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Persistence of Infectious HIV on Follicular Dendritic Cells

Beverly A. Smith,* Suzanne Gartner,† Yiling Liu,‡ Alan S. Perelson,‡ Nikolaos I. Stilianakis,§ Brandon F. Keele,§ Thomas M. Kerkering,¶ Andrea Ferreira-Gonzalez,† Andras K. Szakal,¶ John G. Tew,* and Gregory F. Burton3§

Follicular dendritic cells (FDCs) trap Ags and retain them in their native state for many months. Shortly after infection, HIV particles are trapped on FDCs and can be observed until the follicular network is destroyed. We sought to determine whether FDCs could maintain trapped virus in an infectious state for long periods of time. Because virus replication would replenish the HIV reservoir and thus falsely prolong recovery of infectious virus, we used a nonpermissive murine model to examine maintenance of HIV infectivity in vivo. We also examined human FDCs in vitro to determine whether they could maintain HIV infectivity. FDC-trapped virus remained infectious in vivo at all time points examined over a 9-mo period. Remarkably, as few as 100 FDCs were sufficient to transmit infection throughout the 9-mo period. Human FDCs maintained HIV infectivity for at least 25 days in vitro, whereas virus without FDCs lost infectivity after only a few days. These data indicate that HIV retained on FDCs can be long lived even in the absence of viral replication and suggest that FDCs stabilize and protect HIV, thus providing a long-term reservoir of infectious virus. These trapped stores of HIV may be replenished with replicating virus that persists even under highly active antiretroviral therapy and would likely be capable of causing infection on cessation of drug therapy. The Journal of Immunology, 2001, 166: 690–696.

Abbreviations used in this paper: FDC, follicular dendritic cell; CEM-TART, CEM cells transfected with HIV-1 tat and rev; DC-SIGN, DC-specific ICAM-3 grabbing nonintegrin; HAART, highly active antiretroviral therapy; QC-PCR, quantitative competitive PCR; TCD50, 50% tissue culture-infective dose.

Materials and Methods

Viral preparations

Viral stocks were prepared by propagating HIV-1HXB in H9 cells: HIV-1MC99IIIBRev in CEM-TART (CEM cells transfected with HIV-1 tat and rev) cells, and the primary strains LW1, 92US714, and 91US054 in PHA-stimulated (3-day) PBLs. Virus was harvested at the time of peak reverse-transcriptase and/or p24 production, pooled, filtered through a 0.45-μm membrane, and stored as aliquots in liquid nitrogen until used. HIV-1MC99IIIBRev, HIV-1HXB, 92US714, and 91US054 were obtained from the National Institutes of Health AIDS Research and Reference Agent Program. The LW1 virus was provided by Dr. Suzanne Gartner.
**HIV trapping and retention in vivo on murine FDCs**

Female, BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, ME), 5–8 wk of age, were passively immunized with 0.5 ml tissue culture supernatant from the Chessie-8 hybridoma (murine IgG anti-HIV-1 gp41; average protein concentration 2–10 μg/ml). The next day, HIV-1<sub>inj</sub> (50 μl of a stock cell-free virus suspension containing 28 ng of p24) was injected in each of the hind and fore feet to allow immune complex formation and FDC trapping in multiple draining lymph nodes. Mice were rested for the indicated times, their draining lymph nodes excised, and the FDCs isolated according to previously published procedures (15, 16). Our FDC-enriched preparations routinely contained 25–50% FDCs with contaminants of equal numbers of T and B lymphocytes. In some experiments, FDCs were isolated from mice 1 mo after injection of HIV-1<sub>inj</sub>, and a portion of the cells was subjected to FDC depletion using a mAb directed against murine FDCs (FDC-M1; rat, anti-murine FDC; a generous gift from Dr. Marie Kosco-Vilbois, Serono Pharmaceutical Research Institute, Geneva, Switzerland). Briefly, FDCs were incubated with biotin-conjugated FDC-M1, washed to remove unbound Ab, and depleted using magnetic streptavidin beads (Dynal, Lake Success, NY). This procedure typically removes ≥90% of FDCs, as determined by FACS analysis.

**Rescue of infectious HIV-1 trapped in vivo on FDCs**

Murine FDCs bearing in vivo trapped HIV were used as the only source of virus for infection of target cells. Virus rescue was performed by adding isolated FDCs (100–10,000 per culture) to H9 cells (for primary HIV-1 isolates, 3-day PHA blasts were substituted for H9s) and coculturing for 6 days. Productive infection of the target cells was assessed using DNA PCR (qualitative and quantitative) and p24 Ag capture ELISA.

**Isolation of human FDCs and maintenance of infectious virus in vitro**

Human FDCs were isolated from the tonsils of HIV-uninfected subjects, and in one instance from the lymph node of an HIV<sup>+</sup> subject treated with a combination of a protease and reverse-transcriptase inhibitor. Tonsils were cut into 3–5-mm cubes and digested for 1 h at 37°C in a solution of RPMI 1640 containing collagenase D (10 mg/ml; Boehringer Mannheim, Indianapolis, IN) and DNase I (1% v/v; Sigma, St. Louis, MO). After incubation, free cells were collected and transferred into RPMI 1640 containing 33% FBS, and new enzyme solution was added to the remaining tonsillar tissue and the incubation step was repeated. After the second incubation step, the cells were again collected, centrifuged at 400 × g to remove enzyme, washed, and resuspended in 10 ml of serum-free RPMI 1640. Two milliliters of the resulting cell suspension were gently added to 3-day PHA blasts of continuous Perceoll gradients (25-ml vol), and the mixture was subjected to centrifugation for 25 min at 700 × g. The low density fraction (1.055–1.060 g/ml) containing FDCs, lymphocytes, and macrophages was harvested and washed as before to remove residual Percoll. The cells were then labeled with H12J, a murine, IgM anti-human FDC-specific mAb (kindly provided by Dr. Moon Nahm, University of Rochester, Rochester, NY) and incubated for 2 h at 4°C. After washing the cells to remove unbound Ab, magnetic beads conjugated with rat, anti-mouse IgM (Miltenyi Biotec, Auburn, CA) were added and the FDCs were positively selected using MACS. FDC-enriched preparations using this procedure typically contain 50–80% FDCs with contaminants of T and B lymphocytes. Because FDCs are resistant to radiation, our FDC preparations were subjected to 3000 rad of γ-irradiation before use to minimize the ability of contaminating lymphocytes to support HIV infection/replication. To assess the ability of FDCs to maintain HIV infectivity in vitro, HIV-1<sub>inj</sub> (360 pg p24) alone or in the presence of anti-gp41 (Chessex 8; 0.3 mg) was incubated ± FDCs (10,000) for 1 mo. At various time intervals, H9 target cells were added to the cultures to rescue any remaining infectious virus. After 2 days of coculture, p24 production was determined and values greater than 500 pg/ml were considered positive for infection.

**Determination of HIV t½ on FDCs in vivo**

To estimate the t½ of HIV on FDCs, sucrose double-banded HIV-1<sub>inj</sub> (Advanced Biotechnologies, Columbia, MD) was surface labeled with <sup>125</sup>I. Fifty micrograms of virus were suspended in phosphate buffer and incubated on ice for 45 min with 2 mCi of sodium iodide (Amer sham) in the presence of Iodobeads (Pierce Chemical, Rockford, IL). After removing the Iodobeads, the sp. act. was determined to be 22 μCi/μg HIV. The virus was gently centrifuged through a Sephadex G-25 column to remove unbound label and resuspended to a total volume of 400 μl in PBS. Virus was then defatidically injected into the left or right feet of anti-gp41 passively immunized mice. At 6, 8, 10, and 12 wk, groups of mice were sacrificed and their draining lymph nodes excised. FDC-associated radioactivity was detected by ethidium bromide labeling, followed by Southern blotting onto Hybond-N transfer membranes (Amer sham, Arlington Heights, IL). Detection of HIV γgag was performed using a specific probe (SK 19) and a commercial enhanced chemiluminescence procedure following the manufacturer’s instructions (Amer sham).

Quantitative-competitive PCR (QC-PCR) was used to determine the number of viral DNA copies present. Primers 6575 and 7330C were used in QC-PCR to amplify HIV env and the competitive fragment, p9.6 (17). DNA was isolated from the FDC-target cell cocultures and added to PCR tubes in the presence of an equal volume of working standard (dilutions of p9.6 competitive fragment representing 10<sup>1</sup>, 10<sup>2</super>, 10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> copies of HIV env). ACH-2 cells were used as a positive control. PCR amplification was performed for 30 cycles (denaturation, 94°C × 1 min; annealing, 55°C × 30 s; extension, 72°C × 2 min; after 30 cycles, an additional extension at 72°C for 10 min was performed). Amplicons were analyzed by electrophoresis on 2% agarose gels, and β-globin amplicons were detected by ethidium bromide labeling, followed by Southern blotting onto Hybond-N transfer membranes (Amer sham, Arlington Heights, IL). Detection of HIV γgag was performed using a specific probe (SK 19) and a commercial enhanced chemiluminescence procedure following the manufacturer’s instructions (Amer sham).

Quantitative-competitive PCR (QC-PCR) was used to determine the number of viral DNA copies present. Primers 6575 and 7330C were used in QC-PCR to amplify HIV env and the competitive fragment, p9.6 (17). DNA was isolated from the FDC-target cell cocultures and added to PCR tubes in the presence of an equal volume of working standard (dilutions of p9.6 competitive fragment representing 10<sup>1</sup>, 10<sup>2</super>, 10<sup>3</sup>, 10<sup>4</sup>, and 10<sup>5</sup> copies of HIV env). ACH-2 cells were used as a positive control. PCR amplification was performed for 30 cycles (denaturation, 94°C × 1 min; annealing, 55°C × 30 s; extension, 72°C × 2 min; after 30 cycles, an additional extension at 72°C for 10 min was performed). Amplicons were analyzed by electrophoresis on 2% agarose gels and Southern blotting onto Hybond-N transfer membranes (Amer sham). Detection of specific PCR products was performed using an HIV env-specific FITC-labeled oligonucleotide probe (CTCTAGAGGGGAGCCCAATA) and a commercial enhanced chemiluminescence procedure following the manufacturer’s instructions (Amer sham). After development, hybridized PCR products were scanned and analyzed using Quantscan software. DNA copy numbers were determined from linear regression analysis of density plots of the competitive and experimental PCR products (i.e., the intersection of density plots of the competitive fragment PCR product with the HIV env product).

HIV-1 p24 Ag capture ELISA was performed using a commercial kit (Coulter, Palo Alto, CA), as per the manufacturer’s directions. In each experiment in which p24 was measured, the FDC-trapped HIV (used as the only source of input virus) was subtracted from the total p24 in cultures to determine the quantity of p24 produced.

Infectious virus quantitation was determined by 50% tissue culture infective dose (TCID<sub>50</sub>) assay using FDCs obtained from mice injected 6 days previously with HIV-1. FDCs were diluted (10-fold serial dilutions) over a range of 10,000 to 1 cell and cultured with HIV targets, as above. Determination of TCID<sub>50</sub> units was based on the Reed-Muench accumulative titration method.

**Results**

To assess whether or not FDCs could maintain the infectious nature of trapped HIV for long periods in an in vivo setting, it was first necessary to have a model that would preclude HIV replication. This was needed because ongoing viral replication, even at low levels, might provide newly produced virions that could replete the HIV reservoir on FDCs, thus appearing to prolong the period that infectious virus was detected. Mice provide an excellent model because they are nonpermissive for HIV infection due to their lack of human CD4<sup>+</sup> cells and the inability of mouse cells to support viral replication (18). In previous studies, we determined the experimental conditions needed for FDC trapping of HIV in vivo, although these studies were limited to 5 days or less (15). Therefore, we sought to use the nonpermissive murine model to determine whether FDC-trapped HIV remained infectious in vivo for many months and could thereby serve as a long-term reservoir of infectious HIV. Mice were passively immunized to provide specific Ab needed for immune complex formation and...
FDC trapping, after which HIV was injected. FDCs were then isolated over a 9-mo period and used as the only source of virus in rescue cultures.

Using a qualitative DNA PCR procedure for detection of HIV gag, we found that FDC-trapped virus was infectious at all time intervals examined including 9 mo after injection (Fig. 1). Remarkably, as few as 100 FDCs bore sufficient infectious virus particles to infect the target cells. Although qualitative in nature and somewhat variable in signal intensity (due to independent PCR analysis of samples from each time interval), the hybridization signals on Southern blots suggested that HIV DNA decreased in an FDC dose-dependent manner. Our hypothesis that FDCs maintain virus infectivity is dependent on the absence of viral replication in mice. Although mice are nonpermissive for HIV infection, we wanted to ensure that the specialized microenvironment of the germinal center did not permit infection to occur and thus contribute to the HIV reservoir. Therefore, we injected a mutant virus, HIV-1MC99IIIB-Tat-Rev, in which tat and rev are disrupted so that viral replication can only occur in cells that produce these proteins. After 1 mo in vivo, FDCs bearing mutant virus were harvested and cocultured with CEM cells or with CEM-TART (Fig. 2). Infection of the target cells was monitored using p24 production. Values expressed represent the mean ± the SE of triplicate cultures. Input virus was 0.9, 0.09, and 0.009 ng for 10,000, 1,000, and 100 FDCs, respectively. Production of p24 was not seen in the control CEM cells, but was clearly observable when CEM-TART cells were used as targets, further supporting the concept that viral replication is not necessary for FDCs to retain virus infectivity.

was observed as before with as few as 100 FDCs, but infection was confined to CEM-TART cells. We interpret these data, coupled with our results showing the absence of detectable PCR signal from any of our isolated murine FDC preparations, and previous reports of murine immunity to HIV infection (18) to indicate that viral replication is not contributing to the reservoir of infectious HIV retained on murine FDCs.

To estimate how many copies of HIV DNA were present in cocultures containing FDCs obtained during the first 6 mo of the study, a QC-PCR was used (Table I) (17). HIV DNA was present over a 4-log range, with the amount dependent on the number of HIV-bearing FDCs added to the cultures. As expected, the number of infectious virions rescued in our cocultures declined very slowly over the period of testing, although the rate of decline of rescued infectious particles appeared faster when 10,000 FDCs were used.

FIGURE 1. FDC-trapped HIV-1 remains infectious for at least 9 mo. Southern blots of DNA-PCR-amplified HIV-1 gag product obtained from H9 target cells cocultured with HIV-1IIb trapped and retained on murine FDCs in vivo for the indicated time intervals. FDCs isolated from groups of four mice were added in decreasing numbers (ranging from 10,000 to 100) to 50,000 H9 cells and cocultured for 6 days before DNA isolation. The 6-day culture period before DNA isolation was chosen because it allowed examination of productive infection and not just virus integration, as might be the case if DNA were isolated at 24 h after interaction with FDCs. Positive controls consisted of DNA isolated from 50,000 ACH-2 cells, and negative controls consisted of 50,000 H9 cells alone and 100,000 virus-bearing FDCs in the absence of target cells. All samples were simultaneously PCR amplified for the β-globin gene to ensure equivalent amounts of DNA were present in all lanes and that PCR amplification was occurring (bottom). The HIV-1 gag product (115 bp; top) was detected where FDCs bearing HIV-1 were used as the only source of virus for infection of H9 cells. Amplification of DNA from each time interval was performed individually as the samples became available, and as a consequence, some variation in signal intensity of the ACH-2 positive control was apparent (6- and 9-mo periods particularly). However, quantitative assays (PCR and p24) were used to confirm these qualitative results.

FIGURE 2. Replication-deficient HIV is maintained in vivo in an infectious state on FDCs. HIV-1MC99IIIB-Tat-Rev was injected into mice, as previously described for HIV1IIb. After 30 days in vivo, draining lymph nodes were isolated and FDCs were prepared as before. Decreasing numbers of FDCs bearing replication-deficient virus were cocultured for 6 days with parental CEM cells (lacking Tat and Rev proteins) and with CEM-TART. Infection of the target cells was monitored using p24 production. Values expressed represent the mean ± the SE of triplicate cultures. Input virus was 0.9, 0.09, and 0.009 ng for 10,000, 1,000, and 100 FDCs, respectively. Production of p24 was not seen in the control CEM cells, but was clearly observable when CEM-TART cells were used as targets, further supporting the concept that viral replication is not necessary for FDCs to retain virus infectivity.

Table I. HIV DNA (env) copies present after infection of 50,000 H9 target cells by virus that had been trapped in vivo on FDCs for the indicated period of time after virus injection

<table>
<thead>
<tr>
<th>Time (Weeks)</th>
<th>HIV DNA Copies Present a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50,257 ± 10,930</td>
</tr>
<tr>
<td>6</td>
<td>25,607 ± 8,210</td>
</tr>
<tr>
<td>12</td>
<td>29,624 ± 12,142</td>
</tr>
<tr>
<td>25</td>
<td>4,019 ± 624</td>
</tr>
</tbody>
</table>

 a Mice were injected with HIV, and their FDCs were isolated and cocultured as described in Fig. 1. DNA was isolated from the contents of each well after 6 days of coculture and subjected to QC-PCR analysis. Data represent the mean values ± the SE obtained from triplicate cultures.
To confirm our PCR-based assays for infection, we monitored the amount of p24 produced in our cocultures of H9 target cells with FDCs. We isolated the cells and performed p24 ELISA on lysed...fected by Roche Amplicor was nondetectable (limit of detection <10 FDCs) and purified (>90% FDCs) fractions (16).

Ten thousand FDC-depleted cells, FDC-enriched (i.e., not fractionated), or purified FDCs were then cocultured with H9 target cells. When FDC-enriched preparations were used as the only source of virus, over 120 ng p24/ml was produced in the cultures, but this was reduced to 0.2 ng/ml when cells depleted of FDCs were substituted (Fig. 3). Furthermore, adding HIV-bearing purified FDCs back to the cultures resulted in a restoration of p24 production. These data indicated that infectious virus is associated with FDCs and not with other cells, and is consistent with the hypothesis that FDCs are responsible for maintaining HIV in an infectious state.

To ensure that FDC maintenance of HIV infectivity was a general phenomenon and not confined to a laboratory strain of virus, we also tested three primary HIV-1 strains: LW1, 92US714, and 91US054. LW1 was isolated from an HIV-1 endemic-infected lab worker and is genetically similar to HIV-1<sub>inm</sub> isolated from the H9HTLV-IIIB cell line, with the exception that LW1 is dual tropic (20). Virus was injected into mice as before and harvested for rescue experiments after 3 wk in vivo. To ensure virus infection was ongoing in culture and not a result of residual p24 Ag from input virus, cocultures of FDCs bearing LW1 and PHA-stimulated CD4<sup>+</sup> T cells, infectious virus was rescued, as evidenced by production of 94.6, 32.8, and 12.9 ng/ml p24, respectively. Although there is no way to determine how long the rescued infectious virus had been trapped on FDCs (because some viral replication is possible under HAART), the rescue of infectious virus is consistent with the concept that FDC-associated virus may be a potential problem even in treated subjects.

To further assess virus persistence on FDCs, we<sup>125</sup>I labeled the HIV surface and injected virus as before. Mean values of FDC-associated HIV were as follows: 14.9, 14.5, 14.9, and 8.9 pg/lymph node at weeks 6, 8, 10, and 12, respectively. Although the data are significantly impacted by the last time interval, a virus decay of −0.076 per week and a 1/2 seen previously using conventional protein Ags (2, 19).

Because our murine FDC preparations (i.e., FDC enriched) consisted of 25–50% FDCs with about equal numbers of contaminating B and T lymphocytes (16), we sought to ensure that FDCs and not another cell type were responsible for the maintenance of HIV in an infectious state. We isolated cells from mice injected 1 mo earlier with HIV and depleted the FDC population from one portion by means of a biotin-conjugated anti-mouse FDC Ab and streptavidin-magnetic beads. This procedure generates both FDC-depleted (<10% FDCs) and purified (>90% FDCs) fractions (16).

Production of significant amounts of p24 (<500 pg/ml), which rapidly declined with the time before coculture. However, the addition of FDCs to cultures bearing HIV and specific Ab resulted in a rapid decline of p24 production (<500 pg/ml) which was ongoing in culture and not a result of residual p24 Ag from input virus. Cocultures of FDCs bearing LW1 and PHA-stimulated CD4<sup>+</sup> blasts were monitored for p24 production over 6 days and the final concentrations of p24 were similar to those observed using HIV-1<sub>inm</sub>. We also tested more conventional HIV-1 primary strains 92US714 and 91US054 (R5 and X4 viruses, respectively) (Table III). As with the laboratory strain of virus, as few as 100 FDCs bearing the primary isolates were able to transmit infection.

In the above studies, we used the mouse to study in vivo events in the absence of virus replication that could otherwise falsely influence our results. However, we also wanted to determine whether human FDCs could maintain HIV infectivity. To test this, FDCs were obtained from the tonsils of HIV-uninfected subjects and added to cultures containing HIV ± specific Ab (Fig 5). In the absence of FDCs, virus recovery by H9 cells added within the first few days led to low levels of p24 production (<500 pg/ml), which rapidly declined with the time before coculture. However, the addition of FDCs to cultures bearing HIV and specific Ab resulted in production of significant amounts of p24 (>500 pg/ml) by H9 cell cocultures started throughout a 25-day period consistent with the hypothesis that FDCs maintain virus infectivity.

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>HIV&lt;sub&gt;inm&lt;/sub&gt; Retained In Vivo on FDCs&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p24 (ng) Retained on 100,000 FDCs&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p24 Production (ng/ml) in Cocultures of FDCs and H9&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>58</td>
<td>336 ± 6</td>
<td>10,000 FDCs</td>
</tr>
<tr>
<td>6</td>
<td>55</td>
<td>278 ± 14</td>
<td>1000 FDCs</td>
</tr>
<tr>
<td>12</td>
<td>73</td>
<td>176 ± 3</td>
<td>100 FDCs</td>
</tr>
<tr>
<td>24</td>
<td>14</td>
<td>48 ± 3</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>5</td>
<td>14 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mice were passively immunized and injected with HIV<sub>inm</sub> as described (Fig. 1). FDCs were isolated from groups of four to five mice at the indicated time after injection of virus.

<sup>b</sup> FDCs (1 × 10<sup>6</sup>) were determined to test the amount of associated (trapped) p24. This value was used to calculate the input virus present on 10,000, 1,000, and 100 FDCs.

<sup>c</sup> HIV p24 production was monitored by p24 capture ELISA (Couler) according to the manufacturer’s instructions. Cocultures of the indicated number of FDC bearing virus and 50,000 H9 cells were maintained for 6 days prior to analysis, and the amount of p24 produced was determined by subtracting the input virus (i.e., trapped on FDCs) from the total p24 present. Values expressed are the means ± SE of triplicate cultures.
and a mAb directed against murine FDC (FDC-M1). Ten thousand murine cells was subjected to FDC depletion using magnetic beads the different cell types remains infectious: DC-SIGN maintains protection of HIV infectivity mediated by FDCs is the time that virus bound to grabbing nonintegrin (DC-SIGN), in binding and short-term pro-

FIGURE 3. HIV-1 associated with FDCs and not other cells remains infectious. FDC were isolated from mice 1 mo after injection of HIV-1m5a, and a portion of the cells was subjected to FDC depletion using magnetic beads and a mAb directed against murine FDC (FDC-M1). Ten thousand murine cells (either from the initial or FDC-depleted fraction, H9+ FDCdep) were added to 50,000 H9 target cells and cultured for 6 days, after which the amount of p24 produced by infection was determined by Ag capture ELISA. Note that removal of FDC ablated transfer of infection and that addition of purified FDC (H9+dep FDC, obtained by adding back the FDC that were depleted) appeared to increase the level of p24 produced as expected because the purified FDC would result in higher levels of input virus than in preparations in which contaminating cells were present. The FDC-trapped HIV used as the only source of input virus was present at a concentration of 1.9 ng p24/1 x 10^6 FDC, and this value has been subtracted from each coculture to yield the amount of p24 produced therein. Values expressed are the means of triplicate cultures ± the SE.

Discussion

Our data indicate that in vivo, FDC-trapped HIV remains infectious for long periods. In the mouse, a single exposure to HIV-1 allowed FDC trapping, and this virus remained infectious for at least 9 mo (Fig. 1 and Tables I and II). Even more remarkably, as few as 100 FDCs bore sufficient virus to transfer infection. FDC maintenance of HIV infectivity was not restricted to a single virus isolate (Fig. 4 and Table III), nor was it dependent on ongoing virus replication that could replenish the HIV reservoir (Fig. 2). Human FDCs also appeared to be able to preserve the infectious nature of HIV in an in vitro system in which HIV immune complexes remained infectious for at least 25 days (Fig. 5). Collectively, these data indicate that the FDC network can be a long-term source of replication-competent virus.

Although the mechanism of FDC protection of HIV infectivity is not currently understood, it is an area of active investigation. We hypothesize that stabilization of viral envelope glycoproteins by virus-Ab-FDC-Fc receptor interactions may contribute. Recent studies implicate the adhesion molecule, DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN), in binding and short-term protection of HIV-1 by dendritic cells (21–24). An important difference between the protection of HIV infectivity mediated by DC-SIGN and that mediated by FDCs is the time that virus bound to the different cell types remains infectious: DC-SIGN maintains viral infectivity for <5 days in vitro, whereas FDCs protected infectivity for at least 25 days. The prolonged protection of virus by FDCs is consistent with their function as a long-term reservoir of native proteins (and in the case of HIV-1, infectious virus). Whatever, the mechanism(s) involved, the fact that trapped virus remains infectious for months in an in vivo setting without viral replication indicates that FDCs represent an important reservoir of infectious HIV that could reignite infection when permissive conditions occur.

The use of the murine model to study FDC maintenance of infectivity offers a number of advantages over other models that are permissive to HIV infection and replication. The mouse model permits the controlled introduction of defined amounts of virus that cannot replicate and thus replenish the FDC reservoir of HIV. Furthermore, virus retention on FDCs occurs in a physiological setting with an intact immune system and other conditions (e.g., presence of endogenous proteases and complement proteins) that are not recapitulated in culture. We also have significant experience in working with FDC trapping and retention in the mouse. Although the murine model makes it possible to examine the decay of infectious virus in the FDC compartment uncomplicated by replacement from ongoing replication, the model does have some limitations, including a lack of destruction of the FDC network and the

Table III. Production of p24 from 6-day cocultures of murine FDCs bearing primary isolates of HIV-1 and PHA-stimulated lymphocytes

<table>
<thead>
<tr>
<th>HIV Strain</th>
<th>p24 Production (ng/ml)</th>
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<tbody>
<tr>
<td></td>
<td>100 FDCs</td>
</tr>
<tr>
<td>91US714</td>
<td>243 ± 38</td>
</tr>
<tr>
<td>91US054</td>
<td>213 ± 44</td>
</tr>
</tbody>
</table>

a Virus was injected into mice 21 days prior to isolating FDCs. The data are expressed as the mean p24 (ng/ml) produced in triplicate wells ± the SE.

b The indicated numbers of FDCs were incubated for 6 days with 1 x 10^6 PHA-stimulated lymphocytes. Tissue culture supernatant fluid was assessed for p24 concentration using an Ag capture ELISA (Coulter). Input virus p24 was subtracted from the total p24 to yield p24 production, and the data expressed are the means of triplicate cultures ± the SE.

c HIV-1 primary strains were obtained from the National Institutes of Health, AIDS Research and Reference Reagent Program. Virus stocks were prepared in 3-day, PHA-stimulated lymphocytes with 20 U/ml IL-2 added on day 3 to the end of culture. Stocks were stored in liquid nitrogen until used. Strains 92US714 and 91US054 are R5 and X4 viruses, respectively.

The respective input values for 10,000, 1,000, and 100 FDCs were 6, 0.6, and 0.06 ng of p24 for each primary isolate.
lack of overall immune activation that would occur in a permissive model.

In some of our prior work, we used the mouse to model FDC trapping of HIV immune complexes (with or without neutralizing Abs) for short periods of time (≤5 days) to determine whether trapped HIV was infectious (15). However, this study differs significantly from the earlier one in that our purpose here was to determine whether FDC-trapped HIV (resulting from a single infection of virus) could retain its infectious nature for many months and thus be a potential dangerous reservoir. Our findings indicate that this is indeed the case. The observation that after 9 mo in vivo as few as 100 FDCs provide sufficient virus to cause productive infection of target cells suggests that the FDC reservoir may be very potent.

In addition to the more controlled murine in vivo studies, we also examined the ability of human FDCs to preserve HIV infectivity in vitro. In contrast to HIV alone or in immune complex form, the presence of FDCs resulted in maintenance of HIV infectivity over 25 days. In other experiments examining FDC-mediated maintenance of HIV infectivity, we found that FDCs were unable to maintain virus infectivity in vitro unless specific Ab to determinants present on the virus envelope was present (Burton et al., unpublished). These results contrast with other studies in which HIV binding to FDCs appears to be dependent on adhesion molecules (CD54, CD11a) (25) or complement protein C3 or its fragments (26, 27). However, it should be noted that in one of the latter studies, the presence of specific Ab did increase virus trapping on FDCs (26). The reasons for these apparent discrepancies in the various studies may relate to differences in the experimental systems, such as source of Ab (e.g., polyclonal vs monoclonal, human vs murine), conditions under which immune complexes were formed, FDC isolation procedures, and heterogeneity in FDC populations.

We also examined FDCs isolated from lymphoid tissue obtained from a HAART-treated patient and found that this virus was infectious. However, in this study the use of FDCs obtained from lymphoid tissues of infected subjects is not definitive evidence that FDCs maintain HIV infectivity, because a contaminating infected cell (CD4+ T cell or macrophage) or incomplete suppression of viral replication by HAART (10, 28–30) could not be excluded as a potential source of infectious virus. This latter concern underscores the importance of our nonpermissive murine model to address the issue of FDC maintenance of HIV infectivity.

Although the demonstration that human FDCs increase the maintenance of HIV infectivity in vitro is important, there are a number of reasons that maintenance of viral infectivity does not persist as long as seen in the mouse. First, the ability to maintain FDCs in culture is not well understood and the 25-day period of maintenance may be at the limit of our ability to keep FDCs fully functional in vitro. Furthermore, it is known that cultured FDCs typically lose cell surface markers, some of which may be important in the maintenance of virus infectivity and FDC function (31). It is also known that B cells are important in maintaining FDCs in vivo and contaminating B cells in our cultures would have been destroyed by exposure to 3000 rad of γ irradiation before use (32).

The unique nature of FDC cocultures makes them more complex than typical infectious assays (e.g., TCID50). The amount of viral DNA or p24 detected depends not only on the amount of infectious virus present on added FDCs, but also on the amount of secondary or costimulatory signaling provided by the FDCs (16). In our hands, this signaling augments HIV infection/replication (33). This FDC costimulation is optimal at a ratio of 1 FDC per 10 lymphocytes. Although our FDC–lymphocyte assays are not quantitative for the number of infectious units present, they suggest that >1 TCID50 of HIV was present on 100 FDCs, because this number caused infection at all time points examined. We performed a TCID50 assay using FDCs obtained 1 wk after injection of HIV and determined that 100 FDCs bore 77 TCID50. Using the estimated 9.5-wk t1/2 of HIV decay on FDCs, 3.8 t1/2 would be lost after 9 mo, leaving about 5 TCID50 per 100 FDCs, consistent with the finding that 100 FDCs were able to transfer infection at each time interval examined.

In humans, the FDC reservoir of HIV is estimated at 1.5 × 108 copies of viral RNA per gram of lymphoid tissue (11, 34). HAART dramatically reduces the amount of viral RNA detected on FDCs (34, 35). However, even with HAART, some virus remains after prolonged therapy, and levels below 10,000 copies of viral RNA per gram tissue may not be detected (11, 34, 35). A recent study indicated that even in the absence of detectable viral RNA, p24 protein remained on FDCs (36). This residual p24 may simply be trapped viral protein; however, we reason that it may also represent virus on FDCs that is below the level of detection by in situ hybridization.

The rate of virus decay from FDCs in patients receiving HAART is biphasic: the first phase decays with a t1/2 of 1.7 days, and the second phase with a t1/2 of 14 days (34). These data are in apparent contradiction to the longer decay periods observed in our murine study, although Hlavacek et al. (37), using a stochastic model of decay, calculated that with a beginning virus load of 1011 copies of RNA, virions could still be expected for as long as 10 yr. We hypothesize that differences between the Cavert study and ours may be related to analysis of the FDC network under very different conditions of FDC–virus density. At high virus densities (as would occur in untreated subjects), limited numbers of FDC Fc and complement receptors may be available for binding viral immune complexes, resulting in many less stable, univalent interactions. At low densities (as in our mice), many FDC Fc and complement receptors should be available, and trapped HIV immune complexes could be anchored to FDCs in more stable, highly multivalent states of attachment. This postulate is consistent with mathematical modeling of HIV-1 dissociation from FDCs (37), as well as a model of the kinetics of viral decline in patients on HAART (38).

Importantly, it has been reported that even under HAART, some HIV replication continues (10, 28–30), and we reason that this
may lead to recharging of the FDC reservoir, thus providing a continual source of virus that could renew infection once drug therapy is discontinued. Future intervention strategies may need to target this important reservoir to reduce the risk of reinfection following cessation of drug therapy.

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References