Expression of HIV-1 Intron-Containing RNA in Microglia Induces Inflammatory Responses

Running title: HIV RNA-induced innate immune activation in microglia

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Abstract

Chronic neuroinflammation is observed in HIV$^+$ individuals on suppressive combination antiretroviral therapy (cART) and is thought to cause HIV-associated neurocognitive disorders. We have recently reported that expression of HIV intron-containing RNA (icRNA) in productively infected monocyte-derived macrophages induces pro-inflammatory responses. Microglia, yolk sac-derived brain-resident tissue macrophages, are the primary HIV-1 infected cell type in the central nervous system (CNS). In this study, we tested the hypothesis that persistent expression of HIV icRNA in primary human microglia induces innate immune activation. We established multiple orthogonal primary human microglia-like cell cultures including peripheral blood monocyte-derived microglia (MDMG) and induced pluripotent stem cell (iPSC)-derived microglia. Unlike MDMG, human iPSC-derived microglia (hiMG), which phenotypically mimic primary CNS microglia, were robustly infected with replication competent HIV-1, and establishment of productive HIV-1 infection and de novo viral gene expression led to pro-inflammatory cytokine production. Blocking of HIV-1 icRNA expression, but not multiply spliced viral RNA, either via infection with virus expressing a Rev-mutant deficient for HIV icRNA nuclear export or infection in the presence of small molecule inhibitor of CRM1-mediated viral icRNA nuclear export pathway, attenuated induction of innate immune responses. These studies suggest that Rev–CRM1-dependent nuclear export and cytosolic sensing of HIV-1 icRNA induces pro-inflammatory responses in productively infected microglia. Novel strategies targeting HIV icRNA expression specifically are needed to suppress HIV-induced neuroinflammation.

Importance

Although peripheral viremia can be effectively suppressed with the advent of highly active anti-retroviral therapy, a significant portion of HIV^+ individuals still suffer from neurocognitive disorders. Despite suppressive therapy, HIV persists in various tissues including central nervous system (CNS), leading to chronic inflammation, the chief driver of neurocognitive disorders. While persistent infection has been described in CNS-resident macrophages, microglia, molecular mechanisms of how HIV infection in microglia contributes to neuronal inflammation have remained unclear. In this study, we used multiple primary human microglia-like cellular platforms and demonstrate that HIV-1 intron-containing RNA induces microglial activation and damage. Since current anti-retroviral therapy does not suppress HIV-1 transcription, new therapeutics targeting HIV RNA expression may help to treat HIV-associated neurocognitive disorders.

Introduction

Since the advent of combination antiretroviral therapy (cART), mortality and morbidity of HIV-1 infection has been dramatically reduced. Although prolonged cART can suppress peripheral viremia in HIV^+ individuals under the detection limit for decades, these therapeutic regimens fail to suppress chronic immune activation, the chief driver of HIV-associated non-AIDS complications (HANA) including HIV-associated neurocognitive disorders (HAND) (1, 2). Numerous studies have demonstrated that inflammatory markers associated with myeloid cell activation are strongly and selectively predictive of HAND (3). In vivo, persistent HIV infection has been reported in the central nervous system (CNS)-resident macrophages including perivascular macrophages and microglia (4-7). However, molecular mechanisms of how HIV infection in the CNS-resident macrophages contributes to chronic immune activation have remained unclear.

Recently, we have shown that expression and Rev–CRM1-dependent nuclear export of HIV intron-containing RNA (icRNA) in productively infected peripheral blood monocyte-derived macrophages (MDMs) is the trigger to induce type I interferon (IFN-I)-dependent production of pro-inflammatory cytokines even in the absence of new viral particle production (8). Similar findings have also been reported in monocyte-derived dendritic cells (9), suggesting HIV icRNA expression-induced innate immune activation might be a conserved phenotype in myeloid cells. Numerous studies have documented continued presence of HIV RNA in the CSF even after prolonged cART (3, 10-12). Since cART regimens as constituted presently cannot suppress viral RNA expression from integrated proviruses, it is plausible that persistent expression of HIV icRNA in the CNS-resident microglia and perivascular macrophages contributes to the chronic inflammatory state in the brain of HIV^+ individuals on cART.

Productively infected microglia can contribute to virus persistence and CNS pathology during HIV-1 infection (4, 13), though the extent to which these reservoirs persist and the mechanisms that might allow for virus persistence in these cells in patients on cART remains unclear. HIV infection of microglia has been shown to impact microglial functions including activation status, viability and metabolism (14). In addition, changes in microglial functions have been postulated to contribute to neuropathogenesis by secreting pro-inflammatory cytokines and neurotoxins (15). Activated microglia are also known to cause neurodegeneration directly by damaging synapses or indirectly via activation of other CNS-resident cells such as astrocytes (reviewed in (16)). Microglia play a pivotal role in maintaining brain homeostasis, and microglial dysfunction caused by HIV infection is thought to impact CNS functionality of HIV^+ individuals on suppressive cART. To date, several mechanisms have been proposed to explain how HIV induces microglia activation. For example, HIV proteins Tat, gp120, Nef and Vpr have been shown to activate microglia, leading to alterations in microglial functions and neuronal health (reviewed in (14)). However, the physiological relevance of these findings needs to be carefully considered, since most of the studies have used overexpression of viral proteins or transgenic rodents. Whether such high concentrations of these viral proteins are observed in the CNS of HIV^+ patients on suppressive therapy requires further investigation. While HIV infection of primary human fetal microglia has been reported (17, 18), these cells are not easily accessible, which preclude detailed investigations of the molecular mechanisms of HIV-induced innate immune activation. Overall, the molecular mechanisms of HIV-induced microglia activation in the CNS remain unclear.

In this study, we investigate the role of HIV-1 infection of microglia in promoting neuroinflammation using two model systems, primary monocyte-derived microglia (MDMG) and induced pluripotent stem cell (iPSC)-derived microglia (iCell-MG and hiMG). We report that while HIV-1 infection of MDMGs is attenuated, restriction to infection was alleviated upon SAMHD1 degradation. In contrast, both iCell-MGs and hiMGs were robustly infected with wild type HIV-1, and innate immune activation in these cells was triggered by de novo expression and nuclear export of icRNA via the Rev–CRM1-dependent pathway.

Results

MDMG model of HIV-1 infection in microglia

HIV-1 infection of primary human fetal microglia has been reported (17, 18), though these cells are not easily accessible due to ethical and technical issues. To overcome these limitations, microglia-like cells have been generated in vitro from monocytes and characterized extensively (19-22). We derived microglia-like cells from $CD14^+$ monocytes by culturing in serum-free conditions in the presence of IL-34 and GM-CSF (**Figure 1A**). These cells displayed a unique microglia-like ramified morphology (**Figure 1B**), as previously reported (19, 20). MDMGs have been shown to display similar morphology to that of human primary microglia and express genes that are highly or uniquely expressed in human microglia (19-23). In agreement with these previous findings, expression of *P2RY12* and *Gas6* mRNAs in MDMGs was significantly enhanced compared to those in donor-matched monocyte-derived macrophages (MDMs) (**Figure 1C and D**). Furthermore, expression of P2RY12 and IBA-1 in MDMGs was confirmed by immunofluorescence (**Figure 1E**). We next examined if MDMGs were

susceptible to HIV-1 infection. MDMGs were infected with replication competent CCR5-tropic HIV-1 (Lai/YU-2env), and $p24^{Gag}$ secretion in the culture supernatants was quantified by ELISA. While infection of MDMGs resulted in productive infection and release of progeny virions (**Figure 1F**), the amount of $p24^{Gag}$ in the supernatants was very low. Since MDMGs were differentiated from peripheral blood monocytes in GM-CSF and IL-34 containing media, and GM-CSF has been shown to alter phosphorylation status of SAMHD1 and render MDMs less susceptible to HIV-1 infection (24), we sought to determine the phosphorylation status of SAMHD1 in MDMGs. Western blotting analysis demonstrated that while total SAMHD1 levels were similar, MDMGs expressed significantly reduced levels of phosphorylated SAMHD1, compared to donor-matched MDMs or THP-1 monocytoid cells (**Figure 1G**) (25, 26). We next infected MDMGs and donor-matched MDMs with HIV-1 in the absence or presence of SIV_{mac} Vpx containing virus-like particles (VLPs) that degrades SAMHD1 (27, 28) and enhances HIV-1 infection of myeloid cells (29). In the absence of SIV_{mac} Vpx, MDMGs produced much lower amount of $p24^{Gag}$ in the supernatants than MDMs (**Figure 1H**). Interestingly, pre-treatment of MDMGs with SIV_{mac} Vpx VLPs significantly enhanced $p24^{Gag}$ production (**Figure 1H**), suggesting that abundant expression of anti-viral SAMHD1 in MDMGs restricts efficient infection of these cells by HIV-1.

HIV-1 infection induces immune activation in MDMGs

We have recently shown that infection of MDMs with HIV-1 induces IFN-I-dependent pro-inflammatory responses (8). To investigate whether HIV-1 infection of microglia induces innate immune activation, total RNA isolated from HIV-1 infected-MDMGs in the presence of SIV_{mac} Vpx VLPs was analyzed with a NanoString human

neuroinflammation panel that contains more than 750 target genes covering the core pathways and processes involved in neuroinflammation. Amongst those analyzed, several mRNAs were up-regulated in a HIV-1 infection specific manner, i.e. up-regulation was only seen in HIV-infected untreated MDMGs but not in reverse transcriptase inhibitor (efavirenz, EFV)- or integrase inhibitor (raltegravir, Ral)-treated MDMGs (**Figure 2A, B and C**). Highly up-regulated genes (> mean + 2xSD) compared to mock, EFV- or Ral-treated MDMGs are shown in **Figure 2A, 2B and 2C,** respectively, which include interferon-stimulated genes (ISGs) (e.g. Siglec1/CD169, RSAD2) and pro-inflammatory cytokines (e.g. CXCL10/IP-10, CCL7/MCP-3). To confirm the results from NanoString analysis, IP-10 production in the MDMG culture supernatants was measured by ELISA. We found that IP-10 production was induced upon infection of MDMGs with HIV-1, which was inhibited upon pretreatment of MDMGs with EFV or Ral (**Figure 2D**). HIV-1 intron-containing RNA (icRNA) export into cytosol via the Rev–CRM1-dependent pathway has previously been shown to induce innate immune activation in MDMs and dendritic cells (8, 9). To investigate the role of HIV-1 icRNA export by the Rev–CRM1-dependent pathway in MDMG innate activation, HIV-1 infected MDMGs were treated with a CRM1 inhibitor (KPT-330, selinexor) or MDMGs were infected by an HIV-1 Rev-deficient (dominant negative) mutant (M10) (8, 30). While establishment of infection of MDMGs and HIV-1 multiply-spliced RNA expression was not affected by KPT treatment or M10 infection (**Figure 2E**), production of $p24^{Gag}$ which is transcribed from icRNA, was completely inhibited by KPT-330 treatment or in M10-infected MDMGs (**Figure 2F**). Interestingly, expression of IP-10 mRNA was severely reduced in HIV-1-infected MDMGs upon KPT-330 treatment or in M10-infected MDMGs (**Figure 2G**). These

results suggest that innate immune activation of MDMGs upon HIV-1 infection requires cytoplasmic expression of HIV icRNA exported via the Rev–CRM1-dependent pathway.

iPSC-derived microglia are highly susceptible to HIV-1 infection

Fate mapping analysis suggests that microglia in the brain originate from yolk-sac-derived primitive macrophages during embryonic hematopoiesis (31, 32). Unlike other tissue-resident macrophages such as Kupffer cells and alveolar macrophages, microglia are not replenished with circulating bone marrow-derived monocytes during adulthood (33-35). To better model HIV-1 infection of human primary microglia, we tested if human induced pluripotent stem cells (iPSCs)-derived microglia can be infected with HIV-1. We obtained iPSC-derived microglia, iCell Microglia (iCell-MG), from a commercial source (FUJIFILM Cellular Dynamics) which were generated as previously described (36). iCell-MGs showed heterogeneous morphology (**Figure 3A**) and expressed the macrophage/microglia marker IBA-1 (**Figure 3B**). Flow cytometry analysis revealed robust intracellular expression of the microglia-specific marker P2RY12, and minimal expression on the cell surface (**Figure 3C**). Immunoblotting analysis revealed that, in contrast to MDMGs, the majority of SAMHD1 was phosphorylated in iCell-MGs (**Figure 3D**). We then infected iCell-MGs with replication competent CCR5-tropic HIV-1/YU-2 and monitored $p24^{Gag}$ production in the culture supernatants over 15 days. We found that iCell-MGs persistently produced $p24^{Gag}$, which peaked at 6 days p.i. (**Figure 3E**). Intracellular $p24^{Gag}$ staining revealed that about 20% of iCell-MGs in the culture were productively infected at 6 days p.i. (**Figure 3F**). HIV-1 replication in the infected iCell-MG cultures was inhibited by reverse transcriptase (efavirenz, EFV), integrase (raltegravir, Ral) and CRM1 (KPT-335, verdinexor) inhibitors (**Figure 3E and F**). To investigate if

HIV-1 infection of iCell-MGs induced innate immune activation, we harvested cells on day 6 p.i. and stained them for CD169, a myeloid-cell-specific ISG (37, 38). iCell-MGs upregulated CD169 expression upon infection with HIV-1 (**Figure 3G**) on both infected cells and on bystander uninfected cells, suggesting that low levels of IFN-I was secreted by infected cells similar to that observed in HIV-1-infected MDMs (8). Expression of CD169 was suppressed by pretreatment of iCell-MGs with RT (EFV), integrase (Ral) and CRM1 (verdinexor) inhibitors (**Figure 3H**). Furthermore, IP-10 and CCL2 production was induced by productive infection of iCell-MGs by HIV-1 and inhibited upon treatment by EFV, Ral or verdinexor (**Figure 3I and J**). These results suggest that iPSC-derived microglia are highly susceptible to HIV-1 infection, and that expression and nuclear export of HIV icRNA in infected iCell-MGs triggers innate immune responses in microglia.

Establishment of iPSC-derived microglia/neuron co-culture system

We took advantage of recent descriptions in the literature for generation of microglia from iPSC lines (39). Briefly, iPSC-derived human microglia were derived by co-culturing iPSC-derived yolk-sac primitive macrophages (hiMAC) with iPSC-derived neurons (hiNeuron) (**Figure 4A and B**) (39). Cells in the hiMG–hiNeuron co-cultures expressed significantly higher levels of mRNA of microglia-specific markers TMEM119 (**Figure 4C**), CX3CR1 (**Figure 4D**), and P2RY12 (**Figure 4E**) compared to those in hiNeuron mono-culture or in hiMACs. Immunofluorescence revealed hiMGs expressed macrophage/microglia markers (IBA-1 or TMEM119) (39, 40) and made numerous cell-to-cell contacts with neurons as previously reported (**Figure 4F**) (39). P2RY12 was highly expressed on the cell surface of hiMGs, similar to CNS-resident human microglia

(23, 41), and these cells were clearly distinguishable from hiNeuron (tubulin β3/TUBB3^{+}) by flow cytometry (**Figure 4G**).

HIV-1 infection of hiMGs induces pro-inflammatory responses

hiMG–hiNeuron co-cultures were infected with replication competent HIV-1 Lai/YU-2env, and HIV-1 replication was measured by flow cytometry (intracellular p24^{Gag} expression) or ELISA (p24^{Gag} in the culture supernatants). While hiNeurons were not susceptible to HIV-1, hiMGs were robustly infected with HIV-1 in hiMG–hiNeuron co-cultures (**Figure 5A and B**). Furthermore, establishment of infection in hiMG–hiNeuron co-cultures was blocked by pretreatment with EFV and Ral, and anti-CRM1 inhibitor (KPT-330) (**Figure 5B**). We detected increasing amounts of p24^{Gag} in the culture supernatants over time (**Figure 5C**), which is suggestive of persistent virus replication in hiMG–hiNeuron co-cultures. HIV-1 infection induced increased production of IP-10 (**Figure 5D**) and up-regulated CCL2 secretion (**Figure 5E**). HIV-1 infection in microglia has been postulated to lead to neuronal disorder by disrupting microglia viability and functionality (14). To investigate the impact of HIV-1 infection on microglial functionality and neuronal toxicity, HIV-1-infected hiMG–hiNeuron co-cultures were analyzed for microglial and neuronal viability by flow cytometry on day 6 p.i. Interestingly, the proportion of live microglia in the co-cultures decreased upon HIV-1 infection over time, which was suppressed upon initiation of infections in the presence of HIV-1 inhibitors (EFV and Ral), suggesting that productive HIV-1 infection, but not exposure to HIV-1 particles alone, affected hiMG viability (**Figure 5F**). On the other hand, HIV-1 spread in hiMG–hiNeuron co-cultures did not affect the viability of hiNeurons (**Figure 5G**). These data suggest that hiMGs in the microglia–neuron co-cultures are highly susceptible to HIV-1 infection and that Rev–

CRM1-dependent nuclear export of HIV icRNA in microglia triggers secretion of pro-inflammatory cytokines, which might contribute to neuroinflammation in vivo.

Discussion

HIV infection and innate immune responses

Chronic inflammation is thought to be the chief driver of HAND (2, 42, 43), though underlying mechanisms of persistent neuroinflammation remain unclear. In this study, we demonstrated that HIV-1 infection of microglia induces innate immune activation, resulting in secretion of pro-inflammatory cytokines, up-regulation of ISGs, and microglia cytotoxicity. Considering their long lifespan with self-renewal capacity (31, 44, 45), coupled with the observation that HIV-1^{+} microglia have been detected in cART-suppressed individuals (4), it is highly plausible that persistently infected microglia produce pro-inflammatory cytokines and chemokines, such as IFN-I and IP-10, contributing to a chronic state of neuroinflammation. Previous studies have suggested that IFN-I production contributes to cognitive impairments in HIV-1 infection (46) and neurodegenerative diseases (47, 48). Although multiple roles for chemokines in CNS inflammation have been described, CCL2, specifically, has been shown to modulate neuronal death in a mouse model (49, 50). Elevated levels of IP-10 have been observed in several neurodegenerative diseases including in patients with HAND (51) and are known to affect neuronal viability (52, 53). Since we did not find obvious neuronal cytotoxicity in hiMG–hiNeuron co-cultures in 6 days of infection, future studies will be focused on long-term co-cultures and the consequence of persistent HIV-1 infection in microglia on neuronal cytotoxicity such as synaptic loss and dendrite degeneration (54).

HIV icRNA and innate immune responses

While viral proteins such as Tat, Vpr and gp120 have been hypothesized to contribute to HIV-associated neuroinflammation (14), most of these studies have relied on overexpression of viral proteins or transgenic animals. In this study, we showed that HIV-1-infection-induced activation of microglia in all primary cell culture models was triggered by cytoplasmic export of icRNA, since infection with HIV expressing a Rev mutant deficient for CRM1 interaction (M10) was unable to induce innate immune activation (**Figure 2**), and CRM1 inhibitors suppressed HIV-induced activation in microglia (**Figure 2, 3 and 5**). We have previously shown that HIV icRNA expression alone induces IFN-I-dependent proinflammatory responses in MDMs, even though HIV icRNA expression does not lead to production of new virions or functional viral proteins including gp120 and Vpr (8). Furthermore, the Rev mutant M10, which fails to induce innate immune activation in microglia, expresses multiply-spliced viral RNAs, including those encoding for Tat, suggesting that de novo Tat expression is not the trigger for HIV-induced microglia activation. Interestingly, HIV icRNA (*gag* mRNA) has been detected in the CSF from HIV-1^{+} individuals on cART (3, 10-12), and a highly sensitive RNAScope assay has revealed presence of a significant number of SIV gag mRNA (icRNA) positive cells in the brain of cART-suppressed monkeys (55). We postulate that these viral icRNA expressing cells in the brain, which are most likely microglia, induce pro-inflammatory cytokines and affect neuronal health in cART-suppressed individuals. Several drug candidates that suppress expression or stability of HIV icRNA such as Tat and Rev inhibitors (56, 57), or inhibitors that selectively target CRM1-dependent nuclear export of HIV icRNA (58), might have clinical benefit for suppressing HIV icRNA induced aberrant inflammation and incidence of HAND in cART-suppressed patients.

Establishment of primary human microglia culture system for HIV infection studies.

In order to investigate the role of HIV-1 infection of microglia in HIV-1 neuropathogenesis, and to overcome the limited access to primary microglia, we employed three different in vitro models of primary microglia in this study: MDMG, iCell-MG and hiMG. MDMG expressed microglia-specific markers such as P2RY12 and were poorly susceptible to HIV-1 infection (**Figure 1**). Since peripheral blood monocytes are readily accessible and the protocol for MDMG generation is relatively simple, MDMG is a reasonable model to study HIV-1 biology in microglia. It should be pointed out that infection of MDMG with HIV-1 in the absence of SAMHD1 antagonism was inefficient (**Figure 1**). Further optimization of the generation protocol is warranted, for example using M-CSF instead of GM-CSF in the differentiation conditions, since GM-CSF has been shown to induce anti-viral SAMHD1 expression in MDMs (24) (**Figure 1G**). To better mimic the origin of microglia (yolk-sac-derived), we used two independent iPSC-derived microglia lines and tested their susceptibility to replication competent HIV-1 in vitro. iCell-MGs are commercially available and expressed microglia markers IBA-1 and P2RY12 (**Figure 3**). It should be noted that as opposed to CNS-resident microglia (23, 41), we observed mostly intracellular expression of the microglia-specific marker, P2RY12 in iCell-MGs (**Figure 3B**). iCell-MGs were highly susceptible to HIV-1 infection (**Figure 3**), which is in agreement with previous studies using primary fetal microglia (17). While iCell-MG is a powerful tool to study HIV-1 infection in microglia, the inability to genetically manipulate these cells limits their utility in robust mechanistic approaches.

The third model we used was hiMG–hiNeuron co-cultures that were generated from iPSCs. This system has numerous advantages: (i) hiMGs are highly susceptible to HIV-1 infection (**Figure 5**), (ii) establishment of iPSC-derived microglia and neuron co-cultures allows for the study of intricate interactions between diverse cell types in the context of viral infection and, importantly, the impact of HIV-infection induced microglia activation can be assessed on autologous neurons, (iii) the purinergic receptor, P2RY12, which detects extracellular nucleotides accompanied with CNS injury and regulates microglial homeostasis (41, 59, 60), and plays an important role in communicating with neighboring neurons to protect their functions (61) is robustly expressed on the hiMG cell surface (in contrast to the mostly intracellular expression of P2RY12 in iCell-MGs), (iv) iPSCs are amenable to gene-editing approaches (62), and (v) iPSC lines generated from somatic cells of various individuals including HIV-infected patients make possible studies of HIV infection of microglia from unique genetic backgrounds and their contribution to human disease. A recently published study (while this manuscript was in preparation) described a new cellular platform that consists of iPSC-derived microglia, neurons and astrocyte tri-cultures (63) and have shown that HIV-1 infection of iPSC-microglia in isolation or in tri-cultures resulted in production of pro-inflammatory cytokines including IL-1β and TNFα. Though the mechanism of induction of pro-inflammatory responses in HIV-1 infected microglia was not defined, inflammatory responses were suppressed upon treatment with RT inhibitor (efavirenz) (63). Differentiation protocols for iPSC-derived microglia in this recently published study (63) were similar to those utilized for generation of iCell-MG (iCell Microglia, FUJIFILM Cellular Dynamics) that we tested in this report. While the cytokine-driven differentiation protocol generated iPSC-microglia with similar transcriptional profiles to human primary

microglia (36, 63), our results suggest that iCell-MGs express low levels of P2RY12 on the cell surface, unlike primary human microglia (23, 41). Since the CNS environment is critical for establishing and maintaining microglial cell identity (64), co-culture-dependent terminal differentiation of iPSC-microglia, as described here and by Takata et al (39), may better model primary microglia in the brain.

Impact of innate immune activation on homeostatic functions of microglia

We have shown that HIV-1 infection of microglia promotes microglia cell death and pro-inflammatory cytokine production in the hiMG–hiNeuron co-cultures (**Figure 5**), though significant cytotoxicity of co-cultured neurons was not observed at the time of harvest (6 days p.i.). In contrast, a recent study using non-isogenic iPSC-derived microglia and neurons (from independent lines) demonstrated that infected microglia induce neuronal death, and damaged neurons induce activation of HIV-1 transcription in latently-infected microglia (65). These differences might be the result of divergent experimental setup, as hiMGs in this study were generated by co-culturing hiMACs and hiNeurons from the same iPSC-line, and infections of hiMGs were initiated in co-cultures. Further studies are needed to determine the effects of long-term co-culture of HIV-infected hiMGs and hiNeurons and the consequences of persistent HIV icRNA-induced chronic inflammation on neuronal homeostasis. It has been shown that activation of microglia leads to dysfunctions such as defects in clearing neurotoxins including fibrilar amyloid β and Tau, and promoting a senescent phenotype in microglia (reviewed in (14)). Inclusion of other cell types which have been reported to be HIV-1^{+} in the CNS such as astrocytes and perivascular macrophages (reviewed in (66)) in the hiMG–hiNeuron co-culture might better mimic the brain environment. In addition, human iPSC-derived cerebral organoids

with diverse cell types that interact in a 3D environment is an attractive model to study HIV neuropathogenesis in vitro (67). Future studies will need to assess the effects of persistent HIV-1 infection on homeostatic functions of microglia and contribution to neuronal dysfunction in these 3D cerebral organoid cultures. Finally, our findings highlight the urgent need to develop novel therapeutic strategies targeting cytosolic HIV icRNA expression to reduce HIV-induced neuroinflammation and incidence of HAND.

Materials and Methods

Viruses

HIV-1 replication competent molecular clones, Lai/YU-2env, single-round reporter virus constructs, LaiΔenvGFP (GFP in place of the *nef* orf) and Rev-deficient LaiΔenvGFP-M10, have been described previously (8, 68, 69). Replication competent viruses were derived from HEK293T cells via calcium phosphate-mediated transient transfection (70). Single-round-replication-competent viruses pseudotyped with VSV-G were generated from HEK293T cells via co-transfection of HIV-1Δenv proviral plasmids and VSV-G expression plasmid, and the packaging construct (psPAX2), if necessary (70). SIV_{mac} Vpx containing VLPs were generated from HEK293T cells via co-transfection of $SIV3^{+}$, a SIV packaging plasmid containing SIVmac239 Vpx (29), and VSV-G expression plasmid. Virus-containing cell supernatants were harvested 2 days post-transfection, cleared of cell debris by centrifugation (300 x g, 5 min), passed through 0.45 µm filters, and purified and concentrated by ultracentrifugation on a 20% sucrose cushion (24,000 rpm and 4˚C for 2 hours with a SW32Ti or SW28 rotor (Beckman Coulter)). The virus pellets were resuspended in PBS, aliquoted and stored at -80 °C until use. The capsid content of HIV-1 was determined by a $p24^{gag}$ ELISA (70) and virus titer was measured on TZM-bl by measuring β-gal activity as previously described (71).

Cell culture

HEK293T (ATCC) and TZM-bl (NIH AIDS Reagent Program) were maintained in DMEM (Gibco) containing 10% heat-inactivated FBS (Gibco) and 1% pen/strep (Gibco) (37, 70, 72). THP-1 (NIH AIDS Reagent Program) was maintained in RPMI1640 (Gibco) containing 10% FBS and 1% pen/strep (73). In some experiments, THP-1 cells were

405 stimulated with PMA (Sigma-Aldrich) for 48 hours at 100 nM. All cell lines have been
406 tested for mycoplasma contamination and confirmed negative. Human iPSC-derived
407 microglia were either purchased (iCell Microglia, FUJIFILM Cellular Dynamics) or
408 generated by us (hiMG, see below). iCell Microglia (iCell-MG) were maintained per the
409 manufacturer's instruction. All the reagents used to maintain iCell-MG are listed below:
410 DMEM/F-12, HEPES no phenol red (Gibco, #11039021), B-27 supplement (Gibco,
411 #17504044), GlutaMAX supplement (Gibco, #35050061), insulin-transferrin-selenium
412 (Gibco, # 41400045), MEM non-essential amino acids (Gibco, #11140050), penicillin-
413 streptomycin (Gibco, #15140122), N-2 supplement (Gibco, #17502048), bovine serum
414 albumin (Sigma-Aldrich, #A1470), recombinant human CD200 (ACRO Biosystems,
415 #OX2-H5228), recombinant human IL-34 (PeproTech, #200-34), recombinant human
416 fractalkine (PeproTech, #300-31), human insulin solution (Sigma-Aldrich, #I9278),
417 human TGF-β1 (Miltenyi Biotec, #130-095-066), ascorbic acid (Sigma-Aldrich, #A8960),
418 recombinant human M-SCF (PeproTech, #300-25), and 1-Thioglycerol (MTG) (Sigma-
419 Aldrich, #M6145).
420
421 **Generation of monocyte-derived microglia-like cells and macrophages**
422 To generate monocyte-derived microglia (MDMG), $CD14^+$ peripheral blood monocytes
423 positively-isolated with CD14 Micro Beads (Miltenyi Biotec) (68) were seeded on Geltrex
424 (Gibco) coated tissue culture plates and cultured for 12-14 days in RPMI1640 Glutamax
425 (Gibco) supplemented with 1% pen/strep, 100 µg/ml of IL-34 (Peprotech), and human
426 GM-CSF (10 ng per ml, Miltenyi Biotec). Human monocyte-derived macrophages
427 (MDMs) were derived from $CD14^+$ peripheral blood monocytes by culturing in RPMI1640
428 (Gibco) containing 10% heat-inactivated human AB serum (Sigma-Aldrich) and

429 recombinant human M-CSF (20 ng per ml; Peprotech) for 5-6 days and maintained in
430 the same media.
431
432 **Generation of human iPSC-derived cells**
433 Human iPSCs were generated from human PBMCs by using the STEMCCA
434 polycistronic lentiviral vector (74, 75) followed by the removal of integrated
435 reprogramming cassette using Cre recombinase (76), and were maintained in mTeSR1
436 media (STEMCELL Technologies). Human iPSC-derived primitive macrophages
437 (hiMacs) were generated as previously reported (**Figure 4A**) (39). Briefly, human iPSC
438 colonies were specified to the mesoderm, and induced into hemangioblast and toward
439 hematopoietic precursors followed by differentiation into primitive macrophages by
440 changing culture media every 2-4 days. After differentiation (Day 26), floating cells were
441 collected and used for FACS as described below. In parallel, human iPSC-derived
442 neurons (hiNeurons) were generated from the same batch of iPSCs as previously
443 reported (39). Human iPSCs were dissociated to single cells, plated onto Matrigel-
444 coated 6 well plates, and differentiated into neuronal progenitors (NPCs). NPCs were
445 terminally differentiated into hiNeurons. To generate iPSC-derived microglia cells
446 (hiMGs), $CD45^+$ $CD11b^+$ $CD163^+$ $CD14^+$ $CX3CR1^+$ hiMacs were sorted by FACS as
447 described below and co-cultured with terminally differentiated hiNeurons for 14 days. All
448 the reagents used to generate iPSC-derived cells are listed below: mTeSR (STEMCELL
449 Technologies, #85850), ReLeSR (STEMCELL Technologies, #05872), DMEM/F-12,
450 HEPES (Gibco, #11330057), IMDM (Gibco, #12440061), Stempro-34 SFM (Gibco,
451 #10639-011), neurobasal (Gibco, #21103049), PBS (Gibco, #14190-144), Ham's F-12
452 nutrient mix (Gibco, #11765054), N2 supplement (Gibco, #17502048), B-27

supplement, serum free (Gibco, #17504044), B27 minus vitamin A (Gibco, #12587010), bovine albumin fraction V (7.5% solution) (Gibco, #15260037), primocin (InvivoGen, #ant-pm-2), GlutaMax (Gibco, #35050061), laminin (Gibco, #23017-015), Matrigel hESC-qualified matrix (Corning, #354277), Matrigel membrane matrix (Corning, #354234), poly-L-ornithine solution (Sigma-Aldrich, #P4957), laminin mouse protein, natural (Gibco, #23017015), human transferrin (Roche, #10-652-202-001), glutamic acid (Sigma-Aldrich, #G1251), ascorbic acid (Sigma-Aldrich, #A4544), SB431542 (Tocris, #1614), Y27632 (ROCK inhibitor) (STEMGENT, #04-0012-02), MTG (Sigma-Aldrich, #M6145), accutase (Gibco , #A1110501), polyornithine (Sigma-Aldrich, #P4957), CHIR99021 (Tocris, #4423/10), γ-secretase inhibitor XXI, compound E (Millipore, #565790), recombinant human BDNF (R&D Systems, #248-BD), recombinant human GDNF (R&D Systems, #212-GD), recombinant human BMP-4 (R&D Systems, #314-BP), recombinant human VEGF (R&D Systems, #293-VE), recombinant human EGF (R&D Systems, #236-EG), recombinant human FGF2 (R&D Systems, #233-FB), recombinant human SCF (R&D Systems, #255-SC), recombinant human DKK-1 (R&D Systems, #5439-DK), recombinant human IL-3 (R&D Systems, #203-IL), recombinant human IL-6 (R&D Systems, #206-IL), and recombinant human M-CSF (R&D Systems, #216-MC).

Infection

Cells were spinoculated with HIV-1 (1h at RT and 1100 x g) at various multiplicity of infection (MOI, typically 0.5 to 2), cultured for 2-3 hours at 37˚C, washed to remove unbound virus particles, and cultured for 3-6 days. Infection was quantified by analyzing $p24^{Gag}$ released into the culture supernatants or GFP expression by flow cytometry (BD LSRII). In some experiments, cells were pretreated prior (at least 30 min) to infection

with efavirenz (1 µM, NIH AIDS Reagent Program), raltegravir (30 µM, Selleckchem), or treated 2-3 hours post infection (p.i.) with KPT-330 (1 µM, selinexor, Selleckchem), or KPT-335 (0.1 µM, verdinexor, Selleckchem). DMSO (Sigma-Aldrich) was used as a vehicle control.

RNA analysis

Total mRNA was isolated from 0.5-1x10^6 cells using an RNeasy kit (QIAGEN) and reverse-transcribed using oligo$(dT)_{20}$ primer (Superscript III, Invitrogen). Target mRNA was quantified using Maxima SYBR Green (Thermo Scientific) using the following primer sets: P2RY12 (forward: 5'- CTTTCTCATGTCCAGGGTCAG-3', reverse: 5'-CTGCAGAGTGGCATCTGGTA-3') and GAS6 (forward: 5'-CCTTCCATGAGAAGGACCTCGT-3', reverse: 5'-GAAGCACTGCATCCTCGTGTTC-3'). Primer sequences for GAPDH, HIV spliced RNA and IP-10 were described previously (72). For hiMG-hiNeuron co-culture, target mRNA was quantified using TaqMan Universal PCR Master Mix (ThermoFisher Scientific) and the following primer/probe sets: Hs99999905_m1 (GAPDH), Hs01922583_s1 (CX3CR1), Hs01881698_s1 (P2RY12), and Hs01881698_s1 (P2RY12). The C_T value was normalized to that of GAPDH and represented as a relative value to a control using the $2^{-\Delta\Delta C}{}_T$ method as described (72, 77). NanoSting analysis was performed using a human neuroinflammation kit and total RNAs (100 ng) isolated from MDMGs per the manufacturer's instruction.

ELISA

500 IP-10 and CCL2 production in culture supernatants was measured with a BD Human IP-
501 10 ELISA Set and a BD Human MCP-1/CCL2 ELISA Set, respectively. To quantitate
502 virus production, $p24^{Gag}$ in culture supernatants was quantified by in-house ELISA (8).
503
504 **Flow cytometry**
505 To sort $CD45^+$ $CD11b^+$ $CD163^+$ $CD14^+$ $CX3CR1^+$ hiMacs, cells were stained with
506 Fixable Viability Stain 780 (BD Bioscience, #565388) followed by staining with a PE-
507 conjugated mouse anti-human CD45 antibody (BD Biosciences, #555483, 1:10), an
508 APC-conjugated anti-human CD11b antibody (BioLegend, #301410, 1:20), a BV421-
509 conjugated mouse anti-human CD14 antibody (BD Biosciences, #565283, 1:20), a
510 FITC-conjugated mouse anti-human CD163 antibody (BD Biosciences, #563697, 1:20),
511 and a PerCP/Cy5.5-conjugated anti-human CX3CR1 antibody (BioLegend, #341614,
512 1:20) in the presence of human Fc blocker (BD Bioscience, #564220). Stained cells
513 were sorted with Beckman Coulter MoFlo Astrios. To examine microglia activation, iCell-
514 MGs or hiMG–hiNeuron co-cultures were harvested with Cellstripper (Corning), stained
515 with Zombie-NIR (BioLegend, #423105, 1:250) followed by staining with a BV421-
516 conjugated mouse anti-P2RY12 antibody (BioLegend, 1:50) in the presence of human
517 Fc Blocker (BD Bioscience, #564220). Cells were fixed with 4% PFA (Boston
518 Bioproducts) for 30 min, permeabilized with Perm/Wash (Invitrogen), and intracellular
519 $p24^{Gag}$ expression was detected as described (72) using a FITC-conjugated mouse anti-
520 $p24^{Gag}$ monoclonal antibody (KC57, Coulter, # 6604665, 1:25). As for iCell-MGs, cell
521 surface CD169 expression was also analyzed using a BV605-conjugated mouse anti-
522 CD169 antibody (BioLegend, 1:50). Intracellular tubulin β3 in the hiMG–hiNeuron co-
523 cultures were analyzed with an Alexa 549 or 647-conjugated mouse anti-tubulin β3

antibody (TUJ-1, BioLegend, 1:50). Cells were analyzed with BD LSRII (BD). Data was analyzed with FlowJo software (FlowJo).

Imaging

For MDMGs and iCell-MGs, cells cultured in coverslip chambers (LabTekII) were washed and fixed with 4% paraformaldehyde. Cells were then permeabilized with 0.1% TritonX100, and stained with a rabbit anti-P2RY12 antibody (Sigma-Aldrich, HPA014518, 1:100), or a rabbit anti-IBA1 antibody (Fujifilm Wako, 019-19741, 1:250). Cells were then stained an Alexa594-conjugated anti-mouse-IgG antibody (Invitrogen, # A-11020, 1:200) and DAPI (Sigma-Aldrich). Cell were analyzed with a Nikon SP5 confocal microscope. hiMG-hiNeuron co-culture was fixed, permeabilized, and stained with an mouse anti-beta-Tubulin III antibody (Clone TUJ1, STEMCELL Technologies, #60052, 1:1000), and a rabbit polyclonal anti-TMEM119 antibody (Novus Biologicals, #NBP2-30551, 0.25-2 µg/mL), or a goat anti-IBA-1 antibody (Abcam, #ab5076, 1:500), followed by a Alexa Fluor 488-conjugated donkey anti-mouse IgG (Invitrogen, #A21202, 1:500) and an Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen, #A11012, 1:500) or an Alexa Fluor 594-conjugated rabbit anti-goat IgG (Invitrogen, #A11080, 1:500), respectively, and DAPI (NucBlue, Invitrogen). Cells were analyzed with a Keyence BZ-X710 All-in-one Fluorescence Microscope. Images were analyzed with ImageJ (NIH).

Immunoblot Analysis

To assess expression of host proteins, cell lysates containing 15-30 µg total protein were separated by SDS-PAGE, transferred to nitrocellulose membranes and the membranes were probed with the following antibodies: a mouse anti-SAMHD1 antibody

(Abcam, #ab67820, 1:1000) or a rabbit anti-phosphorylated (Thr 592) SAMHD1 antibody (Cell Signaling, #15038, 1:1000), and specific staining visualized with secondary antibodies, goat anti-mouse-IgG-DyLight 680 (Pierce) or a goat anti-rabbit-IgG-DyLight 800 (Pierce). As loading controls, actin expression was probed using a rabbit anti-actin antibody (Sigma-Aldrich, A2066, 1:5000). Membranes were scanned with an Odessy scanner (Li-Cor).

Statistics

All the statistical analysis was performed using GraphPad Prism 8. *P*-values were calculated using one-way ANOVA followed by the Tukey-Kramer post-test (symbols for *p*-values shown with a line) or the Dunnett's post-test (comparing to mock), symbols for *p*-values shown on each column), One sample t-test (comparing two samples, symbols for two-tailed *p*-values shown with a line), or a Wilcoxon signed rank test (comparing two samples, symbols for two-tailed *p*-values shown with a line). Symbols represent, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. No symbol: not significant ($p \geq 0.05$).

Data availability

The authors declare that the data that support the findings of this study are available within the paper and from the corresponding author upon reasonable request.

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573

574 **Author Contributions**

575 H.A., G.M., and S.G. designed the experiments. H.A., S.J., M.L, and S.P. performed the
576 experiments and analyzed the data. H.A. and S.G. wrote the manuscript.

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Figure Legends

Figure 1. Monocyte-derived microglia (MDMG) are susceptible to HIV-1 infection.

(**A**) Schematic of MDMG differentiation protocol. (**B**) Representative image of MDMs or MDMGs differentiated from the same donor. Bars=20 µm. (**C, D**) Expression of (**C**) P2RY12 (**D**) GAS6 mRNA in MDMGs was quantified by qRT-PCR and normalized to that of MDM generated from the same donor. (**E**) Representative immunofluorescence images of MDMGs stained for nucleus (DAPI, blue) and P2RY12 or IBA-1 (red). Bar=20 µm. (**F**) MDMGs were infected with Lai/YU-2env (replication competent CCR5-tropic HIV-1, MOI=1), and production of $p24^{Gag}$ in the culture supernatant was quantified by ELISA (3 dpi). (**G**) Western blot analysis for total SAMHD1, phosphorylated SAMHD1 expression in MDMGs, MDMs and THP-1 cells. Actin was probed as a loading control. +: PMA-treated THP-1, -: unstimulated THP-1. (**H**) MDMGs and MDMs were infected with HIV-1 (LaiΔenvGFP/VSV-G, MOI=2, in the absence or presence of SIV_{mac}239 Vpx VLPs), and production of $p24^{Gag}$ in the culture supernatant was quantified by ELISA (3 dpi). NT: no-treatment (DMSO), EFV: efavirenz (1µM), Ral: raltegravir (30 µM). The means ± SEM are shown and each symbol represents an independent experiment. *P*-values: One-sample t-test (**C**, two-tailed), the Wilcoxon matched-pairs signed rank test (**D**, two-tailed), or one-way ANOVA followed by the Tukey-Kramer post-test (**F**) or the Dunnett's post-test comparing to mock (**H**). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Figure 2. HIV-1 infection induces innate immune activation in MDMGs.

(**A**) mRNA expression profiles in MDMGs infected with HIV-1 (LaiΔenvGFP/VSV-G, MOI=2, in the presence of SIV_{mac}239 Vpx VLPs) was analyzed using the human neuroinflammation panel (NanoString). Expression of mRNA in HIV-1-infected MDMGs

was normalized to that in mock-infected MDMGs (**A**), in infected MDMGs in the presence of (**B**) efavirenz or (**C**) raltegravir, and genes which were expressed greater than the mean+ 2xSD are shown. (**D**) Production of IP-10 in HIV-1-infected MDMGs (MOI=2, 3 dpi) measured by ELISA. Effects of CRM1 inhibitor (KPT-330) on HIV-1-infected MDMGs or infection of MDMGs with a Rev mutant deficient for icRNA nuclear export (Rev*: M10) on (**E**) viral infection (multiply-spliced viral RNA expression, Rev-independent, shown as ΔCT to GAPDH), (**F**) $p24^{Gag}$ production (Rev-dependent) measured by ELISA, or (**G**) IP-10 mRNA expression (shown as ΔCT to GAPDH). The means ± SEM are shown and each symbol represents an independent experiment. *P*-values: one-way ANOVA followed by the Dunnett's post-test comparing to mock (**D**-**G**). *: $p < 0.05$, **: $p < 0.01$, ****: $p < 0.0001$. NT: no treatment (DMSO), EFV: efavirenz (1 µM), Ral: raltegravir (30 µM), KPT: KPT-330 (Selinexor, 1 µM), Rev*: M10.

Figure 3. iPSC-derived microglia are highly susceptible to HIV-1 infection

(**A**) Representative phase-contrast images of iCell-MGs (FUJIFILM Cellular Dynamics). Bar=20 µm. (**B**) Representative immunofluorescence image of iCell-MGs stained for nucleus (DAPI, blue) and IBA-1 (red). Bar=20 µm. (**C**) Representative flow cytometry profile of iCell-MGs stained for intracellular and surface P2RY12. (**D**) Western blot analysis for total SAMHD1, phosphorylated SAMHD1 expression in iCell-MGs, MDMGs, and MDMs. Actin was probed as a loading control. (**E**) Replication kinetics of HIV-1 in iCell-MGs. Cells were infected with HIV-1 (Lai/YU-2env, replication competent CCR5 tropic HIV-1, MOI=1), and production of $p24^{Gag}$ in the culture supernatant was quantified by ELISA. (**F**-**J**) iCell-MGs were infected with HIV-1 (Lai/YU-2env, MOI=1), and (**F**) HIV-1 infection (intracellular $p24^{Gag}$ expression) and (**G, H**) CD169 expression were analyzed

by flow cytometry. Production of proinflammatory cytokines (**I**) IP-10 and (**J**) CCL2 in the culture supernatants was measured by ELISA (6 dpi). The means ± SEM are shown and each symbol represents an independent experiment. *P*-values: one-way ANOVA followed by the Dunnett's post-test comparing to mock (**F, H-J**), *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. NT: no treatment (DMSO), EFV: efavirenz (1 µM), Ral: raltegravir (30 µM), Ver: verdinexor (KPT-335, 0.1 µM).

Figure 4. Establishment of iPSC-derived microglia/neuron co-culture system. (**A**) Schematic of hiMG generation by co-culturing hiMAC (yolk-sac-derived primitive macrophages) and neurons from human iPSCs. (**B**) Representative phase-contrast image of hiMGs and hiNeurons co-cultured for 11 days. Bar=50 µm (**C**-**E**) Expression of (**C**) TMEM119, (**D**) CX3CR1, and (**E**) P2RY12 mRNA in hiMACs or hiMG–hiNeuron co-cultures was quantified by qRT-PCR and normalized to that of hiNeuron solo culture. *P*-values from one-way ANOVA test for **C**, **D** and **E** were 0.0972, 0.0829, and 0.0814, respectively. (**F**) Representative immunofluorescence images of hiMG–hiNeuron co-cultures stained for nucleus (DAPI, blue), neuron (tubulin beta 3: TUBB3, green) and MG markers IBA-1 or TMEM119 (red). Bars=50 µm. (**G**) Representative flow cytometry profile of hiMG–hiNeuron co-culture stained for neurons (TUBB3) and hiMGs (P2RY12). The means ± SEM are shown and each symbol represents an independent experiment.

Figure 5. HIV-1 infection of hiMGs in hiMG–hiiNeuron co-cultures induces pro-inflammatory responses.

hiMG–hiNeuron co-cultures were infected with HIV-1 (Lai/YU-2env: replication competent CCR5 tropic HIV-1, MOI=1). (**A**, **B**) HIV-1 infection (intracellular $p24^{Gag}$ expression) was analyzed by flow cytometry. (**A**) Representative flow cytometry profile is shown, and microglia ($P2RY12^{+}$) and neuron ($P2RY12^{-}$) populations are highlighted with pink and blue, respectively. (**B**) HIV infected ($p24^{Gag+}$) cells in microglia (pink in **A**) were calculated. (**C**) Replication kinetics of HIV-1 in hiMG–hiNeuron co-culture. Co-cultures were infected with HIV-1 (Lai/YU-2env, replication competent CCR5 tropic HIV-1, MOI=1), and production of $p24^{Gag}$ in the culture supernatant was quantified by ELISA. Production of proinflammatory cytokines (**D**) IP-10 and (**E**) CCL2 was measured by ELISA (6 dpi). Proportion of live cells in (**F**) microglia (pink in **A**) and (**G**) neurons (blue in **A**) was calculated. The means ± SEM are shown and each symbol represents an independent experiment. *P*-values: one-way ANOVA followed by the Dunnett's post-test comparing to mock (**B, D**-**F**), **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$. *P*-value from one-way ANOVA test was 0.9662 for **G**. NT: no-treatment (DMSO), EFV: efavirenz (1 µM), Ral: raltegravir (30 µM), KPT: KPT-330 (selinexor, 1 µM).