in astrocytes. *J Neurovirol* 21:66–80. doi: 10.1007/s13365-014-0304-0 [PubMed: 25522787]
- Martins LJ, Bonczkowski P, Spivak AM, De Spiegelaere W, Novis CL, DePaula-Silva AB, Malatinkova E, Typsteen W, Bosque A, Vanderkerckhove L, Planelles V (2016) Modeling HIV-1 Latency in Primary T Cells Using a Replication-Competent Virus. *AIDS Res Hum Retroviruses* 32:187–93. doi: 10.1089/aid.2015.0106 [PubMed: 26171776]
- Melendez RI, Roman C, Capo-Velez CM, Lasalde-Dominicci JA (2016) Decreased glial and synaptic glutamate uptake in the striatum of HIV-1 gp120 transgenic mice. *J Neurovirol* 22:358–65. doi: 10.1007/s13365-015-0403-6 [PubMed: 26567011]
- Musante V, Summa M, Neri E, Puliti A, Godowicz TT, Severi P, Battaglia G, Raiteri M, Pittaluga A (2010) The HIV-1 viral protein Tat increases glutamate and decreases GABA exocytosis from human and mouse neocortical nerve endings. *Cereb Cortex* 20:1974–84. doi: 10.1093/cercor/bhp274 [PubMed: 20034999]
- Nakagawa S, Castro V, Toborek M (2012) Infection of human pericytes by HIV-1 disrupts the integrity of the blood-brain barrier. *J Cell Mol Med* 16:2950–7. doi: 10.1111/j.1582-4934.2012.01622.x [PubMed: 22947176]
- Nath A, Hartloper V, Furer M, Fowke KR (1995) Infection of human fetal astrocytes with HIV-1: viral tropism and the role of cell to cell contact in viral transmission. *J Neuropathol Exp Neurol* 54:320–30. [PubMed: 7745431]
- Niu F, Yao H, Zhang W, Sutliff RL, Buch S (2014) Tat 101-mediated enhancement of brain pericyte migration involves platelet-derived growth factor subunit B homodimer: implications for human

- immunodeficiency virus-associated neurocognitive disorders. *J Neurosci* 34:11812–25. doi: 10.1523/JNEUROSCI.1139-14.2014 [PubMed: 25164676]
- Oguariri RM, Brann TW, Imamichi T (2007) Hydroxyurea and interleukin-6 synergistically reactivate HIV-1 replication in a latently infected promonocytic cell line via SP1/SP3 transcription factors. *J Biol Chem* 282:3594–604. doi: 10.1074/jbc.M608150200 [PubMed: 17150965]
- Olive PL (2009) Endogenous DNA breaks: gammaH2AX and the role of telomeres. *Aging (Albany NY)* 1:154–6. doi: 10.18632/aging.100025 [PubMed: 20157507]
- Persidsky Y, Hill J, Zhang M, Dykstra H, Winfield M, Reichenbach NL, Potula R, Mukherjee A, Ramirez SH, Rom S (2016) Dysfunction of brain pericytes in chronic neuroinflammation. *J Cereb Blood Flow Metab* 36:794–807. doi: 10.1177/0271678X15606149 [PubMed: 26661157]
- Piekna-Przybylska D, Maggirwar SB (2018) CD4+ memory T cells infected with latent HIV-1 are susceptible to drugs targeting telomeres. *Cell Cycle*. doi: 10.1080/15384101.2018.1520568
- Piekna-Przybylska D, Sharma G, Maggirwar SB, Bambara RA (2017) Deficiency in DNA damage response, a new characteristic of cells infected with latent HIV-1. *Cell Cycle* 16:968–978. doi: 10.1080/15384101.2017.1312225 [PubMed: 28388353]
- Planelles V, Bachelier F, Jowett JB, Haislip A, Xie Y, Banooni P, Masuda T, Chen IS (1995) Fate of the human immunodeficiency virus type 1 provirus in infected cells: a role for vpr. *J Virol* 69:5883–9. [PubMed: 7637036]
- Poli G, Kinter AL, Fauci AS (1994) Interleukin 1 induces expression of the human immunodeficiency virus alone and in synergy with interleukin 6 in chronically infected U1 cells: inhibition of inductive effects by the interleukin 1 receptor antagonist. *Proc Natl Acad Sci U S A* 91:108–12. [PubMed: 7506410]
- Potter MC, Figueroa-Losada M, Rojas C, Slusher BS (2013) Targeting the glutamatergic system for the treatment of HIV-associated neurocognitive disorders. *J Neuroimmune Pharmacol* 8:594–607. doi: 10.1007/s11481-013-9442-z [PubMed: 23553365]
- Roshal M, Kim B, Zhu Y, Nghiem P, Planelles V (2003) Activation of the ATR-mediated DNA damage response by the HIV-1 viral protein R. *J Biol Chem* 278:25879–86. doi: 10.1074/jbc.M303948200 [PubMed: 12738771]
- Rustenhoven J, Jansson D, Smyth LC, Dragunow M (2017) Brain Pericytes As Mediators of Neuroinflammation. *Trends Pharmacol Sci* 38:291–304. doi: 10.1016/j.tips.2016.12.001 [PubMed: 28017362]
- Saleh S, Wightman F, Ramanayake S, Alexander M, Kumar N, Khoury G, Pereira C, Purcell D, Cameron PU, Lewin SR (2011) Expression and reactivation of HIV in a chemokine induced model of HIV latency in primary resting CD4+ T cells. *Retrovirology* 8:80. doi: 10.1186/1742-4690-8-80 [PubMed: 21992606]
- Saylor D, Dickens AM, Sacktor N, Haughey N, Slusher B, Pletnikov M, Mankowski JL, Brown A, Volsky DJ, McArthur JC (2016) HIV-associated neurocognitive disorder--pathogenesis and prospects for treatment. *Nat Rev Neurol* 12:234–48. doi: 10.1038/nrneurol.2016.27 [PubMed: 26965674]
- Schreiber V, Ame JC, Dolle P, Schultz I, Rinaldi B, Fraulob V, Menissier-de Murcia J, de Murcia G (2002) Poly(ADP-ribose) polymerase-2 (PARP-2) is required for efficient base excision DNA repair in association with PARP-1 and XRCC1. *J Biol Chem* 277:23028–36. doi: 10.1074/jbc.M202390200 [PubMed: 11948190]
- Schweighardt B, Atwood WJ (2001) HIV type 1 infection of human astrocytes is restricted by inefficient viral entry. *AIDS Res Hum Retroviruses* 17:1133–42. doi: 10.1089/088922201316912745 [PubMed: 11522183]
- Scutari R, Alteri C, Perno CF, Svicher V, Aquaro S (2017) The Role of HIV Infection in Neurologic Injury. *Brain Sci* 7. doi: 10.3390/brainsci7040038
- Sczepanski JT, Wong RS, McKnight JN, Bowman GD, Greenberg MM (2010) Rapid DNA-protein cross-linking and strand scission by an abasic site in a nucleosome core particle. *Proc Natl Acad Sci U S A* 107:22475–80. doi: 10.1073/pnas.1012860108 [PubMed: 21149689]
- Sengillo JD, Winkler EA, Walker CT, Sullivan JS, Johnson M, Zlokovic BV (2013) Deficiency in mural vascular cells coincides with blood-brain barrier disruption in Alzheimer's disease. *Brain Pathol* 23:303–10. doi: 10.1111/bpa.12004 [PubMed: 23126372]

- Soriano-Sarabia N, Bateson RE, Dahl NP, Crooks AM, Kuruc JD, Margolis DM, Archin NM (2014) Quantitation of replication-competent HIV-1 in populations of resting CD4+ T cells. *J Virol* 88:14070–7. doi: 10.1128/JVI.01900-14 [PubMed: 25253353]
- Sun Y, Huang YC, Xu QZ, Wang HP, Bai B, Sui JL, Zhou PK (2006) HIV-1 Tat depresses DNA-PK(CS) expression and DNA repair, and sensitizes cells to ionizing radiation. *Int J Radiat Oncol Biol Phys* 65:842–50. doi: 10.1016/j.ijrobp.2006.02.040 [PubMed: 16751065]
- Tachiwana H, Shimura M, Nakai-Murakami C, Tokunaga K, Takizawa Y, Sata T, Kurumizaka H, Ishizaka Y (2006) HIV-1 Vpr induces DNA double-strand breaks. *Cancer Res* 66:627–31. doi: 10.1158/0008-5472.CAN-05-3144 [PubMed: 16423988]
- Tardieu M, Hery C, Peudener S, Boespflug O, Montagnier L (1992) Human immunodeficiency virus type 1-infected monocytic cells can destroy human neural cells after cell-to-cell adhesion. *Ann Neurol* 32:11–7. doi: 10.1002/ana.410320104 [PubMed: 1303631]
- Tavazzi E, Morrison D, Sullivan P, Morgello S, Fischer T (2014) Brain inflammation is a common feature of HIV-infected patients without HIV encephalitis or productive brain infection. *Curr HIV Res* 12:97–110. [PubMed: 24862332]
- Thompson KA, Cherry CL, Bell JE, McLean CA (2011) Brain cell reservoirs of latent virus in presymptomatic HIV-infected individuals. *Am J Pathol* 179:1623–9. doi: 10.1016/j.ajpath.2011.06.039 [PubMed: 21871429]
- Thompson KA, McArthur JC, Wesselingh SL (2001) Correlation between neurological progression and astrocyte apoptosis in HIV-associated dementia. *Ann Neurol* 49:745–52. [PubMed: 11409426]
- Tornatore C, Chandra R, Berger JR, Major EO (1994) HIV-1 infection of subcortical astrocytes in the pediatric central nervous system. *Neurology* 44:481–7. [PubMed: 8145919]
- Tweedie D, Sambamurti K, Greig NH (2007) TNF-alpha inhibition as a treatment strategy for neurodegenerative disorders: new drug candidates and targets. *Curr Alzheimer Res* 4:378–85. [PubMed: 17908040]
- Vazquez-Santiago FJ, Noel RJ, Jr., Porter JT, Rivera-Amill V (2014) Glutamate metabolism and HIV-associated neurocognitive disorders. *J Neurovirol* 20:315–31. doi: 10.1007/s13365-014-0258-2 [PubMed: 24867611]
- Vesce S, Bezzi P, Rossi D, Meldolesi J, Volterra A (1997) HIV-1 gp120 glycoprotein affects the astrocyte control of extracellular glutamate by both inhibiting the uptake and stimulating the release of the amino acid. *FEBS Lett* 411:107–9. [PubMed: 9247152]
- Ward IM, Wu X, Chen J (2001) Threonine 68 of Chk2 is phosphorylated at sites of DNA strand breaks. *J Biol Chem* 276:47755–8. doi: 10.1074/jbc.C100587200 [PubMed: 11668173]
- Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MB (1986) Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci U S A* 83:7089–93. [PubMed: 3018755]
- Williams ES, Klingler R, Ponnaiya B, Hardt T, Schrock E, Lees-Miller SP, Meek K, Ullrich RL, Bailey SM (2009) Telomere dysfunction and DNA-PKcs deficiency: characterization and consequence. *Cancer Res* 69:2100–7. doi: 10.1158/0008-5472.CAN-08-2854 [PubMed: 19244120]
- Winkler EA, Sengillo JD, Sullivan JS, Henkel JS, Appel SH, Zlokovic BV (2013) Blood-spinal cord barrier breakdown and pericyte reductions in amyotrophic lateral sclerosis. *Acta Neuropathol* 125:111–20. doi: 10.1007/s00401-012-1039-8 [PubMed: 22941226]
- Yan C, Lu J, Zhang G, Gan T, Zeng Q, Shao Z, Duerksen-Hughes PJ, Yang J (2011) Benzo[a]pyrene induces complex H2AX phosphorylation patterns by multiple kinases including ATM, ATR, and DNA-PK. *Toxicol In Vitro* 25:91–9. doi: 10.1016/j.tiv.2010.09.012 [PubMed: 20888899]
- Yan S, Sorrell M, Berman Z (2014) Functional interplay between ATM/ATR-mediated DNA damage response and DNA repair pathways in oxidative stress. *Cell Mol Life Sci* 71:3951–67. doi: 10.1007/s00018-014-1666-4 [PubMed: 24947324]
- Zhu Y, Gelbard HA, Roshal M, Pursell S, Jamieson BD, Planelles V (2001) Comparison of cell cycle arrest, transactivation, and apoptosis induced by the simian immunodeficiency virus SIVagm and human immunodeficiency virus type 1 vpr genes. *J Virol* 75:3791–801. doi: 10.1128/JVI.75.8.3791-3801.2001 [PubMed: 11264368]
- Zimmerman ES, Chen J, Andersen JL, Ardon O, Dehart JL, Blackett J, Choudhary SK, Camerini D, Nghiem P, Planelles V (2004) Human immunodeficiency virus type 1 Vpr-mediated G2 arrest

requires Rad17 and Hus1 and induces nuclear BRCA1 and gamma-H2AX focus formation. Mol Cell Biol 24:9286–94. doi: 10.1128/MCB.24.21.9286-9294.2004 [PubMed: 15485898]

Zou JY, Crews FT (2005) TNF alpha potentiates glutamate neurotoxicity by inhibiting glutamate uptake in organotypic brain slice cultures: neuroprotection by NF kappa B inhibition. Brain Res 1034:11–24. doi: 10.1016/j.brainres.2004.11.014 [PubMed: 15713255]

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Fig. 1. HIV-1 infected pericytes are susceptible to neuroinflammation condition. **a.** Fluorescent microscope images showing γ H2AX (pink) is detected in nuclei of pericytes infected with HIV-GFP (green) at day 2 post-infection. The nuclei were counterstained with DAPI (blue). A magnified view of the boxed region is shown below. Arrow shows individual γ H2AX foci. **b.** The effects of TNF α and glutamate on DNA damage in pericytes infected with DHIV. Pericytes were exposed to DHIV for 24 h, then the virus was removed and cells were incubated with TNF α (75 ng/ml) or glutamate (250 nM) for another 24 h. The level of DNA

damage was analyzed by calculating the level of γ H2AX using fluorescent microscopy. Top left, fluorescent microscope image showing detection of p24 Gag in a culture of pericytes stained with p24-FITC (green) at day 2 post-infection. Top right, the graph shows relative increases in fluorescence intensities of Alexa Fluor 647 conjugated with monoclonal antibodies detecting phosphorylated H2A (AF 647 - γ H2AX) for infected pericytes (Per/DHIV) treated with TNF α (TNF) or glutamate (GLU), in reference to uninfected and untreated (NT) cultures. Bottom, representative fluorescent microscope images showing increases in γ H2AX signaling in response to glutamate and TNF α in uninfected and infected pericytes. A magnified view of the boxed region is shown below. Arrows show γ H2AX foci. Statistical analysis was done by one-way ANOVA and Tukey's multiple comparisons post-test (significance *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$). Differences in respect to untreated (NT) and uninfected pericytes (Per). Differences between Per/DHIV and Per/DHIV treated with TNF and GLU are also indicated.

Fig. 2.

Reductions in the number of pericytes are observed after HIV-1 infection and treatment with TNF α or glutamate. **a** Graph corresponding to 3 experiments shows reductions in cell number in reference to untreated and uninfected pericytes. Number of cells was calculated using fluorescent microscope by counting DAPI stained nuclei. **b**. Graph corresponding to 3 experiments shows the effect of HIV-1 infection on increased susceptibility to glutamate treatment. Reductions in the number of cells were calculated in reference to untreated cultures (NT, dashed line). Statistical analysis was done by one-way ANOVA and Tukey's multiple comparisons post-test (significance was set at $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$, ****, $P < 0.0001$). Differences in respect to untreated (NT) and uninfected pericytes (Per). Differences between uninfected (Per) and infected (Per/DHIV) treated with TNF and GLU are also indicated.

Fig. 3.

Progressive silencing of HIV-1 expression in pericytes. **a.** Flow cytometry analysis of primary brain vascular pericytes infected with HIV-GFP shows a peak of HIV-1 expression by day 3 post-infection and rapid shutdown of virus expression with over 95% of GFP negative cells by day 10 post-infection. **b.** Representative images of pericytes infected with HIV-GFP and DHIV. By day 2 post-infection (2 dpi), a majority of cells express GFP reporter (green), or stain for p24-FITC (green). By day 7 post-infection most of pericytes turn negative for HIV expression. Nuclei were counterstained with DAPI (blue). **c.** Detection of integrated HIV-1 provirus in pericytes by *Alu-gag* PCR followed by the nested PCR. Agarose gel shows amplified products in the nested PCR with primers specific for U3, R, U5 and UTR *gag* regions of HIV-1. The 411 nt product A was amplified using primers specific for U3 (NF- κ 3 region) and UTR *gag*; the B product (330 nt) - U3 (Sp1 region) and UTR *gag*; the C product (140 nt) - R and U5. **d.** The effect of pro-inflammatory conditions on virus reactivation from latency was analyzed on day 11 post-infection. Latently infected pericytes showed increases in GFP population after treating cells with TNF α (50 ng/ml) and

IL-1 β (50 ng/ml) for 24 h. Numbers in gates indicate percentage of GFP positive population. **e.** Procedure used to generate cultured T_{CM} cells latently infected with HIV-1. **f.** Progressive silencing of the HIV-GFP expression in infected primary memory T cells. **g.** The time course of provirus silencing in memory T cells and pericytes. The graphs show the percentage of infected cells (GFP positive) recorded by flow cytometry starting from day 3 post-infection.

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Fig. 4.

Effect of glutamate on primary brain vascular pericytes latently infected HIV-1. At day 11 post-infection DHIV infected pericytes were exposed to glutamate (250nM) for 24 h and analyzed by flow cytometry for the level of γ H2AX. Representative flow cytometry plots showing the level of γ H2AX in uninfected and latently infected pericytes (+ DHIV) and after glutamate (GLU) treatment. The gate represents the plot area with the highest fluorescence intensity of Alexa Fluor 647 detecting γ H2AX in cells. The numbers indicate the percentage of the γ H2AX positive events recorded in the gate. On right, the graph corresponding to 3 experiments shows increases in the γ H2AX level in uninfected (Per) and latently infected pericytes (Per/DHIV), and in response to glutamate treatment. NT – not treated cells. Statistical analysis was done by one-way ANOVA and Tukey's multiple comparisons post-test (significance *, $P < 0.05$, **, $P < 0.01$). Differences in respect to untreated (NT) and uninfected pericytes (Per). Difference between untreated (NT) Per/DHIV and treated with GLU is also indicated.

Fig. 5.

HIV-1 latency affects DDR in primary brain vascular pericytes. At day 11 post-infection pericytes were exposed to inhibitors of ATM (KU55933, 1 μ M), DNA-PK (NU7441, 1 μ M) and PARP (3-ABA, 6 mM) for 30h and the number of cells was calculated using fluorescent microscope and Vi-CELL counter. Graphs corresponding to 3 experiments show that significant reductions in cell population are observed for latently infected pericytes (DHIV) exposed to inhibitor targeting PARP involved in SSB repair; and to inhibitor targeting DNA-PK involved in DSB repair. Statistical analysis was done by one-way ANOVA and unpaired *t* test analysis (significance *, $P < 0.05$, **, $P < 0.01$, ****, $P < 0.0001$). Left graph, differences in respect to untreated (NT) cells. Right graph, differences between uninfected and infected (DHIV) treated with different drugs.

Fig. 6.

Analysis of HIV-1 latency in primary astrocytes. **a.** Flow cytometry plots showing progressive silencing of the HIV-GFP expression in infected primary astrocytes. **b.** Representative flow cytometry plots showing the level of γ H2AX in uninfected and latently infected astrocytes (+ DHIV) and after glutamate (GLU) treatment. On right, the graph corresponding to 3 experiments shows increase in the γ H2AX level in uninfected (HA) and latently infected astrocytes (HA/DHIV), and in response to glutamate treatment. NT – not treated cells. Statistical analysis was done by one-way ANOVA and Tukey's multiple

comparisons post-test (significance *, $P<0.05$, **, $P<0.01$). Differences in respect to untreated (NT) and uninfected astrocytes (HA). Difference between untreated (NT) HA/DHIV and treated with GLU is also indicated. **c.** At day 11 post-infection astrocytes were exposed to inhibitors of ATM (KU55933, 1 μ M), DNA-PK (NU7441, 1 μ M) and PARP (3-ABA, 6 mM) for 30h and the number of cells was calculated using fluorescent microscope and Vi-CELL counter. Graphs corresponding to 3 experiments show effects of DNA repair inhibitors on population of latently infected astrocytes (DHIV). Statistical analysis was done by one-way ANOVA and unpaired t test analysis (significance *, $P<0.05$, ***, $P<0.001$, ****, $P<0.0001$). Left graph, differences in respect to untreated (NT) cells. Right graph, differences between uninfected and infected (DHIV) treated with different drugs.