Brain Microglial Cells Are Highly Susceptible to HIV-1 Infection and Spread

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Abstract

Macrophages are a target of human immunodeficiency virus type 1 (HIV-1) infection and may serve as an important reservoir of the virus in the body, particularly after depletion of CD4⁺ T cells in HIV/AIDS. Recently, sterile alpha motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1) was identified as the major restriction factor of HIV-1 infection in myeloid cells. SAMHD1 is targeted for proteolytic degradation by Vpx, a viral protein encoded by HIV-2 and many simian immunodeficiency viruses but not HIV-1. In this study, we assessed SAMHD1 restriction in *in vitro* differentiated macrophages and in freshly isolated macrophages from the lungs, abdomen, and brain. We found that infection and spread in *in vitro* cultured monocyte-derived macrophages were highly limited and that Vpx largely relieved the restriction to initial infection, as expected. We observed nearly identical infection and restriction profiles in freshly isolated peripheral blood monocytes, as well as lung (alveolar) and abdominal (peritoneal) macrophages. In contrast, under the same infection conditions, primary brain macrophages (microglia) were highly susceptible to HIV-1 infection despite levels of endogenous SAMHD1 comparable to the other macrophage populations. Addition of Vpx further increased HIV-1 infection under conditions of limiting virus input, and viral spread was robust whether or not SAMHD1 was depleted. These results suggest that HIV-1 infection of peripherally circulating macrophages is effectively restricted by SAMHD1; however, microglia are highly susceptible to infection despite SAMHD1 expression. These data may explain the long-standing observation that HIV-1 infection is often detected in macrophages in the brain, but seldom in other tissues of the body.

Keywords: microglia, macrophage, HIV-1

Introduction

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) primarily infects CD4-positive T lymphocytes and myeloid cells, including macrophages and dendritic cells. Macrophages are a diverse set of terminally differentiated phagocytes, which are found in nearly all tissues in the body. While T cell infection and depletion likely drives HIV disease progression, macrophages may play an important role in viral dissemination and persistence throughout the body, for example, by acting as an intermediary reservoir, picking up and passing on infection during antigen presentation interactions with CD4⁺ T cells.¹ Because they are long-lived cells, macrophages may also serve as a latent reservoir for HIV and could provide a constant source of archived virus populations that rebound after cessation of antiretroviral therapy.^{2–5}

Macrophages encode multiple restrictive mechanisms that can control retroviral infections;⁶ however, the sterile alpha

motif and histidine/aspartic acid domain-containing protein 1 (SAMHD1) appears to be the major restriction factor limiting infection *in vitro* and *in vivo*.^{7–9} SAMHD1 is a deoxynucleoside triphosphohydrolase (dNTPase) that can delay retroviral reverse transcription by reducing cellular dNTP pools.^{10–12} SAMHD1 is targeted for degradation by the accessory protein Vpx, which is encoded by HIV-2 and related simian immunodeficiency viruses (SIVs), promoting E3 ubiquitin ligase-mediated degradation of SAMHD1.^{13,14} Treatment of myeloid cells with Vpx-containing virus-like particles (VLPs) before or at the time of HIV-1 exposure enhances infection by accelerating proviral reverse transcription due to increased dNTP pools.¹⁰ Furthermore, myeloid cells isolated from individuals with genetic deficiencies in SAMHD1 expression support high levels of HIV-1 infection.⁹ Because HIV-1 does not encode any factors that counteract SAMHD1 restriction, there has been some speculation whether infection of myeloid cells is important to HIV-1 infection and AIDS pathogenesis.¹⁵

Monocyte-derived macrophages (MDM) have long been used as a model for macrophage infection by HIV-1.^{16,17} Monocyte precursors, isolated from peripheral blood, are highly resistant to HIV-1; however, *in vitro* differentiation results in macrophages that are susceptible to infection.¹⁸ MDM can live for months in culture, thus proving useful to study HIV persistence in macrophages *in vitro*.¹⁹ To what extent MDM simulate tissue macrophages is not clear.

Many groups have focused on alveolar and peritoneal macrophages (PM) to better characterize HIV-1 infection in differentiated tissue macrophages. Alveolar macrophages (AM), found in the pulmonary alveolus, are specialized macrophages, which are the primary innate immune defense cell in the lung and provide a useful *in vitro* model for HIV infection of lung macrophages.²⁰ Recent studies have shown that AM may harbor HIV-1 in healthy HIV-1 infected individuals who are on highly active antiretroviral therapy, even with undetectable plasma viral load.²¹ PM reside in the abdominal cavity and have recently been of interest as a primary macrophage model for HIV-1 infection.²² Both AM and PM express CD4 and CCR5 and are therefore susceptible to HIV-1 infection and may serve as a viral reservoir during a productive HIV infection.^{22,23} AM infected with HIV-1 display impaired phagocytic function, which may alter pulmonary innate immunity,²⁴ and PM similarly are capable of suppressing T cell activation, thus playing a role in immune homeostasis.²⁵ These natural interactions with T cells may provide an important route of HIV-1 dissemination after infection of alveolar and PM.

Perhaps the most important type of macrophage in the context of HIV/AIDS resides in the brain, a distinct lineage known as microglial cells.²⁶ Microglia predominantly express CD4 and CCR5 with subsets that also express CXCR4, although macrophage-tropic HIV strains that utilize the CCR5 coreceptor predominate in brain infections.^{27–29} Brain macrophages, primarily microglia, are thought to be infected early in the course of the disease and can be a source of continuous infection throughout HIV/AIDS progression.^{30,31} Microglia are long-lived cells, surviving for years, which may allow them to act as a viral reservoir of active and latent infections, thus posing a challenge to antiretroviral therapy.^{31,32} Unlike peripheral macrophages, microglia undergo cell division to maintain cell numbers, further providing opportunity for HIV persistence in the brain. Infection of microglia likely contributes to neurodegeneration in patients with HIV-associated dementia, although the pathogenic mechanism remains to be elucidated.³³

In this study, we sought to characterize HIV-1 infection and restriction by SAMHD1 in primary macrophages isolated from distinct tissues of the body. We demonstrate that alveolar and PM are highly restricted to HIV-1 infection, similar to cultured monocytes and MDM, whereas microglia are highly permissive to HIV-1 infection despite comparable SAMHD1 expression levels. Moreover, we found that HIV-1 established spreading infections in microglia cells and formed giant multinucleated syncytia in the cultures. Under conditions of limiting virus input, depletion of SAMHD1 resulted in marked increase of infection, suggesting that SAMHD1 restriction is active in microglia, but is unable to effectively block infection and spread as it does in peripheral macrophage populations. Our data might help explain evidence suggesting that macrophages in the brain are the primary tissue macrophage infected in HIV/AIDS.

Materials and Methods

Viruses

HIV^{NLAD8} is a macrophage (CCR5) tropic HIV-1 derivative of pNL4-3 containing the ADA envelope (NIH AIDS Reagent Program, Germantown, MD).³⁴ HIV^{NLAD8-GFP-Nef} is a fully replication competent recombinant HIV-1 strain that expresses all viral proteins, as well as GFP, which is expressed of a bicistronic Nef mRNA. It was constructed by replacing the NL4-3 Env glycoprotein in NL43-GFP-IRES-Nef³⁵ with the EcoRI-Bam fragment from NLAD8. HIV^{Laidenv} was generated using the single-round, envelope-deleted provirus pLAI (pLAI-ΔEnv), a molecular clone of HIV-1 generously provided by Dr. Michael Emerman. The reporter virus construct created from pLAI encoding the EGFP gene in place of the nef open reading frame (pLAI-ΔEnv-GFP) was created previously³⁶ and was kindly provided by Masahiro Yamashita (Aaron Diamond AIDS Research Center, New York, NY).

Vesicular stomatitis virus G protein (VSV-G) pseudotyped HIV^{Laidenv} virus stocks were produced by calcium phosphate cotransfection of HEK 293T cells with the proviral construct and an equivalent mass of a plasmid expressing VSV-G driven by the HIV-1 LTR (pLTR-VSV-G) as described previously.³⁷ HIV^{NLAD8} and HIV^{NLAD8-GFP-Nef} were produced by calcium phosphate transfection of HEK 293T cells using constructs described above. Transfected cells were washed 16 h posttransfection, media was replaced 8 h later, and virus-containing supernatants were collected the next morning, ~40 h posttransfection. Supernatants were then centrifuged to clear cellular debris, passed through 0.45 μm filters, aliquoted, and stored in liquid nitrogen before infectivity and p24 assays.

SIV-VLP were generated by calcium phosphate cotransfection of HEK 293T cells with pSIV3⁺ in combination with an equivalent weight of pLTR-VSV-G as above. pSIV3⁺, kindly provided by Andrea Cimorelli, was derived from the SIV-mac251 proviral clone and is deficient in envelope expression and genome packaging (env/psi-).³⁸ SIV-VLP were tested for delivery of Vpx by treatment of MDM for 16 h and subsequent imaging for SAMHD1 expression. SIV-VLP stocks were considered effective if they depleted SAMHD1 expression in greater than 90% of the MDM.

Infectivity of the stocks was determined using LuSIV LTR-Luciferase indicator cells,³⁹ and p24 was quantified by ELISA (XpressBio, Frederick, MD). Infectivity of viruses was titered on MDM, and input viruses for all experiments were used at a titer below saturating level. Final concentration in cultures was 150 ng/ml. When infecting microglia in later experiments, low titer of 15 ng/ml was used. To determine viral production from infected MDM and microglia, cells were washed 16 h postinfection (p.i.) and supernatants were harvested 72 h later and frozen before p24 assay.

Monocyte-derived macrophages

Human peripheral blood mononuclear cells (PBMC) were isolated from healthy donors following Ficoll density gradient centrifugation. CD14-positive monocytes were then isolated by positive selection using anti-CD14 magnetic beads (Miltenyi Biotech, San Diego, CA). CD14-positive monocytes were cultured in Iscove's modified Dulbecco's medium (IMDM) without phenol red (Gibco, Waltham, MA) supplemented with 5% human serum (Valley Biomedical, Winchester,

VA), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen, Carlsbad, CA), and 1,000 U/ml macrophage-colony stimulating factor (M-CSF; R&D Systems, Minneapolis, MN). Cells were treated again with M-CSF 2–3 days post isolation and cultured an additional 4–5 days. MDM were fully differentiated after 5–6 days in culture.

Alveolar macrophages

AM were obtained by bronchoalveolar lavage from healthy volunteers. AM were cultured in IMDM without phenol red (Gibco) supplemented with 5% human serum (Valley Biomedical), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) in the absence of stimulating cytokines. Cells were washed vigorously 1 day postisolation to remove non-adherent cells. AM were infected 1 day after isolation or after 1 week of culture.

Peritoneal macrophages

Deidentified human peritoneal exudates were obtained from the Histology Tissue Procurement Facility of University Hospitals of Cleveland following IRB approved protocols for collection of discarded surgical tissues. Samples from HCV-positive individuals were excluded from the study. Centrifugation was performed to isolate PM as previously described.²² CD14⁺ cells were isolated using CD14⁺ microbeads to eliminate mesothelial cells from the cultures. PM were plated and cultured in IMDM without phenol red (Gibco) supplemented with 5% human serum (Valley Biomedical), 100 U/ml penicillin, and 100 μ g/ml streptomycin (Invitrogen) in the absence of stimulating cytokines for 1 day before infection.

Microglia

Human microglia were purchased from ScienCell (San Diego, CA) and cultured in IMDM without phenol red (Gibco) supplemented with 5% human serum (Valley Biomedical), 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen) in the absence of stimulating cytokines, plated on glass chamber slides for immunofluorescence microscopy, and infected 1 day after plating.

CD4⁺ T cells

PBMC were isolated from healthy donors following Ficoll density gradient centrifugation. CD14-negative cells were then collected by negative selection using anti-CD14 magnetic beads (Miltenyi Biotec). Naive CD4⁺ T cells were isolated by negative selection following manufacturer's instructions (Naive CD4⁺ T cell Isolation Kit II; Miltenyi Biotec). To maintain naive CD4⁺ T cells, cells were cultured in IL-2 (2 U/ml). To generate activated CD4⁺ T cells, cells were stimulated with Dynabeads Human T-Activator CD3/CD28 beads for 3 days following manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). CD4⁺ T cells were cultured in RPMI supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 100 U/ml penicillin and 100 μ g/ml streptomycin (Invitrogen). Dynabeads were magnetically removed from cells before further use.

Flow cytometry

Adherent cells were dissociated from culture plates using 0.25% Trypsin (Gibco) and immunostained for 15 min at 4°C in 1 $\times$ phosphate-buffered saline (PBS) supplemented with 0.5% bovine serum albumin and 2 mM EDTA. Fluorescent anti-CD4 (Clone OKT4; BioLegend, San Diego, CA), CCR5 (Clone 2D7/CCR5; BD Biosciences, San Jose, CA), and HIV p24gag [Clone KC57-FITC (fluorescein isothiocyanate); Beckman Coulter, Brea, CA] were used. Matched isotype controls were used according to the manufacturer's directions and analyzed using an LSR II (Becton Dickinson, Franklin Lakes, NJ) or FACSCalibur (BD Biosciences) flow cytometer. Matched isotype controls overlaid the unstained samples and were therefore not shown.

EdU labeling

Cells were washed with PBS and incubated in culture media without cytokines in the presence of 10 μ M EdU overnight. Cells were then rinsed with PBS, fixed in 4% electron microscopy (EM)-grade formaldehyde (Polysciences, Warrington, PA) in PBS for 20 min at room temperature, and rinsed and stored in PBS. Cells were labeled using the Click-iT EdU Alexa Fluor Labeling Kit (Thermo Fisher Scientific) modified as previously described⁴⁰ and mounted in ProLong Gold Antifade Mountant (Life Technologies, Carlsbad, CA).

Fluorescence microscopy

Cells were fixed with 4% EM-grade formaldehyde (Polysciences) in PBS for 20 min and rinsed in PBS. Cell membranes were extracted with 0.1% Triton X-100 in staining buffer (PBS, 10% normal donkey serum; Jackson ImmunoResearch, West Grove, PA) for 5 min at room temperature. Cells were stained for SAMHD1 (Clone 1A1; OriGene, Rockville, MD) in staining buffer for 15 min at room temperature. Nuclei were stained with Hoechst dye (0.5 μ g/ml; Sigma-Aldrich, St. Louis, MO) in staining buffer for 15 min at room temperature. Coverslips were mounted onto glass slides using ProLong Gold Antifade Mountant (Thermo Fisher Scientific).

Slides were imaged using a DeltaVision widefield fluorescence imaging system (Applied Precision, Inc., Mississauga, ON, Canada) and analyzed using the DeltaVision Softworks analysis software. Quantification of infection was performed using IMARIS analysis software (Bitplane, South Windsor, CT). The total number of cells was quantified using surface approximation of Hoechst nuclear staining, and the number of productively infected GFP⁺ cells was quantified by surface approximation of GFP signals with the lower threshold set by uninfected control cells.

Cell extracts and western blot analysis

Cells were washed with PBS and lysed in 1 ml per 10⁶ cells of radioimmunoprecipitation assay buffer [20 mM Tris (pH 7.5), 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCL (pH 8.1), and 500 mM NaCl] with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). Nuclei were removed by centrifugation at 13,000 rpm for 10 min. Protein was quantified using the Bradford assay, and 15 μ g of total protein was loaded. Extracts were separated by SDS-PAGE,

blotted onto nylon membranes and individually probed with the following antibodies: mouse anti-SAMHD1 (Clone 1A1, OriGene), rabbit anti-phospho-T592 SAMHD1 (Cell Signaling, Danvers, MA), and rabbit anti-GAPDH (Clone EPR16891; Abcam, Cambridge, MA).

Results

We first characterized HIV-1 infection and restriction in MDM, a common source of *in vitro* differentiated primary macrophages used for HIV-1 infection studies. Peripheral blood was obtained from healthy donors, and MDM were prepared by culturing CD14⁺ monocytes in the presence of M-CSF. MDM were infected with HIV^{NLAD8}, a recombinant CCR5-tropic HIV-1 strain expressing all viral proteins, or HIV^{NLAD8-GFP-Nef}, which additionally encodes for GFP on a bicistronic Nef mRNA. Infection with either viral strain re-

sulted in ~3% infection by 4 days p.i. (dpi) (Fig. 1A, B), a baseline level that we have observed over dozens of experiments with HIV^{NLAD8} and HIV^{NLAD8-GFP-Nef}, as well as other HIV-1 isolates. To determine to what extent SAMHD1 restricts infection in MDM, SIV Vpx was delivered by exposing cells to VSV-G pseudotyped SIV-VLP that are defective for genome packaging and reverse transcription and deliver Vpx into target cells upon fusion and cytosolic delivery of the VLP contents.³⁸ Treatment with Vpx-containing SIV-VLP reproducibly resulted in greater than 10-fold increased infection when MDM were infected with either HIV^{NLAD8} or HIV^{NLAD8-GFP-Nef}, with infection levels reaching 32.4% and 35.5%, respectively (Fig. 1A, B). Treatment with SIV-VLP alone or infection in the presence of SIV-VLP lacking Vpx (SIV-VLP^{Vpx-}) did not result in increased HIV infection, indicating that increased GFP expression is a result of Vpx delivery (Supplemental Figure S1; Supplementary Data are available online at

FIG. 1. SAMHD1 restricts HIV-1 infection in MDM. MDM were infected with the HIV-1 strains HIV^{NLAD8} (NLAD8) (A) or HIV^{NLAD8-GFP-Nef} (NLAD8-GFP) (B) alone or in the presence of SIV-VLP (+Vpx) as indicated. Cells were stained and analyzed by flow cytometry 4 and 8 dpi. Circled populations are HIV p24-positive (HIV^{NLAD8}) or GFP-positive (HIV^{NLAD8-GFP-Nef}), CD4 down-regulated productively infected cells. Numbers in the plots indicate percent of total cells infected. (C) MDM were cultured on glass coverslips and infected with HIV^{NLAD8-GFP-Nef} without or with SIV-VLP (+Vpx), fixed 4 or 8 dpi and stained for SAMHD1 (red) and nuclear DNA (blue). Adjacent fields were imaged, stitched together, and rendered into single whole cell volume projections. GFP (green) marks productively infected cells. Scale bar: 15 μ m. (D) MDM were infected with HIV^{NLAD8-GFP-Nef} without or with SIV-VLP (+Vpx) for the indicated time and quantified by flow cytometry as above. MVC (2.5 μ M) was added 5 dpi and every 3 days thereafter where indicated to block the potential spread of infection. Infection was quantified using flow cytometry as in (A). Data shown are representative of at least three independent experiments. dpi, day postinfection; HIV-1, human immunodeficiency virus type 1; MDM, monocyte-derived macrophages; MVC, Maraviroc; SAMHD1, sterile alpha motif and histidine/aspartic acid domain-containing protein 1; SIV-VLP, simian immunodeficiency virus-virus-like particles.

www.liebertpub.com/aid). Over the course of numerous independent experiments, we observed a 12.7 ± 1.5 -fold increase in infection after SIV-VLP treatment regardless of blood donor, viral titer, or viral strain. Because HIV^{NLAD8} and HIV^{NLAD8-GFP-Nef} behaved identically in our hands, we used them interchangeably in subsequent experiments. Figure 1C shows immunofluorescence microscopy of MDM infected with HIV^{NLAD8-GFP-Nef}, demonstrating that SAMHD1 expression was significantly reduced in SIV-VLP treated cells, resulting in greatly increased HIV-1 infection.

Surprisingly, when MDM were cultured for longer periods, we observed virtually no spread of infection in cultures with or without SAMHD1 knockdown (Fig. 1D). When MDM were infected with HIV alone, there was little or no spread of infection in the cultures beyond the initial level of infection, with infection levels staying below 3% over the 3-week time course (Fig. 1D). As a control, we treated MDM with the entry inhibitor Maraviroc (MVC) 5 dpi and every 3 days thereafter to block second round infections. In these experiments, we observed essentially the same level of infection as the untreated infected cells after MVC treatment, indicating that cells infected in the first round persist in the cultures and there is very limited spread in the cultures.

When MDM were infected with HIV^{NLAD8-GFP-Nef} after SIV-VLP treatment, infection levels increased modestly over the course of 3 weeks, from 26% at 5 dpi to 30% after an

additional 6% and 28% after 15 days in culture. When these cultures were treated with MVC, we observed 27% and 26% infection throughout the 3-week time course. To determine whether the modest reduction of infection after MVC treatment was a result of cell-cell spread in untreated cultures, we also infected MDM with VSV-G pseudotyped replication-deficient virus, HIV^{Lai δ env} (VSV-HIV) (Fig. 1D).

This single-round control virus demonstrated a slight increase in infection over time, suggesting that the modest increases observed in replication competent infection are due to slow completion of reverse transcription in the MDM and not a consequence of second round spread. Therefore, when the data from Figure 1 are taken together, ~3% of MDM are naturally permissive for HIV-1 infection and there appears to be no spread beyond the first round of infection. Even when high levels of infection were achieved after SAMHD1 knockdown, we saw no evidence of spread in the cultures.

To characterize HIV-1 infection of primary tissue macrophages, we next obtained macrophages from various tissue sources. First, AM were prepared from bronchoalveolar lavage samples from healthy donors by culturing for 16 h and washing vigorously to remove nonadherent cells. The resultant cultures were >95% CD4-positive macrophages as determined by flow cytometry. In parallel, PBMC were prepared from the same donor, and CD14⁺ monocytes were isolated and differentiated into MDM as described above. We infected AM,

FIG. 2. SAMHD1 restricts HIV-1 infection of monocytes and AM. AM and PBMC were obtained from the same healthy donor. CD14⁺ cells were selected from the PBMC, cultured in the presence of M-CSF overnight (monocytes) and infected (A, top) or cultured an additional 6 days (MDM) before infection (B, top). AM were cultured without cytokines overnight, washed vigorously to remove contaminants, and infected 1 day (A, bottom) or 1 week (B, bottom) after isolation. Cells were infected with HIV^{NLAD8} in the absence or presence of SIV-VLP (+Vpx) as indicated. (A, B) Cells were stained and analyzed by flow cytometry 4 dpi. Circled populations are HIV p24 positive, CD4 downregulated, productively infected cells. Numbers in the plots indicate percent of total cells infected. (C, D) Productively infected cells from (A and B) were quantified and displayed as bar graphs in C (infected 1 day after isolation) and D (infected 1 week after isolation). Data are representative of three experiments. AM, alveolar macrophages; M-CSF, macrophage-colony stimulating factor; PBMC, peripheral blood mononuclear cells.

monocytes, and MDM with HIV^{NLAD8} and measured HIV p24 expression 4 days later by flow cytometry. Figure 2 shows the infection levels when infected 1 day after isolation, the standard protocol for AM (Fig. 2A, C), or after 7 days in culture as typical for MDM infection (Fig. 2B, D). Low levels of infection, below 0.5%, were detected when monocytes and AM were infected with HIV^{NLAD8} 1 day after isolation, and there was a robust increase in infection when cells were treated with SIV-VLP at the time of infection to 10.6% and 6.5% in monocytes and AM, respectively (Fig. 2A, C). When the matched MDM and AM were infected after 1 week in culture, both cell types demonstrated significant increase in baseline infection (Fig. 2B, D). This was expected in the case of MDM, as *in vitro* differentiation has long been known to increase monocyte permissivity to HIV-1 infection.¹⁸ The observation that AM also become more permissive over time underscores the importance of our analysis before long-term culture and suggests that *in vitro* culture impacts the physiology of the macrophages. Nevertheless, the cells remained largely refractory to infection, and treatment with SIV-VLP revealed that both macrophage populations remained restricted by SAMHD1. We observed a reproducible 15.0 ± 3.1 -fold increase in AM and 12.7 ± 1.5 -fold increase in MDM in the SIV-VLP treated cultures, suggesting that SAMHD1 restricts HIV-1 infection in AM to a similar degree as in MDM. However, even taking into consideration donor variation, the absolute level of infection either with or without SAMHD1 knockdown was substantially lower than levels typical of MDM. Thus, despite similar levels of restriction to HIV-1 infection by SAMHD1 in alveolar and MDM, AM display decreased baseline permissivity to HIV-1 compared to MDM.

We next assessed HIV-1 infection and restriction in PM isolated from ascites fluid obtained from patients undergoing therapeutic paracentesis.²² PM were isolated by Ficoll centrifugation and subsequent CD14⁺ bead selection. Figure 3A shows immunofluorescence microscopy of PM infected with HIV^{NLAD8-GFP-Nef} in the absence or presence of SIV-VLP, demonstrating that PM cell morphology is quite similar to MDM, and SAMHD1 is expressed in the cells before SIV-

VLP treatment. PM infected with HIV^{NLAD8-GFP-Nef} alone expressed GFP in 1% of cells at 4 dpi and 2% of cells by 8 dpi, levels similar to MDM (Fig. 3B, compare to Fig. 1B). SIV-VLP treatment resulted in an average 21.9 ± 4.8 -fold increase in infection, confirming that SAMHD1 restricts infection of PM to a similar degree as observed in MDM and AM. While the 22-fold increase after SAMHD1 knockdown was higher than the ~ 13 -fold increase observed in MDM, associated error suggests that the difference is not significant and likely due to donor variability. Thus, PM were comparable to MDM in baseline HIV-1 permissivity and restriction by SAMHD1.

Finally, we examined HIV-1 infection of human microglia. Due to the limited number of cells available, it was not feasible to use flow cytometry to quantify HIV-1 infection in these cells. Therefore, we used the fluorescence microscopy approach described above to quantify restriction in these cells. In initial experiments, microglia were infected with the same amount of HIV^{NLAD8-GFP-Nef} used to infect the other macrophage populations. Strikingly, over 50% of microglia were GFP-positive 3 dpi, and nearly 90% were positive 6 dpi when infected with HIV^{NLAD8-GFP-Nef} in the absence of SIV-VLP treatment (Fig. 4A, B), a far higher infection level than seen in the other macrophage populations. When microglia were cultured for 1 week before plating, similarly high infection levels were observed (data not shown). Treatment of both cultures with SIV-VLP resulted in even higher infection levels; however, the magnitude of the effect of Vpx was minimized due to the high level of infection in untreated cultures (Fig. 4B).

We next infected microglia with a 10-fold lower inoculum of HIV^{NLAD8-GFP-Nef} to characterize SAMHD1 restriction and more easily determine whether HIV-1 infection spreads in these cultures. As shown in Figure 4C and D, when a low titer was used, HIV^{NLAD8-GFP-Nef} alone resulted in 1.8% infection at 4 dpi and increased to 14.3% infection by 8 dpi (Fig. 4C, D). Importantly, addition of MVC 1 dpi blocked the increased infection, indicating that the increase observed is due to subsequent rounds of infection from infected to uninfected cells. Similarly, microglia infected with envelope deficient VSV-HIV resulted in very limited increase of GFP

FIG. 3. SAMHD1 restricts HIV-1 infection of PM. Macrophages (PM) were isolated from peritoneal exudates by Ficoll centrifugation and CD14⁺ magnetic bead selection. PM were infected 1 day after isolation with HIV^{NLAD8-GFP-Nef} (NLAD8) without or with SIV-VLP (+Vpx) as indicated. (A) PM were cultured on glass coverslips, fixed, and stained for SAMHD1 (red) and nuclear DNA (blue) 4 and 8 dpi. Adjacent fields were imaged, stitched together, and rendered into single whole cell volume projections. GFP (green) marks productively infected cells. Scale bar: 15 μ m. (B) Cells were cultured overnight, infected as above, and analyzed by flow cytometry 4 and 8 dpi. The mean percent infected cells from three independent experiments are shown; error bars represent standard error of the mean ($n=3$). PM, peritoneal macrophages.

FIG. 4. Microglia are highly permissive to HIV-1 infection even in the presence of SAMHD1. Human microglia were cultured overnight on chamber slides and infected with HIV^{NLAD8-GFP-Nef} (NLAD8) without or with SIV-VLP (+Vpx) as indicated. Cells were fixed and stained for SAMHD1 (red) and nuclear DNA (blue) and imaged. Adjacent fields were stitched together and rendered into single whole cell volume projections. GFP (green) marks productively infected cells. Representative images of multiple fields are shown. (A, B) Standard titer (150 ng/ml) of virus was used, and cells were analyzed 3 and 6 dpi as indicated. (C, D) Low titer (15 ng/ml = 10% of standard titer) of virus was used, and cells were analyzed 4 and 8 dpi as indicated. (B, D) GFP-positive cells in multiple fields were quantified, including those shown in (A, C), and plotted as average GFP-positive nuclei per field. Bars indicate the standard error of the mean ($n = 3$ fields, $\sim 1,500$ cells). Data are representative of three independent experiments. Scale bars: 15 μm (A), 50 μm (C).

expression, further confirming that the increased infection levels seen in HIV-1 infection were a result of spread in the cultures. This was surprising, as we had not observed any evidence of spreading infections in monocyte-derived, alveolar, or PM, as described above. Moreover, microglia infected with HIV alone formed giant multinucleated syncytia (Fig. 4C), a striking result we have never observed in the other macrophage populations studied here. When microglia were treated with SIV-VLP and infected with HIV^{NLAD8-GFP-Nef}, 37% of microglia were infected by 4 dpi and 71% were infected by 8 dpi (Fig. 4D), a considerable increase over infection levels in microglia without SIV-VLP treatment. Thus, while microglia are significantly more permissive to HIV-1 infection and spread, SAMHD1 can restrict infection under conditions of limited infection.

To determine if the increased spread of infection in microglial cultures is due to increased virus production by microglia upon productive infection, we quantified virus produced in culture supernatants by p24 ELISA (Fig. 5A). Interestingly, the amount of virus produced by microglia was not significantly different than MDM and is slightly lower (Fig. 5A),

further suggesting that microglia are uniquely susceptible to HIV-1 infection as low levels of virus released allow for spreading infection. Moreover, viral protein expression in the infected cells is comparable to that seen in MDM (Figs. 1C and 4A), suggesting that the increased infection and spread is not related to virion production or release in microglia. We next wanted to determine whether increased receptor density on the cell surface allows for increased permissivity of microglia. Thus, we stained for CD4 and CCR5, as higher expression of the coreceptors could result in increased fusion and infection. Figure 5B demonstrates that CD4 and CCR5 were expressed at the same or slightly lower levels than seen on MDM.

We performed additional experiments to determine whether the increased infection was due to microglia cycling or dividing in our cultures. To this end, we exposed the cultures to the thymidine nucleoside analog EdU, which incorporates into replicating DNA and can be visualized by subsequent fluorescent labeling.⁴¹ Surprisingly, microglia incorporation of EdU was identical to MDM and virtually undetectable in the cultures (Fig. 5C). We did observe rare MDM and microglia that were brightly stained with EdU, suggesting a very

FIG. 5. Coreceptor expression, viral release, SAMHD1 expression, and phosphorylation are similar in MDM and microglia. (A) MDM and microglia were infected with NLAD8-GFP-Nef. MDM were infected with standard titer, as in Figure 1, and microglia were infected with low titer as in Figure 4. Cells were washed 16 h p.i., supernatants were collected 72 h later, and p24 was quantified by ELISA. Infection was quantified by flow cytometry (MDM) or fluorescence microscopy (microglia). The graph shows the percent of infected cells (bars) and the corresponding p24 concentration in the supernatant (♦). (B) MDM (dark blue) and freshly isolated microglia (dark red) were stained and analyzed by flow cytometry for cell surface expression of CD4 and CCR5. Unstained MDM (light blue) and microglia (light red) controls are also shown. (C) Activated CD4⁺ T cells, naïve CD4⁺ T cells, MDM, and microglia were cultured in 10 μ M EdU overnight. Cells were fixed; EdU was labeled (red) and stained for nuclear DNA (blue). Adjacent fields were stitched together and rendered into single whole cell volume projections. Representative images of multiple fields are shown. (D) Whole cell extracts were prepared from MDM treated with SIV-VLP delivering Vpx (SIV-VLP^{Vpx+}) or SIV-VLP lacking Vpx (SIV-VLP^{Vpx-}) in addition to untreated MDM, microglia, naïve CD4⁺ T cells, activated CD4⁺ T cells, and THP-1 cells. Extracts were separated on SDS-PAGE and analyzed by western blot using the indicated antibodies.

small amount of cycling cells in the cultures; however, the fraction of EdU positive cells was far below basal MDM infection levels and could not account for the extensive infection levels seen in either MDM or microglial cultures.

As noted above, immunofluorescence analysis suggested that SAMHD1 was present in microglia at levels similar to the other primary macrophage populations studied here. SAMHD1 activity is known to be regulated by phosphorylation of Threonine 592.^{42,43} We performed western blot analysis to

accurately quantify SAMHD1 levels and phosphorylation status. Whole cell extracts were prepared from MDM treated with SIV-VLP^{Vpx+} delivering Vpx and SIV-VLP^{Vpx-} lacking Vpx, as controls. In addition, we prepared whole cell extracts from microglia, naïve, and activated CD4⁺ T cells, and THP-1 cells. As shown in the middle panel of Figure 5D, SAMHD1 expression is virtually identical in untreated MDM, microglia, naïve CD4⁺ T cells, and THP-1. CD4⁺ T cells which were activated by CD3/CD28 stimulation expressed SAMHD1 at

lower levels, which have been shown by other groups and, along with SAMHD1 regulation by phosphorylation, may contribute to permissivity to HIV-1.^{43,44} Thus, differential SAMHD1 levels in microglia and MDM do not account for the increased susceptibility of microglia. Interestingly, SAMHD1 was not phosphorylated in microglia or in MDM and naive CD4⁺ T cells, as expected (Fig. 5D). By contrast, SAMHD1 was phosphorylated in activated CD4⁺ T cells and THP-1, as previously described.⁴³ Thus, these data suggest that differential expression or phosphorylation status of SAMHD1 do not account for the increased HIV-1 susceptibility of microglia.

Taken together, our data indicate that microglia are remarkably more permissive to HIV-1 infection and spread than peripheral macrophages. This permissivity does not appear to be a consequence of surface receptor expression, cell proliferation, or SAMHD1 phosphorylation. Importantly, SAMHD1 knockout increased infection of microglia when virus input was low, suggesting that SAMHD1 is active in these cells, consistent with the lack of phosphorylation observed.

Discussion

In this study, we characterized HIV-1 infection and restriction by SAMHD1 in *in vitro* cultured monocytes and MDM, as well as tissue resident macrophages isolated from the lungs, abdomen, and brain. We demonstrated that ~3% of MDM are permissive to initial HIV-1 infection, and second-round spread is highly limited in these cultures. Knockdown of SAMHD1 by the SIV Vpx protein resulted in a 12-fold increase in HIV-1 infection. Surprisingly, even when SAMHD1 expression remained suppressed, we observed no spread of infection over 3 weeks in culture despite the high level of initial infection in the Vpx treated cells. These results were recapitulated in other macrophage populations from the periphery. Freshly isolated peripheral blood monocytes, PM, and AM were all highly resistant to HIV-1 infection, and SAMHD1 knockdown resulted in a 15- to 20-fold increase in infection, demonstrating that MDM faithfully reproduce HIV-1 infection profiles in primary macrophage populations found in peripheral tissues.

Surprisingly, when we exposed brain microglial cells to the same HIV-1 inoculum as the other macrophage populations, greater than 50% of the cells became productively infected in the first round of infection compared to 3%–5% of peripheral macrophages. After 6 days in culture over 80% of the cells were infected, suggesting that these macrophages were unusually permissive to HIV-1 infection and spread. Subsequent experiments utilizing 10-fold lower HIV-1 inoculum demonstrated that HIV-1 can spread in microglia, where the infection level increased from ~2% to over 14% over the course of the experiment. Under these conditions of limiting HIV-1 input, knockdown of SAMHD1 resulted in markedly increased initial infection that spread to nearly overtake the cultures after 8 days. These results indicate that SAMHD1 does in fact restrict infection in microglial cells; however, it does not effectively contain virus replication and spread in the cells.

In addition to the enhanced permissivity of microglia to HIV-1 infection, we noted that under conditions of limiting initial infection, productively infected multinucleated cells accumulated in the cultures over time, suggesting that the virus can efficiently form syncytia in these cells. This was not

observed in any of the other macrophage populations in this study, suggesting that microglia are highly susceptible to syncytia formation by HIV-1, an observation consistent with numerous studies that have described micronodular lesions in the brain that contain multinucleated giant cells, a hallmark of HIV-associated neurocognitive disorders.^{45,46} We note that in conditions of high initial infection, few multinucleated infected cells accumulated over time, likely due to the limited number of uninfected neighboring cells necessary for the HIV-1 gp120 and CD4 mediated fusion events that generate syncytia.

Quantification of the amount of virus released indicated that MDM produced as much or more viral particles than the infected microglia, suggesting that the spread observed is not a consequence of increased HIV-1 production. Moreover, despite the lack of spread in MDM cultures, the virus released is infectious to cocultured T cells¹ (and unpublished observations). Therefore spread in the microglia cultures cannot be explained by increased virus production or infectivity of the released virions. Similarly, we observed slightly lower surface density of CCR5 and CD4 on microglia compared to the MDM, indicating that receptor density does not account for the microglial permissivity. In addition, we found no evidence that the microglia incorporated EdU into chromosomal DNA, indicating that they were not cycling or dividing in the cultures. Furthermore, we measured SAMHD1 expression and phosphorylation, and SAMHD1 levels of MDM and microglia were remarkably similar. Finally, SAMHD1 was not phosphorylated in either of these macrophage types. Thus, SAMHD1 expression levels and phosphorylation status do not account for the increased susceptibility of microglia to infection.

Because of the very limited availability of microglia, it was not possible for us to assess the levels of dNTP pools in the cells. Although the microglia were clearly not actively dividing or incorporating EdU into chromosomal DNA, it is possible that SAMHD1 is only partially active despite the lack of detectable phosphorylation, and dNTP levels are high enough to support efficient HIV-1 reverse transcription. Importantly, depletion of SAMHD1 resulted in increased infection, suggesting that SAMHD1 is at least partially restrictive in these cells. Regardless of whether dNTP pools are different in microglia, it is clear that other restrictive mechanisms that exist in the peripheral tissue macrophages⁴⁷ are not at play in the microglia, as apparent from the lack of spread observed in other primary macrophages when SAMHD1 is depleted.

In summary, our data support the hypothesis that SAMHD1 expression in peripheral tissue macrophages highly restricts HIV-1 infection and is consistent with the rare identification of infected macrophages in the tissues of infected individuals. However, microglia are highly susceptible to HIV-1 infection and spread *in vitro*, suggesting that these cells may in fact be an important target of infection *in vivo*. HIV-1 infection of these long-lived cells may play a role in maintenance of HIV persistence and poses a high barrier to current therapeutic eradication strategies. Furthermore, our data suggest that the susceptibility of microglia to infection and syncytia formation may explain why brain macrophages are frequently identified in infected individuals postmortem, whether or not they exhibited neurological symptoms before death. Further characterization of HIV-1 infection in microglia is warranted

to better understand the contribution to disease progression and potential curative therapies.

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Author Disclosure Statement

No competing financial interests exist.

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