

Macrophages sustain HIV replication in vivo independently of T cells

Jenna B. Honeycutt, ... , Joseph J. Eron, J. Victor Garcia

J Clin Invest. 2016;126(4):1353-1366. <https://doi.org/10.1172/JCI84456>.

Research Article

AIDS/HIV

Macrophages have long been considered to contribute to HIV infection of the CNS; however, a recent study has contradicted this early work and suggests that myeloid cells are not an in vivo source of virus production. Here, we addressed the role of macrophages in HIV infection by first analyzing monocytes isolated from viremic patients and patients undergoing antiretroviral treatment. We were unable to find viral DNA or viral outgrowth in monocytes isolated from peripheral blood. To determine whether tissue macrophages are productively infected, we used 3 different but complementary humanized mouse models. Two of these models (bone marrow/liver/thymus [BLT] mice and T cell–only mice [ToM]) have been previously described, and the third model was generated by reconstituting immunodeficient mice with human CD34⁺ hematopoietic stem cells that were devoid of human T cells (myeloid-only mice [MoM]) to specifically evaluate HIV replication in this population. Using MoM, we demonstrated that macrophages can sustain HIV replication in the absence of T cells; HIV-infected macrophages are distributed in various tissues including the brain; replication-competent virus can be rescued ex vivo from infected macrophages; and infected macrophages can establish de novo infection. Together, these results demonstrate that macrophages represent a genuine target for HIV infection in vivo that can sustain and transmit infection.

Find the latest version:

<https://jci.me/84456/pdf>



Macrophages sustain HIV replication in vivo independently of T cells

Jenna B. Honeycutt,¹ Angela Wahl,¹ Caroline Baker,¹ Rae Ann Spagnuolo,¹ John Foster,¹ Oksana Zakharova,¹ Stephen Wietgreffe,² Carolina Caro-Vegas,³ Victoria Madden,⁴ Garrett Sharpe,³ Ashley T. Haase,² Joseph J. Eron,¹ and J. Victor Garcia¹

¹Division of Infectious Diseases, Center for AIDS Research (CFAR), University of North Carolina at Chapel Hill (UNC), School of Medicine, Chapel Hill, North Carolina, USA. ²Department of Microbiology, University of Minnesota, Minneapolis, Minnesota, USA. ³Department of Microbiology and Immunology, and ⁴Microscopy Services Laboratory, UNC, Chapel Hill, North Carolina, USA.

Macrophages have long been considered to contribute to HIV infection of the CNS; however, a recent study has contradicted this early work and suggests that myeloid cells are not an in vivo source of virus production. Here, we addressed the role of macrophages in HIV infection by first analyzing monocytes isolated from viremic patients and patients undergoing antiretroviral treatment. We were unable to find viral DNA or viral outgrowth in monocytes isolated from peripheral blood. To determine whether tissue macrophages are productively infected, we used 3 different but complementary humanized mouse models. Two of these models (bone marrow/liver/thymus [BLT] mice and T cell-only mice [ToM]) have been previously described, and the third model was generated by reconstituting immunodeficient mice with human CD34⁺ hematopoietic stem cells that were devoid of human T cells (myeloid-only mice [MoM]) to specifically evaluate HIV replication in this population. Using MoM, we demonstrated that macrophages can sustain HIV replication in the absence of T cells; HIV-infected macrophages are distributed in various tissues including the brain; replication-competent virus can be rescued ex vivo from infected macrophages; and infected macrophages can establish de novo infection. Together, these results demonstrate that macrophages represent a genuine target for HIV infection in vivo that can sustain and transmit infection.

Introduction

HIV, the causative agent of AIDS, is severely species restricted, and, to date, only humans and chimpanzees have been shown to be susceptible to infection (1, 2). The limited species specificity of HIV represents a significant challenge for in vivo experimentation, thus the use of animal models for infection has become increasingly important. Human infection by HIV (and infection by its relative SIV in nonhuman primates) is restricted to cells expressing the CD4 molecule. In addition to CD4, productive HIV infection, meaning infection that leads to the production of viral progeny, requires one of two different G protein-coupled receptors: CCR5 or CXCR4 (3). CD4⁺ T cells have been shown to harbor HIV proviruses and represent the most abundant target for HIV infection in vivo (4, 5). Despite the prevalence of virus in CD4⁺ T cells, it is clear that T cells are not the only targets of HIV infection. In fact, macrophages have been shown to express CD4, CCR5, and CXCR4 and to be susceptible to HIV and SIV infection in vitro and in vivo (6–8). Nonhuman primates and humanized mice have been extensively used to study HIV and SIV infection and pathogenesis in vivo. HIV or SIV infection of macrophages and microglia, the tissue-resident macrophages of the brain, are postulated to substantially contrib-

ute to the establishment and pathogenesis of HIV or SIV infection in the CNS (9–11). The CNS is a location that has been considered to be a sanctuary for the virus, where variants of HIV can replicate and expand independently of contributions from the periphery (12, 13). It has been suggested that the compartmentalization between the blood and CNS is associated with the ability of HIV variants in the CNS to infect cells, such as macrophages, with lower levels of CD4 (14). This is especially problematic in the brain, where resident macrophages, such as microglia and perivascular macrophages, could then be susceptible to infection (9).

Analysis of monocytes from peripheral blood consistently shows very low levels or an outright lack of infection in viremic or aviremic patients (15–17). Evidence of both in vitro virus outgrowth from human monocytes obtained from patients and ex vivo virus outgrowth from tissue macrophages (including the brain or CNS) is also limited. Whereas the ability of HIV to replicate in human macrophages in vitro has been extensively documented, evidence for HIV replication in human macrophages in vivo is limited and, in some instances, indirect (18–20). Analysis of the gut has yielded somewhat conflicting results, as human intestinal macrophages did not support HIV replication ex vivo and were found to be more monocyte-like in receptor expression patterns (20); yet, viral HIV DNA was isolated from CD13⁺ cells sorted from rectal biopsies obtained from antiretroviral therapy-suppressed (ART-suppressed) patients, suggesting a non-T cell origin (21). However, the presence of HIV- or SIV-infected macrophages in a variety of tissues has been clearly documented using IHC and ISH approaches (8, 22–24).

In vivo macrophage infection is currently a topic of intense debate. Specifically, data from Calantone et al. suggest that in SIV-infected nonhuman primates, myeloid cells are not a major

Authorship note: A. Wahl and C. Baker contributed equally to this work.

Note regarding evaluation of this manuscript: Manuscripts authored by scientists associated with Duke University, The University of North Carolina at Chapel Hill, Duke-NUS, and the Sanford-Burnham Medical Research Institute are handled not by members of the editorial board but rather by the science editors, who consult with selected external editors and reviewers.

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: August 28, 2015; **Accepted:** January 14, 2016.

Reference information: *J Clin Invest.* 2016;126(4):1353–1366. doi:10.1172/JCI84456.

Table 1. Characteristics of HIV-1–infected patients

Patient no.	ART treatment	Plasma viral load (copies/ml)	CD4 count	CD4/CD8 ratio	Sex	Age (yr)
01	No	4,300	208	0.15	F	41
02	No	109,910	118	0.30	M	57
03	No	30,730	264	0.86	M	24
04	No	10,816	589	0.90	M	29
05	Yes	<40	1278	0.70	F	51
06	Yes	<40	424	0.94	M	54
07	Yes	<40	282	0.74	M	43
08	Yes	<40	585	1.04	M	27

No, indicates not currently on any ART (no exclusion if previously treated); Yes, indicates currently suppressed on ART; M, male; F, Female; CD4/CD8 ratio, percentage of CD4⁺ T cells in peripheral blood divided by the percentage of CD8⁺ T cells in peripheral blood by flow cytometry.

source of virus (25). Rather, macrophages ingest T cells, which explains the presence of HIV nucleic acids and proteins in macrophage preparations. Further evidence in support of this postulate has also been recently presented by Baxter et al. (26). In this article, the authors document that human monocyte–derived macrophages (MDMs) selectively capture and engulf HIV-infected human T cells and that detection of viral DNA (vDNA) or viral proteins within phagocytes, including macrophages, may not necessarily represent their infection, but may indicate uptake of infected immune cells or their debris (26). However, these authors indicate that uptake of virus by phagocytosis could potentially lead to infection of macrophages. Together, these results strongly suggest that analysis of HIV replication in macrophages *in vivo* is greatly compromised by the extensive presence of T cells. In addition to being significantly more abundant, T cells are also more susceptible to HIV and SIV infection than are macrophages via cell-free or cell-associated virus (26, 27).

In order to establish the susceptibility of human myeloid cells to HIV and their ability to sustain HIV replication and productive infection *in vivo*, we first determined the incidence of HIV infection in peripheral blood monocytes from viremic and suppressed HIV-infected patients. Our analysis demonstrated that, in contrast to the relative abundance of HIV DNA found in T cells, we were unable to detect HIV DNA in peripheral blood monocytes. Analysis of primary tissue macrophages from humans remains difficult, as these samples are not easily accessible, being tissue derived, and viability can suffer as a consequence of handling. Invasive techniques such as bronchoscopy or biopsy are necessary to acquire the samples (28), and it is difficult to expand these cells *ex vivo*. While blood monocytes can be differentiated *in vitro* into macrophages, these cells lose much of their heterogeneity that results from anatomical location, which is critical in mimicking primary tissue macrophages (29, 30). Because of the difficulties associated with using primary tissue macrophages from humans, we used a humanized mouse model, in which the only human cells present capable of supporting HIV replication are human myeloid cells (31–33). NOD/SCID mice transplanted with human CD34⁺ (hCD34⁺) stem cells are reconstituted with human myeloid and B cells and are completely devoid of human T cells. Our analysis of internal organs demonstrated the presence of human macrophages in all tissues analyzed including the brain. Since the only

cells that can support replication in these mice are of myeloid origin, we have designated these as myeloid-only mice (MoM). HIV infection of MoM with select isolates resulted in robust and sustained replication. HIV DNA and/or RNA were found in virtually all tissues analyzed, including the brain. Replication-competent virus could be recovered from tissue macrophages, and the transfer of infected macrophages into uninfected animals resulted in sustained infection. Our results also demonstrate the ability of human macrophages to fully sustain HIV infection and replication *in vivo* in the complete absence of human T cells, confirming the role of macrophages as genuine targets for HIV infection *in vivo*.

Results

Absence of HIV in peripheral blood monocytes isolated from viremic patients or from patients undergoing ART. To determine the frequency of HIV infection in peripheral blood monocytes in humans, we collected samples from 8 patients (see Table 1 for patients' demographic information). Four of these patients were receiving ART and four were not. We then used a multistep protocol for the purification and enrichment of monocytes and T cells using magnetic beads (Figure 1A). Specifically, mononuclear cells (MNCs) were first isolated using Ficoll gradient centrifugation. Human T cells were removed from the MNC preparations using a CD3-specific reagent via positive magnetic selection. After T cell removal, blood monocytes were isolated using a negative selection approach that again removed any residual T cells. This procedure yielded highly purified preparations of monocytes for analysis (Figure 1B). Whole blood cells, purified monocytes, and enriched T cells were analyzed for the presence of HIV-*gag* DNA using nested PCR analysis. Clear evidence of HIV DNA was detected in purified T cell preparations in samples obtained from infected patients, regardless of their treatment status (Figure 1C). No evidence of vDNA was observed in any of the analyzed purified monocyte preparations from these same patients (limit of detection was 2 DNA copies).

To address the frequency at which replication-competent HIV isolates exist in the blood monocytes of HIV-infected patients, we evaluated virus outgrowth from peripheral blood-derived monocytes and T cells isolated from nonsuppressed HIV-infected patients injected *i.v.* into BLT mice. Using patients' purified peripheral blood cells (patients 01–03 in Table 1 and Figure

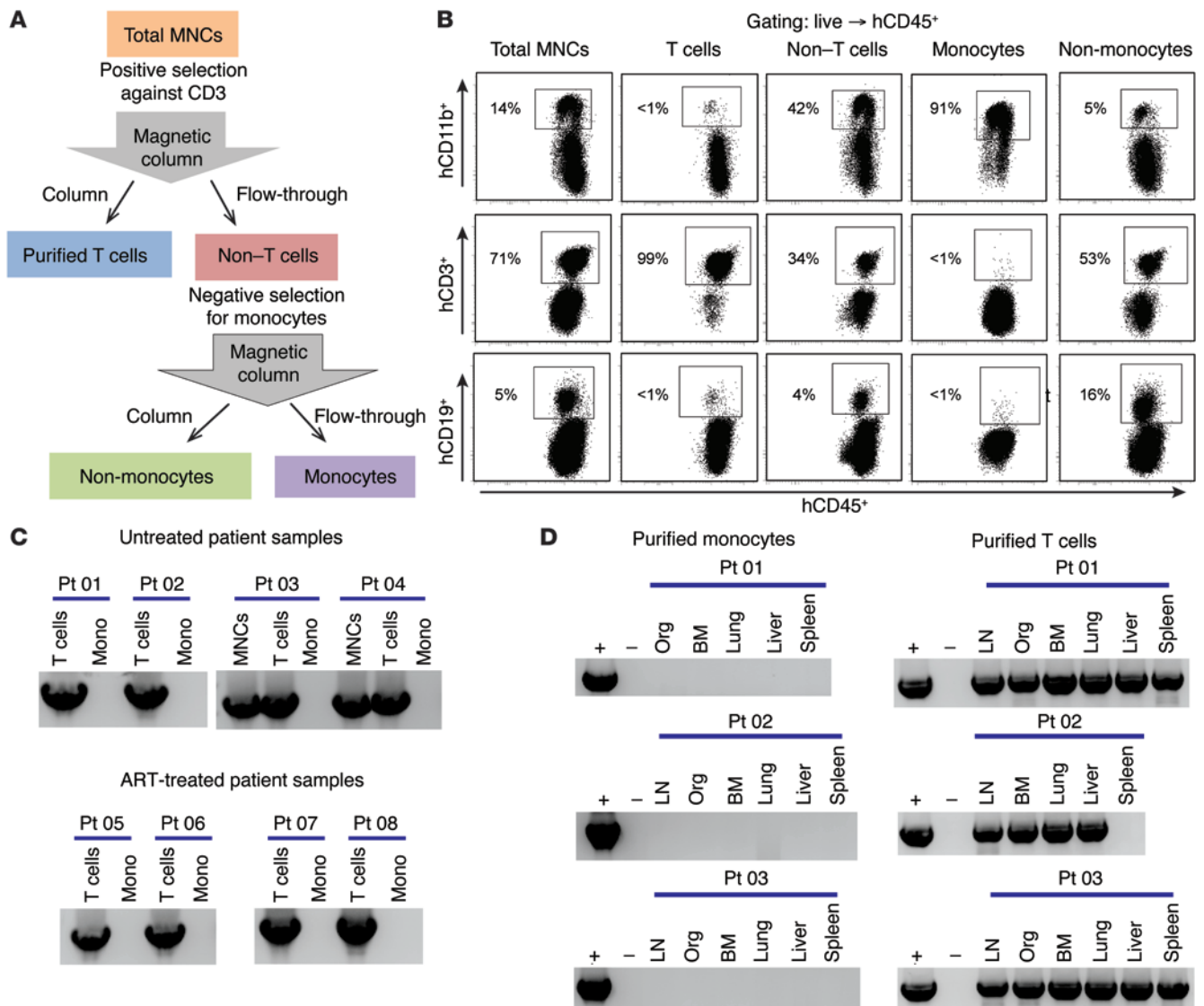


Figure 1. Absence of HIV DNA in monocytes but not in T cells isolated from peripheral blood of infected patients. (A) Schematic of monocyte and T cell purification strategy. Total MNCs were isolated from the peripheral blood of 8 HIV-infected patients (from Table 1). T cells were depleted using positive magnetic selection, and monocytes were enriched using negative magnetic selection. (B) Flow cytometric analysis of total MNCs, T cells, non-T cells, non-monocytes, and monocytes revealed very pure populations of both T cells and monocytes isolated using this protocol (data from patient 04 are shown). (C) One million purified T cells or monocytes (Mono) were analyzed for the presence or absence of HIV-gag DNA using nested PCR analysis of untreated and treated patients. The lower limit of detection for these analyses was 2 copies of HIV DNA. Purified T cells or monocytes from untreated patients 01, 02, and 03 were injected into BLT mice. (D) vDNA analysis demonstrated systemic infection of BLT mice injected with patients' purified T cells but not monocytes. LN, lymph node; Org, human thymic organoid; Pt, patient.

1C), purified monocytes and T cells were injected into BLT mice. After 2 to 8 weeks, mice were harvested and cells from the different tissues of BLT mice were analyzed for the presence of HIV DNA using nested PCR (Figure 1D). Consistent with the lack of HIV DNA monocytes (Figure 1C), we did not observe outgrowth in any of the BLT mice that received patients' purified monocytes.

In contrast, we found replication-competent virus in all BLT mice that received purified T cells. This in vivo outgrowth assay demonstrates the absence of replication-competent virus in monocytes obtained from the peripheral blood of HIV-infected patients. These results further highlight the importance of an animal model in which analysis of tissue macrophages can be readily accomplished.

Reconstitution of NOD/SCID mice with hCD34⁺ hematopoietic stem cells results in systemic reconstitution with myeloid cells and an absence of T cells. In order to establish whether tissue macrophages are infected in vivo, we used an in vivo mouse model designed for this purpose. Preconditioned NOD/SCID mice were administered a bone marrow transplant consisting of hCD34⁺ cells (31–33) and monitored for up to 7 months for human reconstitution as determined by the presence of human cells in peripheral blood (n = 52). Over time, human hematopoietic cells (CD45⁺) in the peripheral blood of these mice increased in numbers that remained stable after approximately 8 to 12 weeks and persisted throughout the duration of the experiments (Figure 2A). Lineage analysis of the human cells present in the peripheral blood of these mice clearly

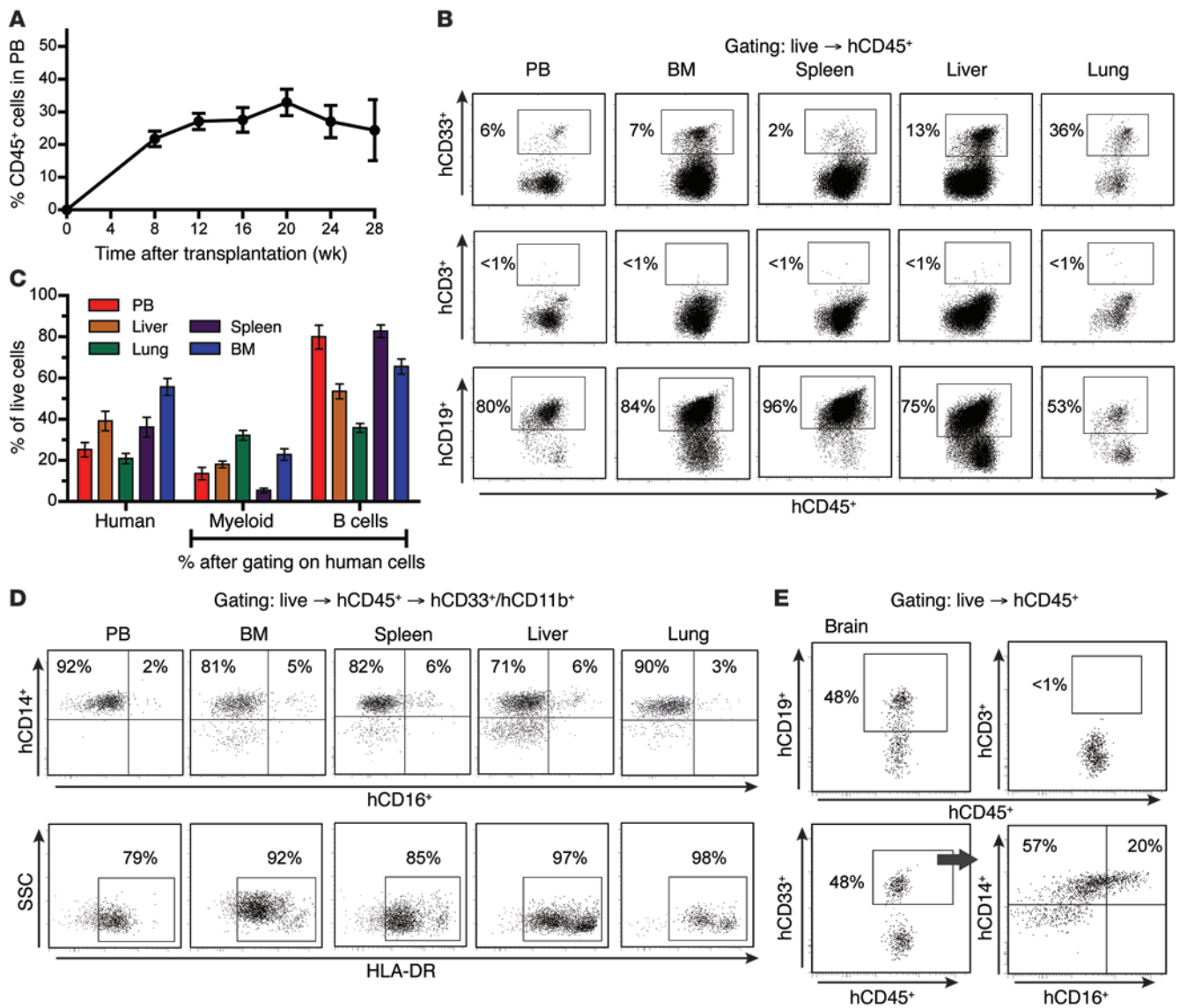


Figure 2. NOD/SCID mice transplanted with hCD34⁺ hematopoietic stem cells are reconstituted with human B cells and myeloid cells but lack T cells. (A) Flow cytometric analysis of the peripheral blood of mice showed a sustained presence of 20% to 30% hCD45⁺ cells over time (28 weeks shown, *n* = 52). (B) Flow cytometric analysis of the bone marrow, spleen, liver, lung, and peripheral blood of a representative mouse demonstrated the presence of human B cells (hCD19⁺) and human myeloid cells (hCD33⁺), with a complete absence of human T cells (hCD3⁺). (C) Average levels of human cell reconstitution in bone marrow (*n* = 36), spleen (*n* = 36), liver (*n* = 35), lung (*n* = 35), and peripheral blood (*n* = 18) and the distribution between myeloid and B cells of the human cells. (D) Phenotypic characterization of the human monocytes and macrophages in the tissues of a mouse reconstituted with hCD34⁺ cells (gating scheme: live → hCD45⁺ → hCD33⁺/hCD11b⁺). (E) Flow cytometric analysis of the brains of MoM demonstrated the presence of human B cells and macrophages, with a lack of human T cells. Percentages in **B**, **D**, and **E** signify the percentage of cells expressing the indicated surface markers for the cell populations designated in the gating strategy. PB, peripheral blood.

indicated the presence of human B and myeloid cells (Figure 2, B and C). No evidence of human T cells in the peripheral blood of these mice was noted at any time during the experiment.

Analysis of the cells present in tissues obtained from transplanted mice confirmed their systemic reconstitution with human hematopoietic cells (Figure 2, B and C). Lineage analysis of the cells present in bone marrow, liver, lung, and spleen demonstrated the presence of human B and myeloid cells. We consistently failed to find T cells in any of the tissues analyzed (Figure 2B). Analysis of hCD14 and hCD16 expression on the cells present in the peripheral blood and tissues of these mice demonstrated the presence

of classical (CD14⁺CD16⁻) and intermediate (CD14⁺CD16⁺) monocyte and macrophage subsets (34, 35), with the classical phenotype representing the majority of the monocytes and macrophages present in these mice (Figure 2D), as is also observed in humans (36). Additionally, the majority of these cells expressed MHC class II, as denoted by expression of HLA-DR (Figure 2D). These results demonstrate the systemic repopulation of these mice with human myeloid cells and B cells in the complete absence of human T cells.

Reconstitution with human cells observed in the brains of NOD/SCID mice transplanted with CD34⁺ cells. HIV infection of the brain and its sequela are hallmarks of AIDS. Macrophages have been

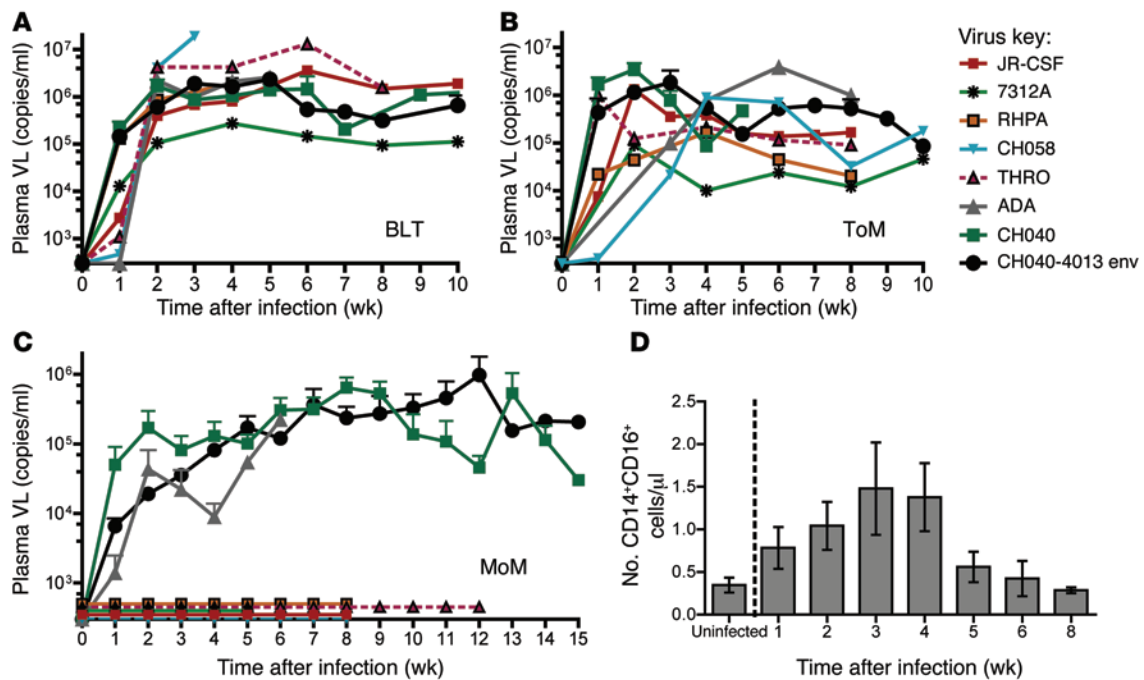


Figure 3. HIV replication can be sustained over time in human macrophages in vivo, but it is limited to very few HIV strains. (A) Efficient replication of all HIV strains tested in BLT mice as determined by viral load analysis. (B) Efficient replication of all HIV strains tested in ToM mice as determined by viral load analysis. (C) Plasma viral load of infected MoM. Only HIV-1 ADA, CH040, and CH040-4013 env (a patient envelope–derived chimeric virus) were detectable in the plasma over time. (D) Increased numbers of CD14⁺CD16⁺ cells (intermediated phenotype monocytes) were detected in the blood of infected MoM ($n = 17$) during the first few weeks of infection compared with the blood of uninfected MoM ($n = 11$). VL, viral load.

shown to be important targets of SIV and HIV infection in the brain (10, 11, 23). Therefore, we investigated the presence of human cells in the brains of reconstituted mice. To minimize potential contamination from cells in the blood in the brain, animals were transcardially perfused with PBS at necropsy. Brain tissue was used to isolate MNCs via Percoll density centrifugation, and isolated cells were analyzed via flow cytometry. Human B cells and macrophages were found in the brains of all mice, and there was no evidence of human T cells in any of the brains analyzed (Figure 2E). Similar to the rest of the tissues analyzed in the previous experiment, macrophages isolated from the brains of these mice had both classical and intermediate phenotypes as determined by CD14 and CD16 expression levels. These results demonstrate reconstitution of the brains of these NOD/SCID mice with human hematopoietic cells.

Analysis of infection by a diverse set of HIV isolates in humanized bone marrow/liver/thymus mice and T cell–only mice demonstrates the ability of these isolates to efficiently replicate in vivo. To establish the fitness of each HIV isolate prior to evaluation of replication in macrophages in vivo, isolates were first evaluated for their replication competency in bone marrow/liver/thymus (BLT) mice and T cell–only mice (ToM). BLT mice are reconstituted with T cells, B cells, and myeloid cells (37, 38), while ToM are devoid of human monocytes and B cells but have a full complement of human T cells (39). Both BLT mice and ToM have been shown to support HIV replication and persistent infection as determined by the presence of latently infected human T cells (39–41). BLT mice and ToM were infected with 8 different viruses: 1 primary HIV-2 isolate (7312A) (42, 43); 1 HIV-1 early–passage isolate (JRCSF) (44, 45); 4 HIV-1 transmitted/founder viruses (THRO, RHPA, CH058, and CH040)

(46); and 1 HIV-1 macrophage–tropic virus (ADA) (47). All viruses utilize CCR5 as a coreceptor for infection (46, 48, 49). In addition, we also evaluated a chimeric virus derived from CH040, in which we replaced its envelope gene with one obtained from the cerebrospinal fluid (CSF) of a patient with moderate CNS disease; this envelope was previously characterized as being macrophage–tropic (Subject #4013, Envelope #C7, denoted as CH040-4013 env) (12). BLT mice and ToM were infected via multiple routes, and viral replication was monitored weekly or biweekly in plasma via viral load analysis as previously described (39, 41, 50–53). All viruses tested, without exception, efficiently replicated in vivo in both BLT mice and ToM (Figure 3, A and B). Each of these viruses maintained detectable levels of viral replication in the plasma of all exposed animals. These results confirm the in vivo replication competence of all viruses used in this study.

Replication of HIV in NOD/SCID mice transplanted with CD34⁺ cells is strictly limited to macrophage–tropic viruses. To investigate HIV infection of myeloid cells in vivo, we used NOD/SCID mice reconstituted with CD34⁺ cells that therefore lacked human T cells. Mice were exposed to a single i.v. dose of the HIV isolates evaluated above in BLT mice and ToM. Virus replication was monitored weekly for up to 15 weeks as a function of plasma viral load as previously described (41, 50–53). We found no evidence of HIV-1 or HIV-2 replication in any of the mice infected with JRCSF, THRO, RHPA, CH058, or 7312A (Figure 3C). Evidence of HIV replication, as determined by the presence of viral RNA (vRNA) in plasma, was observed in CD34⁺ cell–reconstituted NOD/SCID mice exposed to ADA, CH040, and CH040-4013 env only (Figure 3C). All of the viruses that were capable of replicating in mice

Table 2. Viral replication of HIV in various humanized mouse models

	JRCSF	7312A (HIV-2)	THRO	RHPA	CH058	ADA	CH040	CH040-4013 env
BLT	+ (n = 6)	+ (n = 3)	+ (n = 5)	+ (n = 2)	+ (n = 1)	+ (n = 1)	+ (n = 7)	+ (n = 7)
ToM	+ (n = 9)	+ (n = 1)	+ (n = 2)	+ (n = 2)	+ (n = 1)	+ (n = 1)	+ (n = 4)	+ (n = 8)
MoM	– (n = 4)	– (n = 3)	– (n = 3)	– (n = 3)	– (n = 3)	+ (n = 5)	+ (n = 21)	+ (n = 17)

Summary of viruses that were evaluated for replication in MoM, ToM, and BLT mice with regard to plasma viremia and tissue vDNA analysis. For viruses in MoM marked with a minus sign, at least 1 animal was evaluated for the presence of vDNA in the spleen, liver, lung, and bone marrow and was determined to be uninfected.

devoid of T cells did so efficiently, resulting in sustained levels of virus replication in the plasma for the duration of the experiment (up to 15 weeks, the last time point evaluated) (Figure 3C). HIV infection of NOD/SCID mice transplanted with CD34⁺ stem cells led to transiently increased numbers of intermediate phenotype monocytes in the peripheral blood (Figure 3D), similar to what is seen in humans (54). Together, the results presented above demonstrate that all of the isolates tested can replicate efficiently in humanized mice containing human T cells. These results also demonstrate that myeloid cells can support *in vivo* replication of only macrophage-tropic viruses (3 of 8 tested, Table 2). However, the viruses that were capable of replicating in the absence of T cells were able to sustain robust levels of infection that were maintained for the entire experimental period. Sequencing of the V1–V5 region of the viral envelope in MoM infected for 8 to 18 weeks ($n = 3$ for CH040 and $n = 5$ for CH040-4013 env) did not reveal changes in the nucleotide sequence of the original infecting virus. These results, together with the fact that viral load in plasma was readily detectable shortly after exposure, suggest that these viruses are able to replicate efficiently in macrophages *in vivo* without the need of further adaptation. In NOD/SCID mice transplanted with hCD34⁺ cells, the only human cells that are susceptible to HIV infection and that support HIV replication are myeloid cells, hence our designation of these mice as MoM.

Systemic replication of macrophage-tropic viruses in MoM in the absence of T cells. Having established the replication competence of macrophage-tropic viruses in MoM in the complete absence of T cells in peripheral blood, we next determined the presence of infected cells in tissues. For this purpose, cells from the spleen, liver, lung, and bone marrow from infected MoM were analyzed for the presence of HIV DNA. Consistent with systemic infection, HIV DNA was readily found in all tissues examined (Figure 4A). To determine whether systemic viral replication was occurring, tissues from MoM were analyzed for the presence of HIV RNA (Figure 4B). HIV RNA was readily detected in all tissues analyzed. Electron microscopic analysis of the bone marrow from HIV-infected MoM showed the presence of viral budding as well as free virions in this tissue (Figure 4C). To confirm the identity of the infected cells in the liver, lung, and spleen, we used immunohistochemical staining combined with ISH analysis. The presence of HIV RNA in hCD68-expressing cells in these tissues confirmed that the virus detected in the tissues of HIV-infected MoM was indeed being produced by human macrophages (Figure 4D). Together, these results demonstrate systemic replication of macrophage-tropic viruses in MoM.

To determine the frequency at which replication-competent virus exists in the tissues of infected MoM, we performed an *in vitro* outgrowth assay. Cells obtained from different tissues of MoM (bone marrow, spleen, liver, and lung) were cocultured *ex vivo* with CD8-depleted, HIV-negative, PHA-activated allogeneic peripheral blood mononuclear cells (PBMCs). Culture supernatants were analyzed by PCR after 10 days for the presence of vRNA. Coculture of cells from infected MoM with activated allogeneic CD4⁺ cells resulted in viral outgrowth in the majority of wells from all tissues, with as few as 135 total human macrophages leading to infection of the cultures (Figure 4E). This demonstrates that infected macrophages are found in all tissues, and even a small number of macrophages can lead to productive infection of T cells in culture.

Analysis of HIV infection in macrophages in BLT mice. To determine whether tissue macrophages were also infected in BLT mice, we performed ISH analysis of liver, lung, and spleen from a BLT mouse infected with HIV-1 CH040-4013 env using RNAscope technology and staining with anti-hCD68. Dual-labeled hCD68⁺ and HIV RNA⁺ cells were present in the liver, lung, and spleen (Supplemental Figure 1A). To facilitate direct comparison of the levels of infection of macrophages in BLT mice and MoM, we used the cell-sorting method described above for the isolation of purified macrophages. Total MNCs were pooled from the spleen, liver, lung, and bone marrow of an individual BLT mouse or MoM infected with HIV-1 CH040. Mouse and human T cells were then depleted using positive magnetic selection. Human macrophages were further purified using negative magnetic selection essentially as indicated above. Total DNA was then extracted and analyzed for the presence of HIV DNA using quantitative real-time PCR. On average, we found $2,065 \pm 346$ HIV DNA copies per 100,000 human macrophages isolated from infected BLT mice, and, on average, we found 402 ± 162 HIV DNA copies per 100,000 human macrophages isolated from infected MoM (Supplemental Figure 1B, $P = 0.0121$). Experimental details for this section are found in the Supplemental Methods. These results confirm the infection of human macrophages in BLT mice. Consistent with the higher viral load detected in BLT mice, we also observed a higher infection level of macrophages in these mice.

HIV infection is established in the brains of MoM. As indicated above, it has been postulated that HIV infection of the CNS is established and maintained in human myeloid cells (9, 55). SIV infection of macaques leads to accumulation of monocytes and macrophages in the CNS (11); therefore, we wanted to determine the effect of HIV infection on the number of human cells

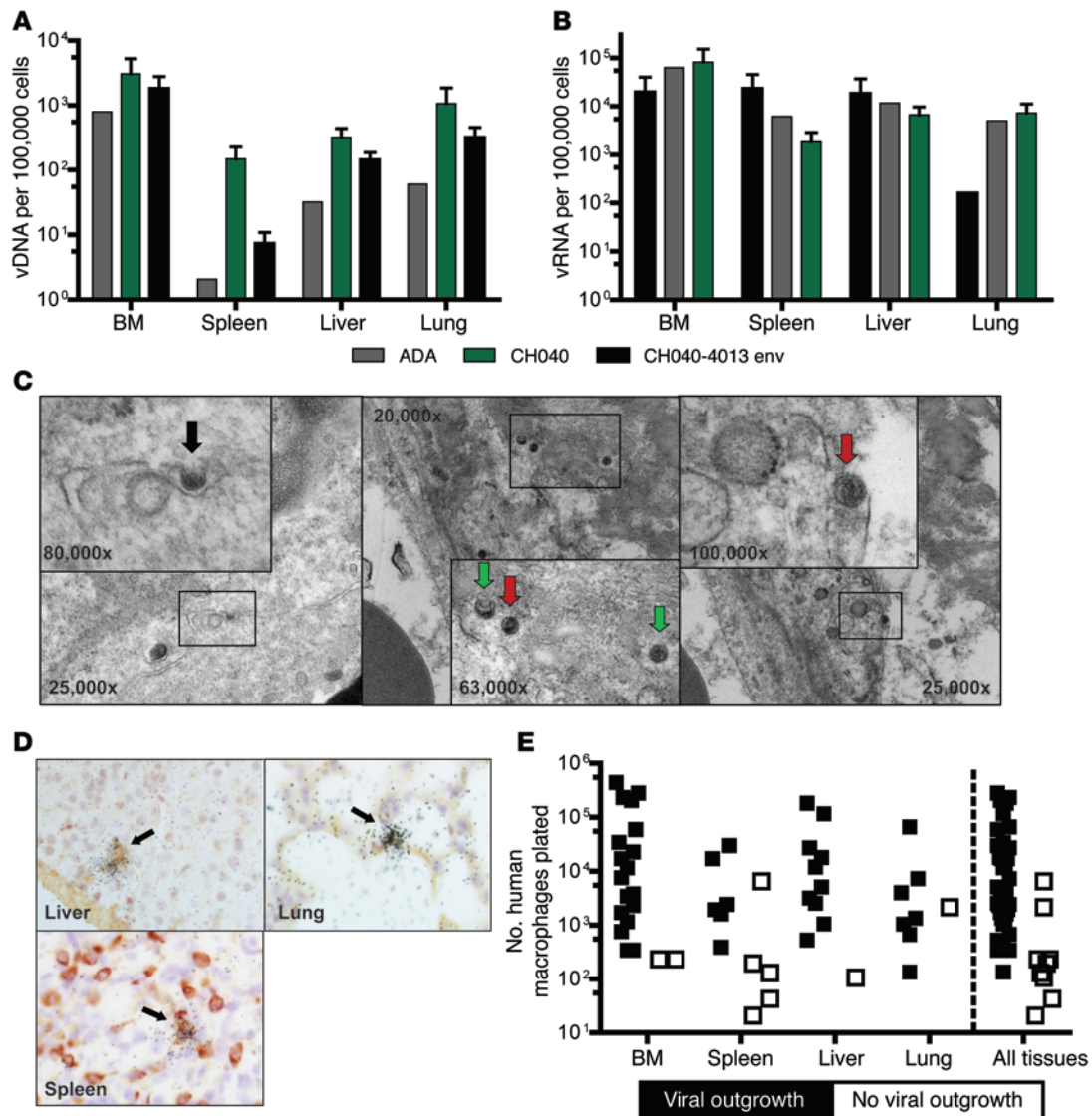


Figure 4. Systemic replication of HIV-1 in the tissues of MoM. (A) Cell-associated vDNA copies per 100,000 total cells and (B) cell-associated vRNA copies per 100,000 total cells were measured from cells isolated from bone marrow (BM), spleen, liver, and lung of MoM infected with ADA ($n = 2$), CH040 ($n = 10$), or CH040-4013 env ($n = 9$). (C) Electron micrograph of infected MoM bone marrow shows the presence of viral budding (black arrows) as well as free virions (green and red arrows) in the tissue. Virions in which the viral core was visible are denoted with red arrows. Original magnification, $\times 20,000$ to $\times 100,000$, as indicated in each image. (D) ISH analysis of the tissues of a MoM infected with CH040 demonstrated the presence of human macrophages (expressing CD68, brown staining) producing HIV RNA transcripts (black granules). Original magnification, $\times 100$. (E) Cells from infected MoM tissues (bone marrow [$n = 20$], spleen [$n = 11$], liver [$n = 11$], and lung [$n = 8$]) were cultured with allogenic donor CD8-depleted feeder cells. Viral outgrowth was determined by the presence (black squares) or absence (white squares) of vRNA in the culture supernatants after 10 days. The number of human macrophages plated in each culture is graphed on the y axis. We were able to measure outgrowth in 41 of 50 total samples (All tissues).

in this tissue. Compared with uninfected controls, the total number of human cells and human macrophages in the brains of infected animals increased significantly from 1,947 to 4,805 ($P = 0.0009$) for total human cells and from 635 to 1,399 for total human macrophages ($P = 0.0055$) (Figure 5A). We also were able to demonstrate by IHC the presence of human macrophages (CD68⁺) throughout the brain, including the cerebellum, caudate putamen, cerebral cortex, ventral striatum, and brain stem, of HIV-infected MoM (Figure 5B).

Having established that human macrophages are present in the brains of infected MoM, we investigated whether HIV infection is established in the brains of these animals. Mice were transcar-

dially perfused with PBS at necropsy to minimize contamination with blood-derived cells, such as monocytes, as well as purge any free virus that might be circulating in the brain. We isolated MNCs from the brains of MoM infected with the 3 macrophage-tropic viruses (ADA, CH040, and CH040-4013 env) and used HIV RNA as a surrogate for HIV replication. vRNA was detected, in cells isolated from 8 of 14 infected animals, representing all 3 macrophage-tropic isolates (Figure 5C). Immunohistochemical analysis demonstrated the presence of HIV-infected cells (p24⁺) in the brains of infected MoM (Figure 5D). HIV p24⁺ cells were located in the cerebellum, cerebral cortex, corpus callosum, midbrain, brain-stem, and caudate putamen of infected MoM.

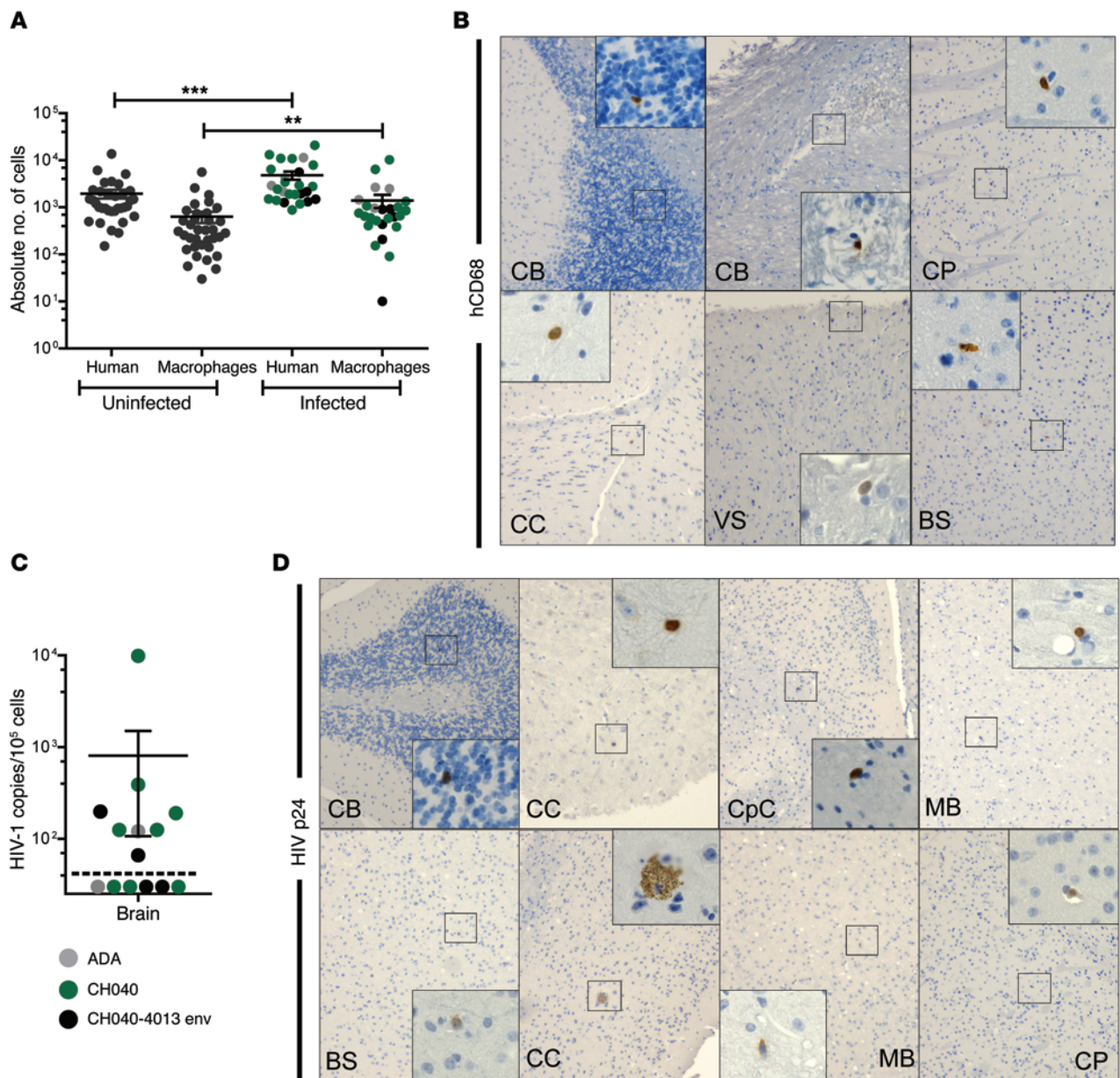


Figure 5. Human cells are present in the brains of MoM and increase in numbers during HIV infection. (A) MNCs from the brains of infected ($n = 25$) and uninfected ($n = 35$) MoM were analyzed via flow cytometry, and the absolute numbers of human cells (hCD45⁺) and human macrophages (hCD33⁺/hCD11b⁺) were calculated per whole brain. There was a significant increase in the overall number of human cells ($P = 0.0009$) and human monocytes and macrophages ($P = 0.0055$) in the brains of infected mice compared with numbers detected in uninfected mice. P values were determined by Mann-Whitney U test. (B) Immunohistochemical analysis of the brain was used to confirm the presence of human macrophages (hCD68) in the brains of infected MoM. (C) Cell-associated vRNA copies per 100,000 total cells were measured in the brains of infected MoM. We were able to detect virus in the brains of 8 of 14 animals (lower limit of detection, 30 copies/ 10^5 cells). (D) Immunohistochemical analysis demonstrated the presence of HIV p24 in the brains of infected MoM. (B and D) Original magnification, $\times 10$ and $\times 40$ (insets). The location of CD68⁺ or p24⁺ cells is indicated by an abbreviation in each panel. BS, brain stem; CB, cerebellum; CC, cerebral cortex; CP, caudate putamen; CpC, corpus callosum; MB, midbrain; and VS, ventral striatum.

Adoptive transfer of HIV-infected macrophages establishes de novo infection. To determine whether the infected myeloid cells present in MoM could establish de novo infection in an uninfected host, we isolated cells from infected MoM and injected them i.v. into uninfected animals. First, bone marrow cells from 3 MoM (infected with CH040 or CH040-4013 env) were injected into BLT mice. This resulted in the development of rapid plasma viremia in all recipient animals (a total of 4 BLT mice), confirming the replication competence of the viruses present in the MoM (Table 3). To

determine whether macrophages in BLT mice are productively infected, tissue macrophages were isolated from the spleen, liver, lung, and bone marrow of a BLT mouse (infected with CH040). T cells were first removed by positive magnetic selection against CD3, and myeloid cells were further enriched by negative magnetic bead selection, using the methodology outlined above for patients' peripheral blood cells (Figure 1A). Purified myeloid cells were then injected i.v. into a MoM. This resulted in sustained virus replication in the recipient animal, confirming that macrophages

Table 3. Human macrophages can establish de novo infection of humanized mice

Donor mouse/mice	Virus	Cell type used	No. of cells injected	No. of HIV DNA ⁺ cells injected	Recipient mouse	Evaluation of infection	
						Plasma VL	Tissue vDNA
MoM #1	CH040	Bone marrow cells	4.00E+06	15,880	BLT #1	+	+
MoM #2	CH040-4013 env	Bone marrow cells	4.00E+06	11,520	BLT #2	+	+
MoM #3	CH040	Bone marrow cells	2.00E+06	1,820	BLT #3	+	ND
					BLT #4	+	ND
BLT #5	CH040	Purified macrophages	3.80E+05	148	MoM #4	+	+
BLT #6, 7 and 8	CH040	Purified macrophages	4.00E+06	4,760	BLT #9	+	+
					MoM #5	+	+

MNCs were isolated from the bone marrow of infected MoM and injected into humanized BLT mice, resulting in systemic infection of the BLT mice. Total MNCs were harvested from the liver, lung, spleen, and bone marrow of BLT mice infected with CH040. Two sequential steps purified tissue macrophages: (1) positive magnetic selection was used to deplete T cells; and (2) negative magnetic selection was used to further purify the pool of human macrophages. Macrophages from infected BLT mice were analyzed for vDNA levels and were then injected into MoM. MoM and BLT mice were evaluated at necropsy for HIV infection over time in the blood and in tissues. Results indicated by a plus sign are samples that tested positive for HIV-1 RNA (plasma viral load) or DNA (for tissue cells). ND, not determined.

present in this infected BLT mouse were productively infected. The presence of productively infected macrophages in HIV-infected BLT mice was confirmed by injecting pooled macrophages purified from 3 BLT mice (infected with CH040) into a MoM and a BLT mouse. The purified macrophages were able to establish de novo infection in both of the recipient animals, confirming our initial observation. These results (Table 3) demonstrate that the virus replicating in macrophages of BLT mice and MoM can efficiently transmit de novo infection to a new host and replicate *in vivo* in the presence or complete absence of human T cells.

Discussion

The study of HIV and SIV replication in macrophages has been confounded, as the presence of viral nucleic acids in these cells has been attributed to phagocytosis of infected T cells and cellular debris (25, 26). Despite the postulated importance of macrophages in HIV infection and AIDS-associated neurological complications, infection of monocytes and macrophages *in vivo* has been questioned (15–17, 25, 26, 56, 57). Our results using monocytes isolated from viremic and aviremic patients are consistent with those showing a lack of infection of these cells in humans (15–17). These outcomes have been ascribed to differences in the susceptibility of monocytes and macrophages to HIV infection during differentiation (56, 58) and highlight the need to study infection of tissue macrophages rather than monocytes.

To investigate *in vivo* HIV infection of macrophages, we took advantage of 2 humanized mouse models that we had previously developed (ToM and BLT mice) and implemented a new model in which only human myeloid cells can serve as targets for HIV infection (MoM). Only macrophage-tropic viruses (ADA, CH040, and CH040-4013 env) were able to sustain infection in the absence of human T cells. Since all isolates tested utilize CCR5 as a coreceptor (46, 48, 49), the ability of these viruses to productively infect MoM is likely related to an ability to infect cells expressing low levels of CD4 on their cell surface (12, 14). It should be noted that the HIV-infected MoM in this study represent animals reconstituted with cells from 24 different human donors; this contrasts with the findings of an *in vitro* study of MDMs, in which donor variation in

susceptibility to infection was identified as a confounding factor (7). We expected ADA, a macrophage-tropic virus, to replicate in MoM. However, we did not expect that CH040, being a transmitted/founder virus, would replicate well in MoM (59–61) and were surprised to see sustained and systemic replication of this virus in MoM. In addition, a chimeric virus in which the envelope gene of CH040 was replaced by one obtained from the CNS of a patient (12) also replicated well in MoM.

Previous experiments to better understand HIV infection of macrophages *in vivo* have been conducted using SIV infection (62). Using rhesus macaques, it has been shown that CD4 depletion (before infection) leads to robust SIV infection of macrophages (63, 64). Additionally, infection of macrophages results in a constant level of viral production and no post-peak drop in viremia (63). In CD8-depleted (after infection) rhesus macaques, increased numbers of monocytes and macrophages entered into the CNS of SIV-infected animals compared with that seen in uninfected animals (11). Similar to the above-cited studies, we noted no post-peak drop in viremia and found higher numbers of human macrophages in the brains of infected MoM compared with numbers in the brains of uninfected MoM. These results can be attributed to HIV infection of macrophages specifically, as MoM are devoid of T cells. HIV replication in MoM could not be ascribed to the phagocytosis of T cells by macrophages, and replication occurred only in the human myeloid cells present.

Despite the lack of infected monocytes in peripheral blood, we found strong evidence of productive macrophage infection in tissues. Replication in macrophages was verified by sustained plasma viremia, immunohistochemical and ISH analyses, electron microscopy, bulk DNA and RNA detection, and *in vitro* and *in vivo* outgrowth assays. Our data demonstrating that macrophages isolated from BLT mice infected with the macrophage-tropic strains are capable of establishing HIV infection when injected into naive recipients indicate that macrophages can be productively infected with HIV in the absence or presence of human T cells. Given the difficulties of procuring human tissue samples that invariably will also contain T cells, the availability of an *in vivo* model such as MoM represents a significant advance in the field. Furthermore, the complete

lack of human T cells in our MoM model dispels the notion that the presence of HIV nucleic acids in macrophages is due to phagocytosis of infected T cells; rather, it confirms that macrophages can sustain infection *in vivo*, which is dependent on specific HIV isolates that can replicate in macrophages like those used in our study.

Monocyte and macrophage transmigration into the brain has been considered to play a central role in establishing HIV infection of the CNS (65). ART has resulted in significant overall benefits to HIV-infected patients, but does not always result in neuropathological improvements (66–68), and this may stem from differences in the tropism of viruses found in the periphery compared with that seen in the CNS (9, 55, 69, 70). Here, we demonstrate that T cells are not necessary to traffic the virus from the periphery to the brain and that macrophages can sustain infection of the CNS. However, several issues remain to be addressed in future work. Given our analysis, we cannot ascertain whether the appearance of HIV⁺ cells in the CNS represents *de novo* infection or the migration of infected cells from other tissues into the brain. If it represents *de novo* infection, it will be important to determine whether the appearance of these HIV⁺ cells is initiated via cell-free or cell-associated virus. The number of infected myeloid cells in the periphery of MoM was very low compared with the relatively high viral load detected in these mice, suggesting that cell-free virus was seeding the infection in the brain of MoM. Our results demonstrating the lack of HIV infection of blood monocytes from humans suggest that infected monocytes might not be directly responsible for the seeding of HIV in the CNS of humans, but rather that cell-free virus or infected T cells in peripheral blood are responsible for introducing HIV into the CNS.

We believe our studies have significant implications for the field. They establish tissue macrophages as true targets of productive HIV infection. Our results imply that tissue macrophages might serve as a reservoir for latent HIV infection in patients, as these cells are capable of sustaining infection over long periods of time. The fact that no infected monocytes were present (or were present at very low levels) in the peripheral blood of patients suggests that future analysis of HIV latency in myeloid cells in humans will be complicated by the need to directly sample tissues in sufficient amounts to perform persistence, latency, and induction analyses. MoM could be used to directly assess the role of tissue-derived macrophages in the persistence of HIV infection *in vivo* during ART, and this will represent an important future direction for this model. Specifically, MoM could be used to address the effectiveness of ART to control viral replication in macrophages. Overall, this model is a unique tool to better understand HIV replication in macrophages *in vivo*.

Methods

Patient blood cell isolations. Approximately 8 ml blood was obtained from deidentified viremic and ART-suppressed patients and separated into 8 sodium citrate CPT tubes (64 ml total; catalog 362761; BD). Samples were spun down, and plasma was isolated from each tube for viral load analysis. MNCs were then collected and washed in PBS. Positive magnetic selection for CD3⁺ T cells (CD3 MicroBeads, catalog 130-050-101; Miltenyi Biotec) was performed. Next, negative magnetic selection for monocytes (Pan Monocyte Isolation Kit, catalog 130-096-537; Miltenyi Biotec) was performed on the non-CD3 cell population to yield a monocyte population that was more than 90% pure.

Generation of humanized mice. Humanized ToM and BLT mice were prepared by implanting allogeneic thymus and liver tissue into 6- to 8-week-old NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (NSG; The Jackson Laboratory) as previously described (39, 41, 51–53). Implants consisted of a 1- to 2-mm piece of fetal liver tissue sandwiched between 2 pieces of fetal thymus. Each implant was seated under the left kidney capsule. In addition to the thymus/liver/thymus implant, BLT mice also received autologous CD34⁺ hematopoietic stem cells (CD34 Microbead Kit, catalog 130-046-703; Miltenyi Biotec). Humanized MoM were prepared by transplanting approximately 1×10^6 human fetal liver or cord blood-derived CD34⁺ hematopoietic stem cells into NOD.CB17-Prkdc^{scid}/J mice (NOD/SCID; The Jackson Laboratory). Prior to humanization, all mice were preconditioned using gamma radiation with 2 Gy (ToM and BLT mice) or 2.5 Gy (MoM).

Flow cytometric analysis of patients' cells and humanized mice. All flow Abs were purchased from BD Pharmingen. The Ab panel used to analyze cells isolated from HIV-infected patients included Abs directed against hCD45 (APC, catalog 555485); hCD3 (FITC, catalog 555339); hCD4 (APC-H7, catalog 560158); hCD8 (PerCP, catalog 347314); hCD19 (PE-Cy7, catalog 557835); and hCD11b (PE, catalog 555388). In all samples, less than 0.1% of T cells were present in the purified monocyte population. To monitor humanization levels in humanized mice, peripheral blood was obtained from animals via submandibular venipuncture and collected into tubes containing EDTA. Whole peripheral blood was stained with the Ab panel listed above, with the exception that an Ab directed against hCD33 (PE, catalog 340679) was substituted for the anti-hCD11b Ab. For tissue and blood analysis from harvested mice, combinations of the above Abs were used. Additionally, Abs directed against hCD14 (FITC, catalog 555397); hCD16 (PE-Cy7, catalog 557744); hCD11b (APC, catalog 340937); hCD45 (APC-Cy7, catalog 557833); and HLA-DR (PerCP, catalog 347364) were used for the animals depicted in Figure 2, D and E. Live cells were distinguished by their forward- and side-scatter profiles as previously described (50). Flow cytometric data were collected on a BD FACSCanto and analyzed with BD FACSDiva software, version 6.1.3.

Tissue harvesting of humanized mice. To prevent blood contamination of tissues (in particular, brain tissues), mice were transcardially perfused with approximately 20 ml room-temperature PBS at necropsy. MNCs were isolated from the bone marrow, spleen, lung, and liver of MoM or BLT mice as previously described for ToM and BLT mice (39, 71, 72). Tissues were minced and/or digested and filtered through a 70- μ m strainer. The liver, lung, and brain MNCs were purified with Percoll density centrifugation. MNCs were washed and counted via trypan blue exclusion, and flow cytometric analyses were performed for the indicated markers.

HIV-1 infections. Stocks of HIV-1 (JRCSF, RHPA, THRO, ADA, CH058, CH040, and CH040-4013 env) and HIV-2 (7312A) were prepared and titered as previously described (73). Briefly, viral supernatants were prepared via transient transfection of 293T cells and were titered using TZM-bl cells (an indicator cell line) as previously described (74). The chimeric virus CH040-4013 env was created by replacing env in CH040 (GenBank database accession no. jn944905, 6396-8863) with the corresponding restriction fragment from subject 4013 (GenBank database accession no. jn562796). Subject 4013 had mild neurological dysfunction (12). To test various HIV strains for macrophage tropism, 360,000 tissue culture infectious units (TCIU) of virus was injected *i.v.* into MoM. BLT mice and ToM were exposed *i.v.* to

360,000 TCIU ADA, CH040, or CH040-4013 env. For the non-macrophage-tropic isolates (RHPA, THRO, JRCSF, HIV-2, and CH058), ToM or BLT mice were exposed i.v. or vaginally to 90,000 to 360,000 TCIU of the various viruses to confirm replication competence.

Analysis of HIV-1 infection. Subsequent HIV DNA analysis of patients' cells and of cells isolated from BLT mice that received patients' cells was performed using nested PCR analysis for HIV-gag. For all patient-derived blood cells, 1×10^6 cells were analyzed. For humanized mouse samples, 1×10^5 to 3×10^6 cells were analyzed, depending on the cell yield from the various tissues. Genomic DNA from MNCs (5×10^5 to 5×10^6 MNCs) from animal tissues was prepared using QIAamp DNA Blood Mini columns (QIAGEN) according to the manufacturer's protocol. Viral DNA was amplified by nested PCR using the Expand High Fidelity PCR System (Roche). The HIV region amplified was a 1.5-kb region in gag (gag: HIV-1JRCSF 617-2358). Amplification of gag was used to assess the presence or absence of HIV-1 gag DNA. Primer sequences were as follows: gag outer forward primer, CTCAATAAAGCTTGCCTTGAGTGC; gag outer reverse primer, CTTCCAATTATGTTGACAGGTGTAGG; gag inner forward primer, GTGTGGAAAATCTCTAGCAGTGCC; and gag inner reverse primer, TAGAAGAGAAGGCTTTCAGCC. Peripheral blood (approximately 100 μ l total) was collected from mice via retro-orbital bleeding using EDTA-coated capillary tubes. Infection of MoM with HIV-1 isolates was determined with a 1-step reverse transcriptase real-time PCR assay (TaqMan Assays-by-Design, custom-designed by Applied Biosystems) according to the manufacturer's instructions (with primers 5'-CATGTTTTCAGCATTATCAGAAGGA-3' and 5'-TGCTTGATGTCCCCCACT-3'; assay sensitivity of ~668 RNA copies/ml). Additionally, at necropsy, MNCs were isolated from tissues and analyzed for the presence of vRNA and vDNA. For viral coculture analysis, human macrophages were plated and allowed to adhere. Within 24 hours of plating, approximately 1 million allogenic, PHA-stimulated feeder cells (CD8-depleted) from healthy donors were added to the cultures as targets for viral outgrowth. After 10 days, culture supernatants were analyzed for the presence of vRNA.

Sequencing of HIV-1 in MoM plasma. For sequence analysis of HIV-1 envelope, vRNA was isolated from plasma obtained from infected MoM using the QIAamp Viral RNA Mini Kit (QIAGEN). Viral cDNA was generated using Superscript III Reverse Transcriptase (Invitrogen) and amplified by nested PCR using the Expand High Fidelity PCR System. The amplified region was a 1.2-kb region including the V1-V5 regions of the viral envelope (CH040 6426-7702; CH040-4013 6426-7702). The primers used for CH040 env amplification included env outer forward primer, CACCACTCTATTTGTGCATCAGATG; env outer reverse primer, GCCCATAGTGCTTCCTGCTGC; env inner forward primer, CACACATGCCTGTGTACCCACAGAC; and env inner reverse primer, CTCTTCTCTTGCCTTGGTGGGTGC. The primers used for CH040-4013 env amplification included env outer forward primer, CACCACTCTATTTGTGCATCAGATG; env outer reverse primer, GCCCATAGTGCTTCCTGCTGC; env inner forward primer, CACACATGCCTGTGTACCCACAGAC; and env inner reverse primer, CTCTTCTCTTGCCTTGGTGGGTGC. Amplified DNA was sequenced and aligned to HIV-1 CH040 or CH040-4013 env to determine whether nucleotide changes had occurred. FinchTV (Geospiza) was used for the sequence analysis of the chromatograms. We used ClustalW (<http://www.genome.jp/tools/clustalw/>) for alignment of the MoM envelopes to the reference sequences.

Electron microscopic analysis of MoM bone marrow. Femurs from CH040-infected MoM were harvested and immersed in fixative containing 4% paraformaldehyde, 2.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer [pH 7.4] with 5 mM calcium chloride), and 1 mM magnesium chloride for several days at 4°C. Femurs were decalcified with a solution of 0.1 M EDTA in 0.1 M sodium cacodylate (pH 7.4) containing 1% glutaraldehyde for 2 weeks at 4°C. After buffer washes, the tissues were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate (pH 7.4) for 1 hour at room temperature and gradually dehydrated with ethanol and propylene oxide, followed by infiltration and embedment in PolyBed 812 epoxy resin (Polysciences Inc.). Ultrathin sections (70-nm) were post-stained with uranyl acetate and lead citrate and observed using a LEO EM910 transmission electron microscope operating at 80 kV (Carl Zeiss SMT). Digital images were taken using an Orius SC1000 CCD camera and Digital Micrograph software, version 2.3.1 (Gatan Inc.).

Immunohistochemical analysis of MoM brains. Brains for immunohistochemical analysis were harvested from MoM and fixed in 4% paraformaldehyde for 24 hours at 4°C, embedded in paraffin, cut into 5- μ m sections, and mounted onto poly-L-lysine-coated glass slides. Following paraffin removal, antigen retrieval (DIVA Decloaker, catalog DV2004; Biocare Medical), and blocking of nonspecific Ig-binding sites (Background Sniper; Biocare Medical), tissue sections were stained with primary Abs overnight at 4°C and developed with a biotin-free HRP polymer system (MACH3 Mouse HRP-Polymer Detection; Biocare Medical). All tissue sections were then counterstained with hematoxylin. Primary Abs directed against CD45 LCA (clone 2B11 + PD7/26; Dako) and CD68 (clone KP1; Dako) were used to determine the presence of human cells and human macrophages in the brains of MoM. HIV-infected cells were detected with an Ab directed against HIV p24 gag (clone Kal-1; Dako). As a control, tissue sections were stained with a mouse IgG1k isotype control (DAK-GO1; Dako). Light microscopic images were taken with a Nikon H550S microscope at $\times 10$ magnification ($\times 40$ magnification for inset images).

ISH analysis. Double-label ISH was performed as previously reported (27), with minor modifications. In brief, formalin-fixed, paraffin-embedded tissue sections were deparaffinized in xylene, treated with hydrogen peroxide in ethanol, pretreated with digitonin and proteinase K, acetylated with acetic anhydride, and dehydrated. After hybridization with HIV 35S riboprobe mixture containing aurin tricarboxylic acid, and after hybridization washes, antigens were retrieved in 10 mM citrate (pH 6), and 0.1 M KCl at 65°C for 4 hours. After blocking, incubation with primary CD68 Ab overnight at 4°C, and incubation with secondary Ab coupled with peroxidase, CD68⁺ cells were stained with DAB. Nuclei were stained with hematoxylin, and the sections were dehydrated and cleared with xylene and cover-slipped with Permount. Light microscopic images were taken with an Olympus BX60 microscope at $\times 20$ magnification.

Adoptive transfer experiments. Infected MoM or BLT mice were harvested and processed as described above. For the first 2 adoptive transfer experiments, bone marrow cells from 2 separate infected MoM were injected i.v. into 2 separate BLT mice. In the third transfer experiment, macrophages were purified from an infected BLT mouse using the magnetic isolation strategy described above for patients' cells and injected i.v. into a MoM. For the last 2 adoptive transfer experiments, macrophages were purified from the pooled cells of 3 infected BLT mice and injected i.v. into a MoM and a BLT mouse. For

the purified macrophage adoptive transfers, MNCs were pooled from the spleen, liver, lung, and bone marrow of infected animals prior to macrophage isolation. Mice that received cells from infected animals were monitored for HIV-1 infection over time as described above.

Statistics. All data were graphed and analyzed using GraphPad Prism, version 5.04 (GraphPad Software). In Figure 2, A and C, Figure 3, A–D, Figure 4, A and B, and Figure 5, A and C, data are represented as the mean \pm SEM. Comparisons of the absolute numbers of cells in the brains of infected and uninfected MoM were performed using a 2-tailed Mann-Whitney *U* test. A *P* value of 0.05 or less was considered statistically significant.

Study approval. HIV-infected individuals were recruited at the UNC Hospital in the Infectious Diseases Department. Samples were collected under UNC IRB number 08-0047, and patients' samples were deidentified prior to cell isolation in the laboratory (UNC IRB number 14-0368). The UNC Office of Human Research Ethics determined that this study did not constitute human subjects research under federal regulations [45 CFR 46.102 (d or f) and 21 CFR 56.102(c) (e)(1)] and required no additional approval. All humanized mice were maintained in a specific pathogen-free facility according to protocols approved by the UNC IACUC.

Author contributions

JBH designed the experiments, processed patient's blood samples, collected and processed blood and tissues samples from mice, prepared viral stocks, performed viral inoculations, performed flow cytometric analyses, and wrote the manuscript. AW performed immunohistochemical analyses of MoM brains. CB performed all patient-derived nested PCR analyses, cell-associated RNA and DNA analyses, and processed blood and tissues from mice. RAS performed cell-associated RNA and DNA analyses and plasma viral load analyses. JF created the CH040-4013 env proviral clone. OZ oversaw IRB protocol submission and approval and also recruited patients for the study. SW and ATH performed ISH analyses. CCV participated in the study's design and patient cell isolations. VM performed electron microscopic analyses of mouse tissues. GS performed viral sequencing analysis. JJE participated in the study's design, IRB protocol submission, and assisted with patient recruitment. JVG conceived the study, designed and coordinated the study, and wrote the manuscript.

Acknowledgments

This work was supported in part by grants from the National Institute of Allergy and Infectious Diseases (NIAID) (AI-096113 and AI-111899) and the National Institute of Mental Health (NIMH) (MH-108179). J.B. Honeycutt was also supported in part by Virology Training Grant T32 AI-007419. This study was also supported by the University of North Carolina at Chapel Hill Center for AIDS Research (UNC CFAR) (P30-AI50410). The funders had no part in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We thank Nancie Archin for providing the CD8-depleted feeder cells for the coculture analysis and Laura Buckman for analysis of the brain images. We thank I. Chen for providing pYK-JRCSF (catalog 2708); F. Gao and B. Hahn for providing HIV-2_{7312A} (catalog 3511); and J. Kappes and C. Ochsenbauer for providing CH040 and CH058 (catalogs 11740, 1174, 1175, and 11856) via the AIDS Research and Reference Reagent Program. We thank Ron Swanstrom for providing the 4013 env for use in our studies. We thank Rick Meeker and Ronald Falk for their comments and suggestions for this manuscript and David Carroll of the North Carolina Translational and Clinical Sciences Institute (NC TraCS) for his editorial assistance. We thank the members of the UNC Microscopy Services Laboratory (MSL) for their assistance in obtaining the electron microscopic images. We also thank former and current members of the Garcia laboratory, the husbandry technicians at the UNC Division of Laboratory Animal Medicine, and the members of the Immunology Core of the UNC CFAR and the Lineberger Comprehensive Cancer Center Animal Histopathology Core Lab for their support of this work. Last, we thank all of the patients who provided blood samples for our analysis.

Address correspondence to: J. Victor Garcia, Division of Infectious Diseases, Center for AIDS Research, University of North Carolina at Chapel Hill, School of Medicine, CB# 7042, Genetic Medicine Building, 120 Mason Farm Rd., Chapel Hill, North Carolina 27599-7042, USA. Phone: 919.843.9600; E-mail: victor_garcia@med.unc.edu. Or to: Joseph J. Eron, Division of Infectious Diseases, Center for AIDS Research, University of North Carolina at Chapel Hill, School of Medicine, CB #7030, Bioinformatics Building, 130 Mason Farm Rd., Chapel Hill, North Carolina 27599-7030, USA. Phone: 919.966.2536; E-mail: joseph_eron@med.unc.edu.

- Sawyer SL, Wu LI, Emerman M, Malik HS. Positive selection of primate TRIM5a identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A*. 2005;102(8):2832–2837.
- Sharp PM, Hahn BH. Origins of HIV and the AIDS pandemic. *Cold Spring Harb Perspect Med*. 2011;1(1):a006841.
- Gorry PR, Ancuta P. Coreceptors and HIV-1 pathogenesis. *Curr HIV/AIDS Rep*. 2011;8(1):45–53.
- Ho DD, Neumann AU, Perelson AS, Chen W, Leonard JM, Markowitz M. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. *Nature*. 1995;373(6510):123–126.
- Chun TW, Engel D, Berrey MM, Shea T, Corey L, Fauci AS. Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A*. 1998;95(15):8869–8873.
- Gorry PR, Francella N, Lewin SR, Collman RG. HIV-1 envelope-receptor interactions required for macrophage infection and implications for current HIV-1 cure strategies. *J Leuk Biol*. 2014;95(1):71–81.
- Bol SM, van Remmerden Y, Sietzema JG, Kootstra NA, Schuitemaker H, van't Wout AB. Donor variation in in vitro HIV-1 susceptibility of monocyte-derived macrophages. *Virology*. 2009;390(2):205–211.
- Li Y, et al. SIV Infection of Lung Macrophages. *PLoS One*. 2015;10(5):e0125500.
- Joseph SB, Arrildt KT, Sturdevant CB, Swanstrom R. HIV-1 target cells in the CNS. *J Neurovirol*. 2015;21(3):276–289.
- Williams KC, et al. Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. *J Exp Med*. 2001;193(8):905–915.
- Nowlin BT, Burdo TH, Midkiff CC, Salemi M, Alvarez X, Williams KC. SIV encephalitis lesions are composed of CD163(+) macrophages present in the central nervous system during early SIV infection and SIV-positive macrophages recruited terminally with AIDS. *Am J Pathol*. 2015;185(6):1649–1665.
- Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R. HIV-1 replication in the central nervous system occurs in two distinct cell types. *PLoS Pathog*. 2011;7(10):e1002286.
- Churchill M, et al. Longitudinal analysis of nef/long terminal repeat-deleted HIV-1 in blood and cerebrospinal fluid of a long-term survivor who developed HIV-associated dementia. *J Infect Dis*. 2004;190(12):2181–2186.

14. Joseph SB, et al. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. *J Virol*. 2014;88(4):1858-1869.
15. Josefsson L, et al. Majority of CD4⁺ T cells from peripheral blood of HIV-1-infected individuals contain only one HIV DNA molecule. *Proc Natl Acad Sci U S A*. 2011;108(27):11199-11204.
16. Shen Y, et al. Blood monocytes from most human immunodeficiency virus type 1-infected patients do not carry proviral DNA. *Clin Diagn Lab Immunol*. 1994;1(5):531-537.
17. Spivak AM, Salgado M, Rabi SA, O'Connell KA, Blankson JN. Circulating monocytes are not a major reservoir of HIV-1 in elite suppressors. *J Virol*. 2011;85(19):10399-10403.
18. Zalar A, et al. Macrophage HIV-1 infection in duodenal tissue of patients on long term HAART. *Antiviral Res*. 2010;87(2):269-271.
19. Watkins BA, et al. Specific tropism of HIV-1 for microglial cells in primary human brain cultures. *Science*. 1990;249(4968):549-553.
20. Shen R, et al. Macrophages in vaginal but not intestinal mucosa are monocyte-like and permissive to human immunodeficiency virus type 1 infection. *J Virol*. 2009;83(7):3258-3267.
21. Yukl SA, et al. A comparison of methods for measuring rectal HIV levels suggests that HIV DNA resides in cells other than CD4⁺ T cells, including myeloid cells. *AIDS*. 2014;28(3):439-442.
22. Takahashi K, Wesselingh SL, Griffin DE, McArthur JC, Johnson RT, Glass JD. Localization of HIV-1 in human brain using polymerase chain reaction in situ hybridization and immunocytochemistry. *Ann Neurol*. 1996;39(6):705-711.
23. Wiley CA, Schrier RD, Nelson JA, Lampert PW, Oldstone MB. Cellular localization of human immunodeficiency virus infection within the brains of acquired immune deficiency syndrome patients. *Proc Natl Acad Sci U S A*. 1986;83(18):7089-7093.
24. Igarashi T, et al. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4⁺ T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): Implications for HIV-1 infections of humans. *Proc Natl Acad Sci U S A*. 2001;98(2):658-663.
25. Calantone N, et al. Tissue myeloid cells in SIV-infected primates acquire viral DNA through phagocytosis of infected T cells. *Immunity*. 2014;41(3):493-502.
26. Baxter AE, et al. Macrophage infection via selective capture of HIV-1-infected CD4⁺ T cells. *Cell Host Microbe*. 2014;16(6):711-721.
27. Zhang Z, et al. Sexual transmission and propagation of SIV and HIV in resting and activated CD4⁺ T cells. *Science*. 1999;286(5443):1353-1357.
28. Davies JQ, Gordon S. Isolation and culture of human macrophages. *Methods Mol Biol*. 2005;290:105-116.
29. Eligini S, et al. Human monocyte-derived macrophages spontaneously differentiated in vitro show distinct phenotypes. *J Cell Physiol*. 2013;228(7):1464-1472.
30. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol*. 2008;8(12):958-969.
31. Islas-Ohlmayer M, et al. Experimental infection of NOD/SCID mice reconstituted with human CD34⁺ cells with Epstein-Barr virus. *J Virol*. 2004;78(24):13891-13900.
32. Palucka AK, et al. Human dendritic cell subsets in NOD/SCID mice engrafted with CD34⁺ hematopoietic progenitors. *Blood*. 2003;102(9):3302-3310.
33. Cravens PD, Melkus MW, Padgett-Thomas A, Islas-Ohlmayer M, Del PMM, Garcia JV. Development and activation of human dendritic cells in vivo in a xenograft model of human hematopoiesis. *Stem Cells*. 2005;23(2):264-278.
34. Yang J, Zhang L, Yu C, Yang XF, Wang H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res*. 2014;2(1):1.
35. Ziegler-Heitbrock L, et al. Nomenclature of monocytes and dendritic cells in blood. *Blood*. 2010;116(16):e74-e80.
36. Ziegler-Heitbrock L, Hofer TP. Toward a refined definition of monocyte subsets. *Front Immunol*. 2013;4:23.
37. Melkus MW, et al. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat Med*. 2006;12(11):1316-1322.
38. Wege AK, Melkus MW, Denton PW, Estes JD, Garcia JV. Functional and phenotypic characterization of the humanized BLT mouse model. *Curr Top Microbiol Immunol*. 2008;324:149-165.
39. Honeycutt JB, Wahl A, Archin N, Choudhary S, Margolis D, Garcia JV. HIV-1 infection, response to treatment and establishment of viral latency in a novel humanized T cell-only mouse (TOM) model. *Retrovirology*. 2013;10(1):121.
40. Denton PW, et al. Targeted cytotoxic therapy kills persisting HIV infected cells during ART. *PLoS Pathog*. 2014;10(1):e1003872.
41. Denton PW, et al. Generation of HIV latency in BLT humanized mice. *J Virol*. 2012;86(1):630-634.
42. Robertson DL, Hahn BH, Sharp PM. Recombination in AIDS viruses. *J Mol Evol*. 1995;40(3):249-259.
43. Gao F, et al. Genetic diversity of human immunodeficiency virus type 2: evidence for distinct sequence subtypes with differences in virus biology. *J Virol*. 1994;68(11):7433-7447.
44. Cann AJ, et al. Human immunodeficiency virus type 1 T-cell tropism is determined by events prior to provirus formation. *J Virol*. 1990;64(10):4735-4742.
45. Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen IS. Dual infection of the central nervous system by AIDS viruses with distinct cellular tropisms. *Science*. 1987;236(4803):819-822.
46. Parrish NF, et al. Phenotypic properties of transmitted founder HIV-1. *Proc Natl Acad Sci U S A*. 2013;110(17):6626-6633.
47. Gendelman HE, et al. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. *J Exp Med*. 1988;167(4):1428-1441.
48. Decker JM, et al. Effective activation alleviates the replication block of CCR5-tropic HIV-1 in chimpanzee CD4⁺ lymphocytes. *Virology*. 2009;394(1):109-118.
49. Deng HK, Unutmaz D, KewalRamani VN, Littman DR. Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature*. 1997;388(6639):296-300.
50. Zou W, et al. Nef functions in BLT mice to enhance HIV-1 replication and deplete CD4⁺CD8⁺ thymocytes. *Retrovirology*. 2012;9:44.
51. Watkins RL, Zou W, Denton PW, Krisko JF, Foster JL, Garcia JV. In vivo analysis of highly conserved Nef activities in HIV-1 replication and pathogenesis. *Retrovirology*. 2013;10:125.
52. Watkins RL, Zou W, Denton PW, Krisko JF, Foster JL, Garcia JV. In vivo analysis of highly conserved Nef activities in HIV-1 replication and pathogenesis. *Retrovirology*. 2013;10(1):125.
53. Zou W, et al. Nef functions in BLT mice to enhance HIV-1 replication and deplete CD4⁺CD8⁺ thymocytes. *Retrovirology*. 2012;9(1):44.
54. Thieblemont N, Weiss L, Sadeghi HM, Estcourt C, Haeflner-Cavillon N. CD14^{low}CD16^{high}: a cytokine-producing monocyte subset which expands during human immunodeficiency virus infection. *Eur J Immunol*. 1995;25(12):3418-3424.
55. Bednar MM, et al. Compartmentalization, Viral Evolution, and Viral Latency of HIV in the CNS. *Curr HIV/AIDS Rep*. 2015;12(2):262-271.
56. Ellery PJ, et al. The CD16⁺ monocyte subset is more permissive to infection and preferentially harbors HIV-1 in vivo. *J Immunol*. 2007;178(10):6581-6589.
57. Almodovar S, et al. HIV-1 infection of monocytes is directly related to the success of HAART. *Virology*. 2007;369(1):35-46.
58. Rich EA, Chen IS, Zack JA, Leonard ML, O'Brien WA. Increased susceptibility of differentiated mononuclear phagocytes to productive infection with human immunodeficiency virus-1 (HIV-1). *J Clin Invest*. 1992;89(1):176-183.
59. Wilen CB, et al. Phenotypic and immunologic comparison of clade B transmitted/founder and chronic HIV-1 envelope glycoproteins. *J Virol*. 2011;85(17):8514-8527.
60. Salazar-Gonzalez JF, et al. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. *J Exp Med*. 2009;206(6):1273-1289.
61. Ochsenbauer C, et al. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. *J Virol*. 2012;86(5):2715-2728.
62. Zaritsky LA, Dery A, Leong WY, Gama L, Clements JE. Tissue-specific interferon α subtype response to SIV infection in brain, spleen, and lung. *J Interferon Cytokine Res*. 2013;33(1):24-33.
63. Ortiz AM, et al. Depletion of CD4(+) T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. *J Clin Invest*. 2011;121(11):4433-4445.
64. Micci L, et al. CD4 depletion in SIV-infected macaques results in macrophage and microglia infection with rapid turnover of infected cells. *PLoS Pathog*. 2014;10(10):e1004467.
65. Spudis S, Gonzalez-Scarano F. HIV-1-related central nervous system disease: current issues in pathogenesis, diagnosis, and treatment. *Cold Spring Harb Perspect Med*. 2012;2(6):a007120.
66. Heaton RK, et al. HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology*. 2010;75(23):2087-2096.

67. Robertson KR, et al. The prevalence and incidence of neurocognitive impairment in the HAART era. *AIDS*. 2007;21(14):1915-1921.
68. Cysique LA, Brew BJ. Prevalence of non-confounded HIV-associated neurocognitive impairment in the context of plasma HIV RNA suppression. *J Neurovirol*. 2011;17(2):176-183.
69. Sturdevant CB, Joseph SB, Schnell G, Price RW, Swanstrom R, Spudich S. Compartmentalized replication of R5 T cell-tropic HIV-1 in the central nervous system early in the course of infection. *PLoS Pathog*. 2015;11(3):e1004720.
70. Sturdevant CB, et al. Central nervous system compartmentalization of HIV-1 subtype C variants early and late in infection in young children. *PLoS Pathog*. 2012;8(12):e1003094.
71. Denton PW, et al. Systemic administration of antiretrovirals prior to exposure prevents rectal and intravenous HIV-1 transmission in humanized BLT mice. *PLoS One*. 2010;5(1):e8829.
72. Honeycutt JB, Sheridan PA, Matsushima GK, Garcia JV. Humanized mouse models for HIV-1 infection of the CNS. *J Neurovirol*. 2015;21(3):301-309.
73. Wei BL, Denton PW, O'Neill E, Luo T, Foster JL, Garcia JV. Inhibition of lysosome and proteasome function enhances human immunodeficiency virus type 1 infection. *J Virol*. 2005;79(9):5705-5712.
74. Wahl A, et al. Human breast milk and antiretrovirals dramatically reduce oral HIV-1 transmission in BLT humanized mice. *PLoS Pathog*. 2012;8(6):e1002732.