Efficient HIV-1 transmission from macrophages to T cells across transient virological synapses

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Abstract
Macrophages are reservoirs of HIV-1 infection, proposed to transmit virus to CD4+ T cells, the primary target of the virus. Here we report that human monocyte-derived macrophages (MDM) rapidly spread HIV-1 to autologous CD4+ T cells resulting in productive infection. Transmission takes place across transient adhesive contacts between T cells and MDM, which have the features of a virological synapse including co-polarization of CD4 on the T cell with HIV-1 Gag and Env on the macrophage. We propose that an infected MDM can infect at least one T cell every six hours. Since HIV-1-infected macrophages can survive for many weeks, these results highlight the central role played by macrophages in HIV-1 infection and pathogenesis.
Introduction

Macrophages are central players in HIV-1 pathogenesis: they are among the first cells infected by the virus, and have been proposed to spread infection to the brain and to form a long-lived virus reservoir\(^1,2\). Two recent studies have shown that HIV-1 in macrophages assembles in a newly-identified intracellular compartment that is a large and complex invaginated plasma membrane domain\(^3,4\). The function of this compartment is unknown, but presumably HIV-1 may be stored here, protected from effector elements of the humoral immune system.

In direct T cell-T cell spread of HIV-1, we and others have demonstrated that HIV-1 forms a ‘virological synapse’ (VS)\(^5\)\(^-\)\(^8\). The VS is a multi-molecular complex that forms at the interface between HIV-1-infected and uninfected, receptor-expressing cells. Its assembly is driven by gp120-CD4-coreceptor interactions and depends upon stable cell-cell junctions maintained by adhesion molecules\(^5,9\). Direct T cell-T cell spread is likely to be the dominant mode of viral spread between these cells in culture, and probably in vivo\(^7\). HIV-1 also appears to spread from dendritic cells to T cells via a VS\(^10\). HIV-1-infected MDM can also transmit virus to PBL\(^2,11\), but the underlying mechanism has not been elucidated.

Here, we investigated the transfer of HIV-1 from MDM to autologous CD4\(^+\) T cells, and found that transfer of virus is rapid, and takes place across transient VS between T cells and MDM.

Materials and methods

Human MDM were generated and infected with HIV-1\(_{BaL}\) as described\(^4\), and maintained in X-VIVO medium (Lonza, Walkersville, MD) containing 1% autologous heat-inactivated and filtered serum. Autologous CD4\(^+\) T cells were negatively selected from PBL (Miltenyi-Biotec, Bergisch-Gladbach, Germany). Samples were fixed and stained as described\(^3,12\), and were mounted in ProLong Gold antifade reagent (Invitrogen, Carlsbad, CA), and analyzed at RT using a non-inverted Zeiss LSM5 Pascal microscope, linked to Pascal software V4.2SP1. Images were acquired using a 63x oil immersion objective (1.4 aperature) and processed using Adobe Photoshop V8.0.
Staining/washing buffer contained 5% human and goat serum. Antibodies used were L12013 (CFAR, UK) with donkey-anti-mouse-FITC (Jackson ImmunoResearch, Suffolk, UK), mouse-anti-Gag-p17 (NIBSC, 4C9) with goat-anti-mouse-IgG2a-alexa647 (Invitrogen), and biotinylated-2G1214 (endogenous biotin blocked with biotin-block (Invitrogen)) with streptavidin-Tritc (Jackson). Samples for electron microscopy (EM) were prepared and analyzed as described using a JEM-2000EX microscope (JEOL, Tokyo, Japan), or with a Tecnai F-30 microscope (FEI, Eindhoven, Netherlands).

For flow cytometry (FC), T cells were collected, fixed and stained for CD3 and CA-p24 as described. Inhibitors were 13B.8.2 (Coulter, Fullerton, CA), Q4120 (CFAR), T20 (NIH, USA), 2D7 (BD Biosciences, San Jose, CA) (all 10 µg/ml), AZT (NIH, 5 µM) and Tak-779 (NIBSC, 300 nM).

For quantitative real-time PCR (qPCR) samples, DNA was extracted as described. \( pol \)-primers: TGGGTTATGAACCTCCATCCTGA (sense), TGTCATTGACAGTCCAGCTGTCT (anti-sense). Reference-gene: human \( \beta \)-globin (AACTGGGCATGTGGAGACAGA (sense), CTAAGGGTGGAATAGACCAATG (anti-sense).

**Results and Discussion**

We co-cultured HIV-1-infected MDM (Figure 1A), with autologous CD4\(^+\) T cells for 6 hours, followed by fixation and staining for LSCM analysis. The T cells were pre-stained with CD4 non-blocking antibody, and after fixation and permeabilization, samples were stained for HIV-1 Gag and Env (Figure 1B,C). Since staining for HIV-1 Env in MDM is complicated by human antibodies binding efficiently to human Fc-receptors, we blocked with human serum and used biotinylated 2G12 to reduce background. A high proportion of MDM (>80%) in the co-culture had one or more T cells attached via the tip of a pseudopod-like extension of the T cell. In the majority of MDM in which HIV-1 Gag and Env were detected (10-30%, depending upon donor), these viral antigens colocalized with CD4 in the adherent T cells (1B,C). Colocalization of Gag, Env and CD4 was observed within 1 hour, and the proportion of MDM-T cell conjugates with colocalization of these antigens was maximal at 5-7 hours. Based on these data, we could not exclude the possibility that at least a proportion of the viral antigens were...
accumulating within the MDM at the contact site with the T cell, but virus was not infecting the T cell. However, we noted that the T cell contacts with the MDM were transient, and many T cells detached from the MDM after becoming Gag-positive, confirming that Gag was transferring to the T cell. To investigate this further, we carefully aspirated detached T cells from the MDM-T cell co-cultures, and analyzed them by LSCM (Figure 1D). We observed that 5-10% of the T cells were Gag-positive at 6 hours post co-culture. This confirmed that the interactions between MDM and T cells were short-lived, and combined with the data from 1B+C suggested that HIV-1 is efficiently transmitted to CD4+ T cells across a transient VS-like structure. This was reinforced by EM analysis of the contact zone between HIV-1-infected MDM and CD4+ T cells, which was characterized by tight adhesive junctions between the cells with regular gaps or pockets (Figure 1E). We found no evidence of cell-cell fusion, consistent with the lack of syncytium formation observed by LSCM. HIV-1 was detected within vesicular MDM-compartments similar to those previously described (1st panel Figure 1E), and virus was observed both proximal to, and in contact with, adherent T cells (2nd and 3rd panel). Further research will be required to determine how this virus-containing compartment in MDM relocates to the VS.

To quantify viral transmission, we harvested all T cells from co-cultures with MDM at various times, and analyzed the cells by FC. We stained for CD3 and intracellular HIV-1 Gag p24 in order to determine the percentage of HIV-1-positive T cells, and found that significant numbers (p<0.002 (ANOVA), as compared to t=0) of Gag-positive T cells could be detected after 1 hour (Figure 2A). After 12 hours, >12% of 5×10^5 T cells were Gag-positive. With an estimated average of 3×10^4 infected MDM per well, based on LSCM analysis and counting, we propose that each infected MDM is able to infect at least one T cell every 6 hours. To investigate the requirement for cell-cell spread compared to cell-free virus spread, we performed an experiment using a transwell system in which infected MDM were separated from T cells by a virus-permeable membrane. Figure 2B shows that after 10 hours co-culture, 8.5% of T cells that were in contact with HIV-1-infected MDM were infected. By contrast, cell-free virus infection resulted in 0.5% of HIV-1 infected cells, indicating that that cell-cell viral spread is >10-fold more efficient than cell-free spread. The presence of RT inhibitor AZT did not
reduce the percentage of Gag-positive T cells, demonstrating that the Gag measured by FC fully represents transmitted virus, and not newly produced Gag in the T cells. To investigate the role of HIV-1-receptor interactions, we tested antibody inhibitors of gp120-CD4 (13B.8.216, Q412013) and gp120-CCR5 (2D717), a small molecule inhibitor of gp120-CCR5 interaction (TAK-77918), and a fusion inhibitor (T2019). Viral transmission was substantially inhibited by CD4-blocking antibodies (Figure 2B) without any obvious reduction in the amount of MDM-T cell clustering, as confirmed by LSCM analysis (not shown). Blocking CCR5 with either 2D7 or TAK-779 gave more subtle but nevertheless significant inhibition, whereas T20 reduced transmission by more than 50%, indicating that gp41-mediated fusion plays a role in transmission by MDM, just as in dendritic15 and T cell5-mediated transmission. The inability of CCR5 antagonists to potently inhibit the presence of a HIV-1 Gag signal in the T cells might be interpreted as representing a proportion of CCR5-independent entry, in accord with another recent study8. However, another possibility is that HIV-1 particles released from infected MDM attach to the surface of T cells rendering them Gag-positive, but entry is inhibited due to CCR5 antagonists or T20. Part of the Gag signal in this experiment therefore may represent extra-cellular virus. We therefore set out to investigate whether MDM-mediated HIV-1 transfer established a productive infection in T cells. To do this we carefully aspirated detached T cells from MDM-T cell co-cultures at 0, 6, 12, and 24 hours, extracted DNA and performed qPCR for HIV-1 pol (Figure 2C). Our results demonstrated significant (p<0.02) de novo viral DNA synthesis within 6 hours of co-culture, rising to >20-fold above baseline at 24 hours, indicating that HIV-1 transmission results in rapid initiation of viral entry and reverse transcription in CD4+ T cells. Finally, productive infection of the T cells was confirmed in a replication-assay, where T cells harvested from MDM-T cell co-cultures were depleted of any residual MDM, cultured for an additional 5 days and supernatant p24 measured (Figure 2D).

Our data are consistent with a central role of MDM as an in vivo viral reservoir that efficiently seeds large numbers of T cells with HIV-1 via cell-cell spread across a VS. This finding has implications for viral pathogenesis in all immune tissues that contain cells of the macrophage lineage, with particular relevance to dense secondary lymphoid tissue.
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Author contribution:
FG designed and performed the research, and wrote the paper; SW performed research; QJS designed research and wrote the paper.

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References


Figure legends

**Figure 1: Macrophages transmit HIV-1 to CD4⁺ T cells across a virological synapse-like structure.** Human MDM were differentiated on glass coverslips for seven days prior to infection with 6 ng CA-p24 of the HIV-1<sub>Bal</sub> isolate for a further 7 days. MDM were subsequently co-cultured with 5×10⁵ PHA/IL-2-activated autologous CD4⁺ T cells that were pre-stained for CD4. Unattached T cells were carefully removed by aspiration at various times after co-culture and incubated on poly-L-lysine-treated coverslips for 1 hour followed by fixation in 4% PFA. MDM were gently washed with warm RPMI, and were fixed together with remaining attached T cells. Samples were subsequently permeabilized and stained for Gag and Env, and analyzed by LSMC. (A) HIV-1-infected MDM in the absence of T cells. We used clone C49 to stain Gag-p17 which recognizes p17 cleaved from p55, representing mature virions. The right hand panel is a magnified image of the boxed region from the merged image. A white line is drawn to indicate the MDM cell membrane in A and B. (B and C) MDM-T cell co-cultures stained for CD4, Gag and Env (D) Detached CD4⁺ T cells stained for HIV-1 Gag and CD4. White scale bars in A-D: 10 µm. (E) EM analysis of the contact zone between HIV-1-infected MDM and CD4⁺ T cells. Cells were co-cultured for 5 hours in the presence of trace amounts of PHA (0.06 µg/ml) to stabilize clustered cells for sample preparation. M represents MDM; T represents CD4⁺ T cell, * represents virus-containing vacuolar compartment; V represents HIV-1 particles; arrows point to closely-apposed plasma membranes of MDM and T cells. Note that many of the cell and viral membranes appear dense and strongly contrasted by the presence of ruthenium red label. Black scale bars: 1 µm.

**Figure 2: HIV-1 transmission to CD4⁺ T cells is receptor and fusion dependent, and results in productive infection.** (A) HIV-1-infected MDM were co-cultured with autologous CD4⁺ T cells for 1-12 hours, followed by harvesting of the T cells with cold 5 mM EDTA/PBS. Cells were subsequently stained for CD3 and intracellular CA-p24 and analyzed by FC to determine the percentage of HIV-1 positive T cells. Data shown represent means of three independent experiments and error bars represent SEM. (B)
HIV-1-infected MDM were co-cultured with CD4+ T cells that had been pre-incubated with several inhibitors (1 hr, 37°C), followed by harvesting of the T cells after 10 hours, and staining for FC as in A. Alternatively, T cells were not added to MDM directly, but were separated by a transwell (0.3 μm pore size, Costar) to prevent cell-cell contact. Data represent the mean of triplicates in a single experiment and error bars represent + 1 SD. *p<0.02; **p<0.03; ***p<0.0001; ****p<0.00001, ANOVA. (C) Detection of HIV-1 reverse transcription using qPCR. HIV-1-infected MDM were co-cultured with autologous CD4+ T cells for 0, 6, 12 and 24 hours, followed by gentle aspiration of the T cells with warm RPMI, lysis, and DNA isolation and purification. qPCR using HIV-1 pol primers was performed to measure de novo viral DNA synthesis. Data were normalized to human β-globin. Data represent the mean of triplicates in a single experiment and error bars represent + 1 SD and * p<0.02, ANOVA (D) Replication of HIV-1 after transmission. CD4+ T cells were collected from 12 hour co-cultures with HIV-1-infected MDM, depleted of all MDM with CD14 beads (Milteny Biotec), and cultured for an additional week (10^5/well/250 μl). Viral replication was detected by p24 released into the supernatant by ELISA. Data represent the mean of quadruplicates in a single experiment and error bars represent + 1 SD.
Figure 1, Groot et al
Figure 2, Groot et al
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