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Macrophages sustain HIV replication in vivo independently of T cells

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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 CD4+ 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 contribute 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
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

### Table 1. Characteristics of HIV-1–infected patients

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

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.
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 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 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.
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+ hematopoietic stem cells (37). HIV infection of the brain and its sequela are hallmarks of AIDS. Macrophages have been
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 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.

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 (intermediate 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.
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 BLT mouse or MoM 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 ± 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

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

<table>
<thead>
<tr>
<th></th>
<th>JRCSF</th>
<th>7312A (HIV-2)</th>
<th>THRO</th>
<th>RHPA</th>
<th>CH058</th>
<th>ADA</th>
<th>CH040</th>
<th>CH040-4013 env</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLT</td>
<td>+ (n = 6)</td>
<td>+ (n = 3)</td>
<td>+ (n = 5)</td>
<td>+ (n = 2)</td>
<td>+ (n = 1)</td>
<td>+ (n = 7)</td>
<td>+ (n = 7)</td>
<td></td>
</tr>
<tr>
<td>ToM</td>
<td>+ (n = 9)</td>
<td>+ (n = 1)</td>
<td>+ (n = 2)</td>
<td>+ (n = 2)</td>
<td>+ (n = 1)</td>
<td>+ (n = 4)</td>
<td>+ (n = 8)</td>
<td></td>
</tr>
<tr>
<td>MoM</td>
<td>- (n = 4)</td>
<td>- (n = 3)</td>
<td>- (n = 3)</td>
<td>- (n = 3)</td>
<td>- (n = 3)</td>
<td>+ (n = 5)</td>
<td>+ (n = 21)</td>
<td>+ (n = 17)</td>
</tr>
</tbody>
</table>

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.
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 transcardially 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 isolates (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.
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

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

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, ×10 and ×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.
Table 3. Human macrophages can establish de novo infection of humanized mice

<table>
<thead>
<tr>
<th>Donor mouse/mice</th>
<th>Virus</th>
<th>Cell type used</th>
<th>No. of cells injected</th>
<th>No. of HIV DNA* cells injected</th>
<th>Recipient mouse</th>
<th>Evaluation of infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>MoM #1</td>
<td>CH040</td>
<td>Bone marrow cells</td>
<td>4.00E+06</td>
<td>15,880</td>
<td>BLT #1</td>
<td>+</td>
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<tr>
<td>MoM #2</td>
<td>CH040-4013 env</td>
<td>Bone marrow cells</td>
<td>4.00E+06</td>
<td>11,520</td>
<td>BLT #2</td>
<td>+</td>
</tr>
<tr>
<td>MoM #3</td>
<td>CH040</td>
<td>Bone marrow cells</td>
<td>2.00E+06</td>
<td>1,820</td>
<td>BLT #3</td>
<td>+</td>
</tr>
<tr>
<td>MoM #4</td>
<td>CH040-4013 env</td>
<td>Purified macrophages</td>
<td>3.80E+05</td>
<td>148</td>
<td>MoM #4</td>
<td>+</td>
</tr>
<tr>
<td>MoM #5</td>
<td>CH040</td>
<td>Purified macrophages</td>
<td>4.00E+06</td>
<td>4,760</td>
<td>MoM #5</td>
<td>+</td>
</tr>
</tbody>
</table>

MoM#5 CH040 Purified macrophages 3.80E+05 148 MoM #4 + +
MoM #5 CH040 Bone marrow cells 4.00E+06 4,760 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.

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). 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-Prkdcscid Il2rgtm1Wjl/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 x 10^6 human fetal liver or cord blood–derived CD34+ hematopoietic stem cells into NOD.CB17-Prkdcscid/l 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 hCD4 (APC, catalog 555485); hCD3 (FITC, catalog 555339); hCD4 (APC-H7 , catalog 560158); hCD8 (PerCP, catalog 347314); hCD19 (PE-Cy7 , catalog 557833); 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 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-μm 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 (JRC57, RHA3, 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 virus was injected i.v. into MoM. BLT mice and ToM were exposed i.v. to
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).

For all patient-derived blood cells, 1 × 10^6 cells were analyzed. For humanized mouse samples, 1 × 10^10 to 3 × 10^10 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, CTCATAAAGGGTCCCTGATGTC; gag outer reverse primer, GCTTCAATATGTTGACAGTGGAC; gag inner forward primer, GTGGTGAATACTCTCAGCTG; and gag inner reverse primer, TAGAAGAGAGCCTTCTACGCC. Peripheral blood (approximately 100 μl) 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’-CATGTTTTTCAGCATATCGAAGGA-3’ and 5’-TGCTTTGATGTCCTCACCCT-3’; assay sensitivity of ~668 RNA copies/ml). Additionally, at necropsy, MNCs were isolated from tissues and analyzed for the presence of vRNA and cDNA. 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 cDNA.

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, CACACCCTATTTTCGTCATGAGC; env outer reverse primer, GCCCATAGTGCTTCTCCGTGC; env inner forward primer, CACACCCTATTTTCGTCATGAGC; env inner reverse primer, CACACCCTATTTTCGTCATGAGC; and env inner reverse primer, CACACCCTATTTTCGTCATGAGC. The primers used for CH040-4013 env amplification included env outer forward primer, CACACCCTATTTTCGTCATGAGC; env outer reverse primer, GCCCATAGTGCTTCTCCGTGC; env inner forward primer, CACACCCTATTTTCGTCATGAGC; and env inner reverse primer, CACACCCTATTTTCGTCATGAGC. 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.
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 ± 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)(l)] 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.

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