HIV-1 Uses Dynamic Podosomes for Entry into Macrophages

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ABSTRACT Macrophages are one of the major targets of human immunodeficiency virus 1 (HIV-1) and play crucial roles in viral dissemination and persistence during AIDS progression. Here, we reveal the dynamic podosome-mediated entry of HIV-1 into macrophages. Inhibition of podosomes prevented HIV-1 entry into macrophages, while stimulation of podosome formation promoted viral entry. Single-virus tracking revealed the temporal and spatial mechanism of the dynamic podosome-mediated viral entry process. The core and ring structures of podosomes played complex roles in viral entry. The HIV coreceptor CCR5 was recruited to form specific clusters at the podosome ring, where it participated in viral entry. The podosome facilitated HIV-1 entry with a rotation mode triggered by dynamic actin. Our discovery of this novel HIV-1 entry route into macrophages, mediated by podosomes critical for cell migration and tissue infiltration, provides a new view of HIV infection and pathogenesis, which may assist in the development of new antiviral strategies.

IMPORTANCE Macrophages are motile leukocytes and play critical roles in HIV-1 infection and AIDS progression. Podosomes, as small dynamic adhesion microdomains driven by the dynamic actin cytoskeleton, are mainly involved in cell migration of macrophages. Herein, we found that HIV-1 uses dynamic podosomes to facilitate its entry into macrophages. Single-virus imaging coupled with drug assays revealed the mechanism underlying the podosome-mediated route of HIV-1 entry into macrophages, including the dynamic relationship between the viral particles and the podosome core and ring structures, the CCR5 coreceptor. The dynamic podosome-mediated entry of HIV-1 into macrophages will be very significant for HIV-1 pathogenesis, especially for viral dissemination via macrophage migration and tissue infiltration. Thus, we report a novel HIV-1 entry route into macrophages mediated by podosomes, which extends our understanding of HIV infection and pathogenesis.

KEYWORDS endocytosis, HIV-1, macrophages, podosome, virus entry

AIDS, caused by human immunodeficiency virus 1 (HIV-1), is currently an incurable disease (1, 2). Macrophages are one of the major targets of HIV-1 and play crucial roles in the pathogenesis of AIDS (3, 4). During AIDS progression, macrophages contribute greatly to viral persistence (5), dissemination (6), and transmission (7). Therefore, to develop new antiviral strategies, it is very important to understand the interactions between HIV and macrophages in detail. However, several critical issues, such as the mechanism of HIV-1 entry into macrophages, remain to be clarified.

Several kinds of distinct, specialized structures protrude from the plasma membranes of primary macrophages, including filopodia, ruffles, podosomes, and blebs (8). These surface specializations, formed by the induction of dynamic actin and microfilaments, contribute to the phagocytosis, component exchange, and migration of

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macrophages (9). Some viruses utilize these specialized structures to facilitate their infection (10). It has been reported that herpes simplex virus 1 virions associate with the filopodia on the plasma membrane, followed by their actin-dependent uptake (11). Vaccinia virus particles associate with and move along the filopodia to the cell body, where they are internalized after inducing the extrusion of large transient membrane blebs (12). Human adenovirus serotype 3 uses membrane ruffles and macropinosomes for its infectious entry (13).

It has also been reported that specialized surface structures participate in HIV-1 infection (10, 14). Sowinski et al. demonstrated that membrane nanotubes provide a novel route for HIV-1 transmission (15). Membrane ruffles play an important role in HIV-1 entry into macrophages (16). HIV-1 also promotes the formation of some specialized structures, such as filopodia, in virus-infected lymphocytes (17). In HIV-infected macrophages, HIV-1 alters the podosomes and reprograms the migration of macrophages using the Nef protein, which is critical for viral dissemination and pathogenesis (18, 19).

Here, we found that HIV-1 uses dynamic podosomes to facilitate its entry into human monocyte-derived macrophages (hMDMs). Podosomes, parts of the actin cytoskeleton, are small dynamic punctate adhesion microdomains located at the plasma membrane in hMDMs and are mainly involved in cell migration and matrix degradation (20–22). In this study, we used fluorescent imaging and drug inhibition assays to demonstrate that podosomes participate in HIV-1 entry into hMDMs. Podosome stimulation also greatly promoted viral entry. Single-virus tracking revealed the dynamic HIV-1 entry process mediated by podosomes, including the dynamic relationship between the viral particles and the podosome core and ring structures, the CCR5 coreceptor, etc. We also demonstrate that podosomes facilitate HIV-1 entry by using a rotation mode, triggered by dynamic actin. Thus, we report a novel HIV-1 entry route into hMDMs mediated by podosomes, which extends our understanding of HIV infection, dissemination, and pathogenesis.

RESULTS

The podosome is involved in HIV-1 entry into hMDMs. HIV-1 particles were labeled with Vpr-mCherry. The incorporation efficiency and the effect of HIV-1-mCherry virus infectivity are shown in Fig. 1. The majority of mCherry fluorescence (approximately 95%) was colocalized with anti-p24 conjugated with fluorescein isothiocyanate (FITC) (Fig. 1A), which demonstrated that mCherry was successfully incorporated into HIV-1 virions. The infectivity of HIV-1-mCherry was quantified by infecting TZM-bl cells and hMDMs. We found that HIV-1-mCherry has similar infectivity to wild-type HIV-1 virus in TZM-bl cells (Fig. 1B) and hMDMs (Fig. 1C). During the imaging of HIV-1 infection of hMDMs, we observed that the HIV-1 particles were associated with F-actin-rich podosomes during viral entry. As shown in Fig. 2A, when we labeled the F-actin in the hMDMs with LifeAct-EGFP (enhanced green fluorescent protein), small actin-enriched punctate microdomains, podosomes, were visible on the basal face of the cell plasma membrane. The podosomes were confirmed by labeling with the cortactin-iRFP (near-infrared red fluorescent protein) and Wiskott-Aldrich syndrome protein (WASP), which are specific podosome markers. In addition, the actin-enriched podosomes colocalized exactly with cortactin and WASP (Fig. 2B and C). The punctate podosomes labeled with cortactin-iRFP also colocalized with the HIV particles (Fig. 2D).

To investigate the effects of podosomes on viral entry, the genetic knockdown in hMDMs for regulation of podosome function was monitored. Here, we use small interfering RNA (siRNA [which has been used successfully with high efficiency in hMDMs]) to target formin-like 1 (FMNL1 [which is associated with macrophage podosomes]) and vinculin (a podosome ring-associated protein) to perform podosome-mediated viral entry (23). A correlating pattern of decreased FMNL1 and vinculin protein expression was observed (Fig. 3A) following incubation of cells with 200 nM siRNA oligonucleotides for about 72 h to target FMNL1 or vinculin. We further examined viral entry into

FIG 1 Characterization of HIV-1-mCherry. (A, left) Fluorescent colocalization image of HIV-1-mCherry with immunostained anti-p24-FITC (scale bar, 1 μ m). (Right) Colocalization efficiency analysis of the HIV-1-mCherry signal with anti-p24 or DiO signal. The majority of mCherry fluorescence (approximately 95%) was colocalized with anti-p24-FITC. (B) Virus infectivity assay of HIV-1-mCherry in TZM-bl cells with a luciferase reporter system. For this experiment, the mean values and SD are shown. *P* values were determined by an unpaired *t* test. NS, not significant. (C) Virus infectivity of HIV-1-mCherry in hMDMs for the intracellular p24 levels was measured by p24 assay. The infectivity of HIV-1-mCherry were similar to that of wild-type HIV-1. For this experiment, values are means $\pm$ SD (*n*=3 donors). A one-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used for statistical analysis. NS, not significant.

hMDMs following genetic knockdown by detecting intracellular p24 after the cells were incubated with HIV-1_{AD8} particles. The results showed that viral entry was significantly decreased (Fig. 3B) following FMNL1 and vinculin knockdown. Viral replication was then quantified by analyzing the supernatant p24 levels, to measure the virus released, at 1 to 7 days after infection. The results showed that the release of the virus after the replication of HIV-1_{AD8} was also reduced at 5 to 7 days following FMNL1 or vinculin knockdown (Fig. 3C). Furthermore, hMDMs were pretreated with the drug SU6656 or LY294002 (no cytotoxicity [Fig. 4]) 1 h before viral infection. SU6656 is a broad inhibitor of SRC kinases and inhibits the formation of podosomes (24). LY294002 is a phosphatidylinositol 3-kinase (PI3 kinase) inhibitor and induces the loss of podosomes (25). In the drug-treated hMDMs, the numbers of dynamic podosomes were significantly reduced (Fig. 3D) and their life span time was increased compared with those of untreated hMDMs (Fig. 3E). We also examined viral entry into hMDMs pretreated with SU6656 or LY294002 by detecting intracellular p24 after the cells were incubated with HIV-1_{AD8} particles. The results showed that viral entry was significantly inhibited by SU6656 or LY294002 (Fig. 3F). Viral replication was then quantified by analyzing the supernatant p24 levels at 1 to 7 days after infection. The results showed that the release of the virus after the replication of HIV-1_{AD8} was also significantly reduced by these inhibitors at 5 to 7 days postinfection (Fig. 3G). All of these observations suggest that cellular podosomes participate in HIV-1 entry into hMDMs.

Dynamic podosome formation promotes HIV-1 entry into hMDMs. We then used a scratch assay to investigate the effect of podosome stimulation on HIV infection. A scratch was made on a confluent monolayer of hMDMs in a cell culture dish

FIG 2 Colocalization imaging of a podosome with HIV-1 in an hMDM. (A) Colocalization imaging of a LifeAct-EGFP-labeled podosome with HIV-1-mCherry in an hMDM (scale bar, 10 μ m). (Right) Enlarged image of white rectangular regions in points 1 and 2. (B) Fluorescent colocalization image of the podosome core (LifeAct-EGFP-labeled F-actin) with cortactin-iRFP in an hMDM (scale bar, 10 μ m). (Rectangular box) Enlarged image of the podosome and cortactin-iRFP in the rectangular region. (Right) Fluorescence intensity profiles of F-actin-enriched podosome core and cortactin-iRFP. (C) Fluorescent colocalization image of the podosome core (phalloidin-Alexa Fluor 488-labeled F-actin) with WASP-Alexa 647 in an hMDM (scale bar, 10 μ m). The rectangular box contains an enlarged image of the podosome and WASP-Alexa Fluor 647 in the rectangular region. (Right) Fluorescent intensity profiles of F-actin-enriched podosome core and WASP-Alexa Fluor 647. (D) Colocalization imaging of a LifeAct-EGFP-labeled podosome and cortactin-iRFP with HIV-1-mCherry in an hMDM (scale bar, 10 μ m). (Right) Enlarged image of white rectangular regions in point 3.

because wounding induces cellular migration and stimulates podosome formation (26, 27). As shown in Fig. 5A, the hMDMs were stimulated to migrate into the region of the scraped space (Fig. 5A; region r1). Cellular polarization and dynamic podosome formation at the edges of the wound facilitated this migration (Fig. 5B and C). We analyzed dynamic podosome formation and virus associated with cell in three regions, r1, r2, and r3, corresponding to at, near, and far from the scratch, respectively. Close to the scratch, podosome formation was more nonuniform, with a polarized distribution pattern, and virus associated with cell also increased, corresponding to podosome

FIG 3 The podosome is involved in HIV-1 entry into hMDMs. (A) FMNL1 and vinculin expression were investigated by immunoblotting after hMDMs were treated separately with FMNL1 and vinculin siRNA-targeting oligonucleotides for 72 h. The anti- β -actin antibody was used as a loading control. (B) The intracellular p24 levels were quantified to test viral entry 3 h after infection with HIV-1_{AD8} (100 ng p24) in FMNL1 or vinculin knockdown cells. (C) Viral release (detected as the p24 levels in the supernatant) was quantified to test viral replication 1 to 7 days after infection with HIV-1_{AD8} (100 ng p24) in FMNL1 or vinculin knockdown cells. (D) Statistical analysis of the number of dynamic podosomes per hMDM with or without treatments with the podosome-related inhibitors LY294002 and SU6656 for 1 h. (E) Effect of podosome-related inhibitors LY294002 and SU6656 on podosomal life span. (F) After the cells were pretreated with LY294002 and SU6656 for 1 h, the intracellular p24 levels were quantified to test viral entry 3 h after infection with HIV-1_{AD8} (100 ng p24). (G) After the cells were pretreated with LY294002 and SU6656, viral release (detected as the p24 levels in the supernatant) was quantified to test viral replication 1 to 7 days after infection with HIV-1_{AD8} (100 ng p24). Values are means $\pm$ SD ($n=3$ donors). A one-way analysis of variance (ANOVA) with Tukey's *post hoc* test was used for statistical analysis.

stimulation (Fig. 5D to F). There were more viral particles (Fig. 5G) in the cells in region r1 than in region r2 or r3, corresponding to the dramatic migration and dynamic podosome formation in the cells of region r1 (Fig. 5H). These results suggest that the stimulation of dynamic podosomes might promote HIV-1 entry (see Movie S1 in the supplemental material).

We also used several angiogenic factors, such as transforming growth factor β (TGF- β) (28), vascular endothelial growth factor (VEGF) (29), and platelet-derived growth factor (PDGF) (30), to stimulate podosome formation. The numbers of dynamic podosomes increased (Fig. 5I) in the hMDMs after treatment with TGF- β , VEGF, or PDGF. Therefore, the internalized viruses increased significantly in hMDMs treated with TGF- β , VEGF, or PDGF (Fig. 5J). The viral release from hMDMs treated with these angiogenic factors after HIV-1_{AD8} replication also increased significantly (Fig. 5K) at 5 to 7 days postinfection. These results suggest that the formation of dynamic cellular podosomes promotes the HIV-1 infection of hMDMs.

Single-virus tracking of dynamic podosome-mediated HIV-1 entry into hMDMs.

The dynamic viral entry process mediated by podosomes was tracked in real time at the single-particle level. As shown in Fig. 6, the typical dynamic entry of single HIV-1-mCherry particles via podosomes enriched with LifeAct-EGFP-labeled F-actin was tracked in a live hMDM. The viral particle moved toward the podosome, attached to the podosome, and then entered the cell (Fig. 6A and B; see Movie S2 in the supplemental material). A kymograph analysis and fluorescence intensity and velocity profiles were used to quantitatively demonstrate and characterize this dynamic process (Fig.

FIG 4 Cytotoxicity by treatment with different inhibitors in hMDMs. Cells were treated with different inhibitors (10 μ M SU6656, 10 μ M LY294002, 5 μ M maraviroc, 10 μ g/ml T20, 2 μ g/ml Cyto D, or 2 μ M Lat A) for 1 h, cell toxicity was analyzed by LIVE/DEAD viability/cytotoxicity kit (live cells, green; dead cells, red). The cytotoxicities of these inhibitors were compared with those in cells in the positive cytotoxic group, which were pretreated with 0.01 μ M H_2O_2 for 1 h. The fluorescent images were monitored with the UltraView VoX spinning-disk confocal system (PerkinElmer) using a 10 $\times$, 0.3-NA air-immersion lens objective. Scale bar, 200 μ m.

6C to E). The viral profiles for velocity and fluorescence intensity showed that the instantaneous velocity increased rapidly (Fig. 6D; green rectangular region) as the HIV-1-mCherry fluorescent signal decreased (Fig. 6E; green rectangular region), suggesting that the virus entered the hMDM in an active movement pattern. Real-time three-dimensional (3D) imaging also revealed the dynamic features of the virus entry involving the podosome (Fig. 7A and B; see Movie S3 in the supplemental material).

This podosome-mediated mode of entry was also demonstrated with total internal reflection fluorescence microscopy (TIR-FM) imaging. TIR-FM imaging can preclude any possible disturbance by intracellular viral particles because it selectively excites the fluorophores in the cellular environment very close to the solid surface of the cell without exciting fluorescence in regions far from the surface (31). The signal detected was only within about 100 to 200 nm from the cell surface, which exactly matches the height of hMDM podosome protrusions. With TIR-FM imaging, we also observed that single fluorescent viral particles attached to the podosomes and entered the cells, whereupon the viral fluorescent signals rapidly disappeared (Fig. 7C and D; see Movie S4 in the supplemental material). A total of 620 events of viral entry mediated by podosomes were observed in TIR-FM imaging.

The dynamic viral entry process was also studied after the hMDMs were treated with the podosome inhibitor SU6656 or LY294002. In the drug-treated cells, we did not observe the typical dynamic motion of viral entry that we had observed in untreated cells. Compared with the typical dynamic motion of viral entry, the viral particles still moved toward the cellular podosome after treatment with the podosome inhibitor SU6656 or LY294002, but the virus only attached to the podosome. The instantaneous velocity of the virus did not increase rapidly, which normally precedes its entry into the cell, after treatment with SU6656 or LY294002 (see Movie S5 in the supplemental material). Figure 6F and G show the imaging results for cells treated with SU6656. Kymograph analysis and fluorescence intensity and velocity profiles quantitatively demonstrated and characterized this dynamic process (Fig. 6H to J). The velocity (Fig. 6I) and fluorescence intensity profiles (Fig. 6J) of the inhibitor-treated hMDMs showed that there was no change in their instantaneous velocity and that the HIV-1-mCherry

FIG 5 Dynamic podosome promotes HIV-1 entry into hMDMs. (A) hMDMs were stimulated to migrate into the scraped space in a monolayer cell culture (scale bar, 100 μ m). (B) Cellular polarization and dynamic podosome formation at the leading edge of the scraped area was driven by dramatic actin polymerization and depolymerization. (C) Images 1, 2, and 3 are amplifications of the red rectangular regions in panel B. (D to F) Migration of hMDMs induced by cellularly dynamic podosomes promoting HIV-1 entry (scale bar, 10 μ m). Arrows indicate the moving cells with HIV-1 particles at the edge of scraped space. Cell nuclei were stained with Hoechst 33324. (G) Statistical analysis of the mean value from viruses that associated with the cells in regions r1, r2, and r3 in panel A. (H) Statistical analysis of the mean number of dynamic podosomes in regions r1, r2, and r3 in panel A. (I) Statistical analysis of the number

(Continued on next page)

fluorescent signal remained constant, suggesting that the virus did not enter the cells after treatment with SU6656.

Ring structure plays an important role in podosome-mediated viral entry. HIV entry is associated with podosomes. However, although the viral particles were usually observed to be attached to F-actin-constituted podosomes, they did not completely colocalize with them (Fig. 8A and B). Podosomes consist of two major structures: a central “core,” consisting of a tightly packed actin bundle running perpendicular to the plasma membrane, and a membrane-bound “ring” subdomain, which is enriched in integrins and various signaling and adaptor proteins (32). In our experiment, LifeAct-EGFP-labeled F-actin puncta represented the podosome core. Figure 8B shows an amplified 3D image of one HIV-1 particle attached to the podosome core. It is clear that the red fluorescent viral signal is located on the edge of the podosome core (green). The distance between the virus and the center of the podosome core was calculated statistically. A diagram of the distance distribution (Fig. 8C) showed that the average distance was about 0.6 to 0.8 μm . Therefore, we speculated that the virus colocalizes with the podosome ring for viral entry.

Therefore, LifeAct-EGFP and α -actinin-iRFP were used to label the podosome core and ring, respectively, in hMDMs. As shown in Fig. 8D and E, fluorescent images clearly showed the podosome structure, consisting of the actin-rich core (green) and the ring (purple). The distance between the podosome core and the ring was calculated by plotting the fluorescence intensity and was about 0.8 μm (Fig. 8F), which is consistent with the maximum distribution of the distance between HIV-1-mCherry and the podosome core (Fig. 8C). When HIV-1-mCherry particles were added to hMDMs with dually labeled podosomes, the virus localized at the podosome ring during its entry into the hMDMs. Figure 8G and H show a viral particle (red) typically colocalized with the podosome ring structure (purple). The dynamic motion involved in the entry of the virus into cells at the site of the podosome ring structure was tracked with time-lapse imaging and analyzed with kymography (Fig. 8J and K; see Movie S6 in the supplemental material). The fluorescence intensity profiles showed that the HIV-1-mCherry signal decreased (Fig. 8I, bottom left) after an increase in its instantaneous velocity (Fig. 8I, bottom right). This indicates that active movement occurs during viral entry, which is consistent with the observations of the viral entry process described above in Fig. 6.

CCR5 coreceptor is recruited and clusters at the podosome ring during viral entry. HIV-1 entry requires the recruitment and clustering of a receptor and coreceptor (14, 33). The dynamic rearrangement of actin may facilitate this process. Therefore, we investigated whether the podosome is associated with receptor and coreceptor recruitment during viral entry. Here, we mainly visualized the relationship between the podosome and CCR5 coreceptor and CD4 receptor after infection with HIV-1 in hMDMs. CD4 and CCR5 were immunostained with an Alexa Fluor 647-labeled antibody. CCR5 molecules were aggregated and attached to the podosome when the hMDMs were incubated with HIV-1_{AD8} (Fig. 9A, middle), differing from the randomly dispersed distribution of CCR5 molecules in virus-uninfected hMDMs (Fig. 9A, upper). CD4 receptors also aggregated and attached to the podosome when the hMDMs were incubated with HIV-1_{AD8} (Fig. 10B and C). The time courses of CD4 (Fig. 10A) and CCR5 (Fig. 11A) localization imaging show that CD4 and CCR5 may also be localized or recruited around podosomes induced by HIV-1_{AD8} at 2 to 3 h. HIV-1-mCherry colocalized with the CCR5 clusters and was attached to the podosomes (Fig. 9D). More interestingly, there were often two fluorescent spots of CCR5 clusters distributed on either side of the podosome core (Fig. 9B, amplified in Fig. 9A, as shown by circled spots and

FIG 5 Legend (Continued)

of dynamic podosomes per cell with or without TGF- β , VEGF, or PDGF treatments for 1 h. (J) Intracellular p24 levels were quantified at 3 h of infection with HIV-1_{AD8} (100 ng p24) after the cells were treated with TGF- β , VEGF, or PDGF for 1 h to test viral entry. (K) Cells were stimulated with TGF- β , VEGF, or PDGF for 1 h. Then viral release (detected as p24 levels in the supernatant) was quantified at 1 to 7 days by infection with HIV-1_{AD8} (100 ng p24) to test viral replication. Values are means $\pm$ SD ($n=3$ donors). A one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

FIG 6 Single-particle tracking of dynamic podosome-mediated HIV-1 entry into hMDMs. (A) Colocalization of F-actin-rich podosome with an HIV-1-mCherry particle. Scale bar, 10 μ m. (B) Sequential images of individual HIV-1 particle entry events involving podosomes. The HIV-1-mCherry particle moved toward the margin of the podosome core. (C) Kymograph analysis for the entry of a viral particle into an hMDM mediated by a podosome (scale bar, 100 s). (D and E) Velocity and fluorescence intensity profiles of viral entry (fluorescent punctum 1 and another five puncta, 2 to 6) into an hMDM mediated by a podosome. (F) Colocalization of an F-actin-rich podosome with an HIV-1-mCherry particle in an hMDM pretreated with SU6656 (scale bar, 10 μ m). (G) Sequential images show an individual HIV-1 particle attached to a podosome, but after treatment with SU6656 for 1 h, the instantaneous velocity of virus did not increase rapidly to allow viral entry into the cell. (H) Kymograph analysis shows a viral particle approaching to the podosome without entering the hMDM (scale bar, 50 s). (I to J) Velocity and fluorescence intensity profiles (six fluorescent puncta 1' to 6') showed that there was no rapid increase in the instantaneous velocity of the virus, and that the HIV-1-mCherry fluorescent signal remained constant, suggesting that the virus did not enter the cells that were pretreated with SU6656.

arrowheads; see Movie S7 in the supplemental material). The distance between the CCR5 cluster and the podosome core was about 0.8 μ m (Fig. 9C), which is exactly the distance between the podosome core and the podosome ring, as shown in Fig. 8E and F. Statistical analysis of the clustering effect of the CCR5 also showed that predominantly one to two CCR5 clusters are distributed around each podosome, which were triggered in response to HIV infection (Fig. 12A, middle). As a control, we also investigated the relationship between CXCR4 and the podosome. Unlike CCR5, the CXCR4 molecules did not form clusters when hMDMs were incubated with HIV-1_{AD8} (Fig. 13).

FIG 7 Real-time three-dimensional (3D) and TIR-FM imaging of HIV-1 entry into an hMDM via a dynamic podosome. (A) Colocalization image of the cellular podosome with HIV-1-mCherry particles. The right panels show enlarged images for the rectangular regions in panel A, including a 3D top view and 3D side view. (B) Sequential 3D (top view and side view) images of observation of one typical virus particle (rectangular regions in panel A) entering cells involving dynamic podosome. (C, top) Colocalization image of a LifeAct-EGFP-labeled podosome with HIV-1-mCherry in a macrophage by TIR-FM imaging (scale bar, 10 μ m). (Bottom) Sequential images of one typical HIV-1-mCherry (rectangular region) entering a macrophage via dynamic podosome. (D) Fluorescent intensity profiles show that HIV-1 entry according to viral fluorescent signals (HIV-1-mCherry) rapidly decreases.

We also used a CCR5 inhibitor, maraviroc, to treat the hMDMs. The inhibition of HIV infection by maraviroc was found to be dose dependent (Fig. 12B). In addition, when viral particles were added to hMDMs pretreated with 5 μ M maraviroc (no cytotoxicity [Fig. 4]), CCR5 no longer aggregated in clusters (Fig. 9A, bottom). The spots of colocalized viruses and podosomes were also significantly reduced (Fig. 9E, left). During time-lapse imaging, we found that the viral particle only wandered around the cellular podosome but did not attach to it after treatment with maraviroc (Fig. 9E, right). Thus, viral entry and the release of progeny virus were also significantly inhibited in the maraviroc-treated hMDMs (Fig. 12C and D).

We used HIV-1_{YU2r}, another macrophage-tropic virus, or HIV-1_{NL-43r}, a T-tropic viral strain, to infect hMDMs. HIV-1_{YU2} also induced CCR5 clustering around podosomes at 2 to 3 h (Fig. 11B). While HIV-1_{NL-43} does not use CCR5 as a coreceptor, when these viral particles were added to hMDMs, CCR5 did not aggregate into clusters (Fig. 14A and B), and the entry of HIV-1_{NL-43} into the hMDMs was less efficient than that of HIV-1_{AD8} (Fig. 14C). We also found that the CCR5 clustering still occurred (Fig. 11C), following HIV-

FIG 8 The podosome ring plays an important role in podosome-mediated viral entry into hMDMs. (A and B) Two- and three-dimensional images of HIV-1-mCherry (red) attached to the podosome core (green) in hMDMs. (C) Diagram of the distribution of the distances between the virus and the podosome core. (D) Fluorescent image of the podosome core (green) and podosome ring structure (purple) in hMDMs (scale bar, 10 μ m). (E) Amplified image of the podosome core and podosome ring structure in the region of interest in panel D (scale bar, 1 μ m). (F) Graph of the distance between the core and ring structure in an intact podosome. (G) Colocalization analysis of the podosome core and podosome ring with HIV-1-mCherry (scale bar, 10 μ m) in hMDMs. (H) Amplified image of the virus (red) in the white rectangular region of panel G (scale bar, 1 μ m). (I) Fluorescence intensity profiles of the podosome core (F-actin) and podosome ring (α -actinin-iRFP) are consistent with viral entry with an increase in the instantaneous velocity of the virus. (J and K) Kymograph analysis of the dynamic entry of this viral particle into an hMDM at the site of the podosome ring (scale bar, 100 s).

1_{AD8} infection of hMDMs treated with T20 (no cytotoxicity [Fig. 4]). The result suggested that inhibition of fusion did not affect CCR5 clustering.

The podosome facilitates HIV-1 entry via a rotation mechanism. After finding that CCR5 clusters are distributed on the sides of the podosome cores, we investigated how the podosome dynamically mediates HIV-1 entry. Therefore, we tracked in detail the real-time movement of HIV-1-mCherry particles attached to podosomes. Interestingly, we found that the podosome pushes HIV-1-mCherry entry via a rotation mode (see Movie S8 in the supplemental). As shown in Fig. 15A and B, sequential images show that as the viral particle is internalized into the hMDM, it rotates around the podosome core. The instantaneous velocity of the virus increases rapidly, in parallel with the loss of the HIV-1-mCherry fluorescent signal (Fig. 15C; green rectangular region), indicating that the virus enters the cell with an active movement pattern. This typical entry mode was observed and analyzed

FIG 9 CCR5 coreceptors are recruited to cluster on the podosome to facilitate HIV-1 entry into hMDMs. (A) Colocalization analysis of the podosome and the CCR5 coreceptor (the circles are typical CCR5/podosome clusters) in untreated hMDMs or in maraviroc-treated hMDMs after preincubation with HIV-1_{AD8}. (B) The enlarged region of interest for aggregated CCR5 molecules (arrows in panel A) around the podosome core after the cells were infected with HIV-1_{AD8}. (C) Profile diagram of the distances between the core and two clusters of CCR5 molecules in a typical podosome. (D) Fluorescence image of the podosome core, CCR5 coreceptor molecules, and HIV-1-mCherry (scale bar, 10 μ m). Arrows indicate representative colocalization images of the cellular podosome, CCR5 coreceptor molecules, and HIV-1-mCherry. To the right are enlarged images of white rectangular regions in points 1 and 2. (E) Colocalization images of the cellular podosome and HIV-1-mCherry in cells with or without maraviroc treatment (scale bar, 10 μ m). Sequential images in the amplified white rectangles (point 3) show that the viral particle just wandered around the cellular podosome but did not attach to it in the cell treating with maraviroc. At point 4, the viral particle associated with the podosome and entered the cell without maraviroc treatment.

in more than 100 viral particles. A statistical analysis to quantify the frequency of this rotation between 0 and 90°, 90 and 180°, 180 and 270°, and 270 and 360° rotation in 100 observed cases is shown in Fig. 15G. It was also shown that 300 ± 38 s is required for one rotation to push a viral particle into a hMDM. Because the podosome core is composed of F-actin, we also used the dynamic actin inhibitors latrunculin A (Lat A) and cytochalasin D (Cyto D) (no cytotoxicity [Fig. 4]) to treat the hMDMs. In the Lat A-treated cells, the podosome-mediated viral rotation entry process was not observed (Fig. 15D to F). When we

FIG 10 CD4 receptors are recruited to cluster on the podosome to facilitate HIV-1 entry into hMDMs. (A) CD4 receptor localization on hMDMs with respect to podosomes induced with HIV-1_{AD8} (100 ng p24) were imaged by immunofluorescence at 0, 1, 2, and 3 h postinfection. (B) Colocalization image of the podosome and the CD4 receptor (the circles are typical CD4/podosome clusters) in hMDMs after preincubation with HIV-1_{AD8} at 3 h. (C) The enlarged regions of interest for aggregated CD4 molecules (arrows in panel B) around the podosome core after the cells were infected with HIV-1_{AD8}. Cell nuclei were stained with Hoechst 33324 (scale bar, 10 μm).

measured intracellular p24, we found that viral entry was significantly inhibited in these drug-treated cells (Fig. 15H), and viral release was also significantly reduced by these inhibitors after 5 to 7 days (Fig. 15I).

DISCUSSION

Macrophages are motile leukocytes and are thought to play a critical role in the dissemination of HIV throughout the host (34, 35). Here, we have shown that the podosome, a kind of protrusive structure involved in the mesenchymal motility of hMDMs, mediates HIV-1 entry into hMDMs. Our experiments showed that through directly silencing podosome components, podosome inhibitors and actin inhibitors significantly affected HIV entry into hMDMs. Podosome stimulation in a scratch assay or by angiogenic factors promoted viral uptake.

The dynamic mechanism underlying this new podosome-mediated route of HIV-1 entry into hMDMs was observed with single-particle tracking. Podosomes consist of an F-actin core and a ring structure. During HIV entry, the virus dynamically colocalizes with the podosome ring and is transported into the hMDM by the dynamic podosome, which involves an active movement pattern. The chemokine receptor CCR5 is recruited and clusters at the podosome ring, where it facilitates HIV-1 entry via the podosome. Real-time imaging showed that the podosome drives HIV-1 entry via a rotation mechanism. It was reported that podosomes can undergo rotational movements (36). The

FIG 11 Time course of imaging of CCR5 coreceptor clustering induced by HIV-1_{AD8} or HIV-1_{YU2} in hMDMs. (A) CCR5 coreceptor localization on hMDMs with respect to podosomes induced with HIV-1_{AD8} (100 ng p24) were imaged by immunofluorescence at 0, 1, 2, and 3 h postinfection. (B) CCR5 coreceptor localization on hMDMs with respect to podosomes induced with HIV-1_{YU2} (100 ng p24) were imaged by immunofluorescence at 0, 1, 2, and 3 h postinfection. (C) CCR5 coreceptor localization on T20 treatment hMDMs with respect to podosomes induced with HIV-1_{AD8} (100 ng p24) were imaged by immunofluorescence at 0, 1, 2, and 3 h postinfection. Cell nuclei were stained with Hoechst 33324 (scale bar, 10 μ m).

podosome core may exert a pushing force toward the podosome ring, facilitating vortical viral entry by actin polymerization.

Previous reports have demonstrated that HIV enters hMDMs via an endocytic route and endosomal fusion (37). Here, we depict an HIV particle entering the cytoplasm through a podosome. As shown in Fig. 16, the virus particle is attracted to the ring of the podosome, and viral binding activates the CCR5 coreceptors to be recruited to cluster at the site of the podosome ring. Through a rotation mode triggered by the dynamic actin core, podosomes facilitate the ability of the HIV-1 particle to enter

FIG 12 Statistical CCR5 cluster distribution and the effect of maraviroc on HIV-1-mCherry infection in hMDMs. (A) Statistical counts of different numbers of CCR5 clusters distributed around each podosome with or without HIV infection of hMDMs and with maraviroc-treated hMDMs. One thousand podosomes were counted in different groups of hMDMs, respectively. (B) Concentration course of the effect of maraviroc on HIV-1_{AD8} (100 ng p24) entry into hMDMs. (C) Analysis of virus particles mediated by podosomes for entry into hMDMs or maraviroc-treated hMDMs. Virus particles were counted in 1,000 images of single cells. (D) Effect of maraviroc on HIV-1_{AD8} virion release. The cells were pretreated with or without maraviroc for 1 h. Then cells were infected with HIV-1_{AD8} (100 ng p24), and virion release (detected as p24 levels in the supernatant) was quantified for 1 to 7 days to test viral replication. Values are means $\pm$ SD ($n=3$ donors). A one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

hMDMs, be engulfed in an endosome, and then release the viral core for a productive infection.

Several specialized structures, including membrane nanotubes, ruffles, and filopodia, have been reported to facilitate HIV infection (10, 14). Here, dynamic podosomes were shown to provide a novel route for HIV-1 infection of macrophages. Considering the critical role of macrophages in HIV infection, the dynamic podosome-mediated entry of HIV-1 into macrophages is very significant for HIV-1 pathogenesis. Podosomes are mainly involved in macrophage mobility (24). The HIV Nef protein regulates podosome formation, and podosomes enhance the mesenchymal migration and tissue infiltration of macrophages (38). Macrophage migration and tissue infiltration contribute to viral dissemination in the blood vessels, lymph nodes, brain, and other tissues (39, 40). The macrophages present in tissues are also potential reservoirs for HIV-1 (41). Therefore, HIV-1 not only modifies the podosome, but also uses the podosome to enter macrophages, forming a positive feedback process of HIV infection. It seems that HIV has developed a clever podosome-based mechanism to facilitate viral infection and dissemination by modulating the host cell.

Podosomes facilitate viral entry because of their constituent molecules and functions. As a small dynamic adhesion microdomain driven by the dynamic actin cytoskeleton, podosomes may provide good binding and endocytic sites (37), with enriched receptors and energy for viral entry. Our results show that the podosome recruits CCR5 and CD4 molecules, which form clusters when the podosome is engaged in HIV-1

FIG 13 Imaging of the cellular podosome and cellular CXCR4 coreceptor. Colocalization imaging of the cellular podosome core and cellular CXCR4 coreceptor molecules in hMDMs with or without HIV-1_{AD8} (100 ng p24) treatment. Cell nuclei were stained with Hoechst 33324 (scale bar, 10 μ m).

entry. It was reported that CCR5 coreceptor is associated with the disarrangement of podosomes and adhesion complex molecules in osteoclast (42). In terms of the migration of macrophages in brain tissues, early reports (43) have shown that the CCR5 receptor is not merely a chemokine coreceptor, but regulates the avidity and redistribution of the adhesion molecules required for HIV entry into the central nervous system. This presumably amplifies the effects of chemokines on the transendothelial migration and recruitment of inflammatory cells into the central nervous system. Therefore, podosome-related viral infection may play a very complex and critical role in HIV-1 pathology. While CCR5 clustering induced by HIV is complex and might be triggered by HIV exposure or viral protein signaling. HIV-1 Nef protein could regulate podosome formation (18, 38). It was also demonstrated that Nef targets CCR5 and CD4 for endocytosis and degradation, which inhibits viral superinfection (44). Thus, the effect of HIV-1 protein (such as gp120 or Nef) on CCR5 clustering around podosome remains to be studied in the future.

In conclusion, we have identified a podosome-mediated entry route for HIV-1 into hMDMs and demonstrated the underlying dynamic process and mechanism in real time. This study extends our understanding of HIV infection and pathogenesis, which may assist the development of new antiviral strategies.

MATERIALS AND METHODS

Plasmids. The HIV-1_{AD8}, HIV-1_{YU2}, and HIV-1_{NL-43} are kindly provided by Yuntao Wu (George Mason University) (45). pAD8 or pNL-43 were used to assemble HIV-1_{AD8} or HIV-1_{NL-43} viral particles. pAD8 and pVpr-mCherry were used to assemble fluorescent HIV-1-mCherry viral particles. To construct pmCherry-C1-Vpr, the coding sequence of Vpr was amplified from the HIV-1 proviral plasmid pAD8 with PCR, using specific primers, and cloned into the pmCherry-C1 vector. The forward primer was 5'-GCTAAGCTTATGGAACAAGCCCCAGAAGAT-3' and the reverse primer was 5'-GTAGGATCCT-TACTAGGATCTACTGGCTCCATT-3'. The pLifeAct-EGFP, p- α -actinin-iRFP and p-cortactin-iRFP plasmids were used to label F-actin, α -actinin, and cortactin, respectively, to visualize the podosomal core and ring structure in hMDMs.

Cell culture and transfection. 293T cells and TZM-bl cells (obtained from the Animal Biosafety Level 3 facility of Wuhan University) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) in a 5% CO₂ environment at 37°C. Human monocytes were isolated from the peripheral blood of healthy donors (3 donors for each corresponding experiment) and differentiated as previously described (37). Briefly, the monocytes were isolated by centrifugation through Ficoll-Paque Plus (GE Healthcare) and resuspended in ice-cold phosphate-buffered saline (PBS). The monocytes (5×10^5) were then distributed on 35-mm glass bottom dishes in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), antibiotics, and 10 ng/ml human macrophage colony-stimulating

FIG 14 Analysis of different HIV-1 phenotypes of viral particles that induced clustering of CCR5 molecules and entered hMDMs. (A) Colocalization imaging of the cellular podosome and CCR5 coreceptor molecules in hMDMs incubated with HIV-1_{AD8} or HIV-1_{NL-43}. Cell nuclei were stained with Hoechst 33324 (scale bar, 10 μ m). (B) Quantification and statistical comparison of the colocalization/clustering of CCR5 and podosomes. (C) The number of HIV-1_{NL-43} viral particles that entered the macrophages was lower than the number of HIV-1_{AD8} particles in the macrophages after the macrophages were incubated with the virus for 2 h. The number of viral particles that entered macrophages was determined by statistical counting of cellular fluorescent points (HIV-1-mCherry). Values are means $\pm$ SD ($n=3$ donors). A one-way ANOVA with Tukey's *post hoc* test was used for statistical analysis.

factor (hM-CSF; Cell Signaling Technology) and incubated in a humidified 5% CO₂ atmosphere. hMDMs at days 6 to 8 of differentiation were used in the experiments. Some macrophage skeleton-related proteins, such as F-actin and α -actinin, corresponding to the podosome core and ring structure, respectively, were labeled with overexpressed fluorescent proteins to visualize viral entry. Briefly, the suspended hMDMs (1×10^5) were transfected by nucleofection (nucleofected) with 2.0 μ g of the pLifeAct-EGFP plasmid or pLifeAct-ECFP (enhanced cyan fluorescent protein) plasmid or 2.0 μ g of the p- α -actinin-iRFP plasmid DNA using an Amaxa human macrophage Nucleofector kit (Lonza Nucleofector 2b).

siRNA knockdown in hMDMs. hMDMs were transduced with small interfering RNA (siRNA) molecules targeting FMNL1 and vinculin (GenePharma) by nuclear transfection. The target sequences were as follows: FMNL1, 5'-AGGUCAUUGCUGAGAAGUATT-3'; vinculin, 5'-CUGGCUUGCAGAUCCAAAATT-3'; and the negative control, 5'-UUCUUCGACGUGACGUTT-3'. hMDMs were prepared as described above. The suspended hMDMs (1×10^5) were transduced with siRNA (200 nM) using an Amaxa human macrophage Nucleofector kit (Lonza Nucleofector 2b). hMDMs were cultured for additional 72 h and then harvested for Western blot analysis or p24 enzyme-linked immunosorbent assay (ELISA). For Western blot analysis, 50 μ g total cellular protein was loaded onto SDS-PAGE gels for electrophoresis. Separated proteins were transferred to a PVDF membrane (Millipore), blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) buffer, and probed with mouse monoclonal antibody against FMNL1 (Santa Cruz) and vinculin (Abcam). The anti- β -actin (Proteintech) was used as a loading control.

Virus preparation. To produce HIV-1_{AD8}, HIV-1-mCherry, HIV-1_{YU2}, and HIV-1_{NL-43}, 100-mm plates of virus-producing 293T cells were transfected with the appropriate plasmids using Lipofectamine 2000

FIG 15 The podosome facilitates HIV-1 entry via a rotation mode. (A) Colocalization of HIV-1-mCherry with cellular podosomes (scale bar, 10 μ m). (B) Sequential images of the entry of a typical HIV-1-mCherry viral particle into an hMDM after rotating around the podosome core (scale bar, 1 μ m). (C) Instantaneous velocity of the virus increased rapidly as the fluorescent signal of HIV-1-mCherry decreased (green rectangular region). (D) Imaging of HIV-1-mCherry on a cellular podosome in cells treated with Lat A (scale bar, 10 μ m). (E) Sequential images of a typical HIV-1-mCherry viral particle show that it does not undergo rotational entry into the hMDMs when the cells are treated with Lat A (scale bar, 1 μ m). (F) Velocity and intensity profiles showed that there was no rapid increase in viral velocity and no loss of the fluorescent HIV-1-mCherry signal after the cells were treated with Lat A. (G) Statistical distribution of degrees of rotation to quantify the frequency of this rotation occurrence in 100 observed cases. (H) Intracellular p24 levels were quantified after infection with HIV-1_{AD8} (100 ng p24) for 3 h, when the cells were treated with Cyto D or Lat A. (I) After the cells were treated with Cyto D or Lat A, viral release (measured as the p24 level in the supernatant) was quantified 1 to 7 days after infection with HIV-1_{AD8} (100 ng p24) to test viral replication. Values are means $\pm$ SD ($n=3$ donors). A one-way ANOVA with Tukey *post hoc* test was used for statistical analysis.

reagent. The plasmid transfections and virus experiments were performed in biosafety level 2 (BSL2) or biosafety level 3 (BSL3) laboratories. The HIV-1-mCherry virus was produced by cotransfecting 11.0 μ g of pAD8 and Vpr-mCherry plasmid DNA. Virus incorporation efficiency of Vpr-mCherry was identified with colocalization of HIV-mCherry with anti-p24-FITC immunostaining.

Viral infectivity assay. Viral infectivity was measured in TZM-bl cells (HeLa-derived indicator TZM-bl cells expressing CD4, CXCR4, and CCR5) with a luciferase reporter system, as previously described (37, 46). The HIV-1 and HIV-1-mCherry titers were measured with an endpoint dilution of the luciferase activity assay, measured 48 h after infection with the luciferase enzyme assay system with reporter lysis buffer (Beyotime), according to the manufacturer's instructions. The median tissue culture infective dose (TCID₅₀) was calculated by the Reed-Muench method. A cutoff value of 2.5 times the background relative luciferase units (RLU) was used to quantify the positive samples. Viral infectivity in hMDMs for the intracellular p24 levels was measured by p24 assay. The amount of p24 was quantified with an ELISA kit (Livzon Diagnostics Co.), according to the manufacturer's instructions.

In vitro podosome analysis and immunofluorescent imaging. hMDMs (1×10^5) were cultured on 35-mm glass bottom dishes with RPMI 1640 medium containing 10% heat-inactivated FCS and used to stimulate podosome formation with TGF- β , VEGF, or PDGF. To visualize the podosome cores in fixed macrophages, F-actin was labeled with Alexa Fluor 488-phalloidin (Invitrogen). To visualize the podosomes, macrophages were fixed with 4% paraformaldehyde and stained with phalloidin and an anti- α -actinin antibody or with overexpressed LifeAct-EGFP and α -actinin-iRFP. We also used macrophage

FIG 16 Schematic model for the dynamic podosome-mediated entry of HIV into hMDMs. HIV-1 is attracted to the ring of the podosome. Viral binding activates the CCR5 coreceptors to be recruited to cluster at the site of podosome ring. Through a rotation mode triggered by the dynamic actin core, podosomes facilitate HIV-1 entry into hMDMs and help to release the viral core via endosomal fusion.

migration into the scraping space to measure cell motility rapidly to investigate podosome formation upon HIV-1_{AD8} entry. Individual podosome-positive cells were characterized by the colocalization of the cortactin or WASP (monoclonal antibodies; Santa Cruz) with F-actin in a dot-like distribution. Importantly, all manual quantification was performed in a double-blind manner. Volocity quantitation (PerkinElmer) software of image recognition module was used to count podosomes and cellular fluorescent points (HIV-1-mCherry).

After the cells were infected with or without HIV-1_{AD8}, HIV-1_{YU2}, HIV-1_{NL-43} or HIV-1-mCherry or underwent treatment with T20 (10 μ g/ml), the cellular CCR5, CD4, and CXCR4 molecules were detected with an immunofluorescence technique. In brief, hMDMs were plated onto 35-mm glass bottom cell culture dishes. hMDMs were plated onto 35-mm glass bottom cell culture dishes and infected with or without HIV-1_{AD8}, HIV-1_{YU2}, HIV-1_{NL-43} or HIV-1-mCherry for 3 h. The cells were washed with PBS (pH 7.4), fixed with freshly prepared 4.0% paraformaldehyde at room temperature for 15 min, and permeabilized for 15 min with 0.1% Triton X-100. The cells were then washed with PBS, incubated in 10% FBS as a blocking agent to prevent nonspecific staining, and incubated with mouse monoclonal antibodies directed against CCR5 (R&D Systems), CXCR4 (Santa Cruz), and CD4 (Novus Biologicals), followed by an Alexa Fluor 647-labeled or rhodamine-labeled rabbit anti-mouse IgG secondary antibody (Cell Signaling Technology). The fluorescent images were monitored with the UltraView VoX spinning-disk confocal system (PerkinElmer) using a 60 $\times$, 1.4-NA oil-immersion lens objective or a 10 $\times$, 0.3-NA air-immersion lens objective. The images were captured with an EM-CCD (Hamamatsu C9100-14). The colocalization of the fluorescent signals for CCR5, CXCR4, and CD4 with those of the podosome core was analyzed with Volocity 6.3.0.

Time-lapse imaging and analysis. To image HIV-1 in live cells, 10^3 to $10^{3.5}$ TCID₅₀ of virus were used to infect 1×10^5 cells, unless otherwise specified. The viruses and cells were incubated in 35-mm glass bottom dishes for 30 min at 4°C to allow virus adsorption to the cells, and then moved to a cell culture chamber (Tokai Hit; 37°C, 5% CO₂) on a microscope. Image acquisition started when the temperature of the medium reached 37°C. We also performed control experiments validating that viruses were outside the cells at the start of imaging. Fluorescence was detected with an UltraVIEW VoX spinning-disk confocal system (PerkinElmer) using a Nikon Eclipse Ti-E microscope with a 60 $\times$, 1.4-NA oil-immersion lens objective. Some time-lapse experiments were performed with the UltraVIEW VoX TIR-FM system. To minimize any photobleaching of the fluorescent proteins, live cells were usually imaged at 2-, 4-, or 10-s intervals for 2 to 40 min. In some experiments, cellular three-dimensional slice images were captured at 0.2- μ m intervals on the z-axis by using piezoelectric ceramic motor. Quantification of podosome life span was measured manually using Volocity software for podosomes appearing and disappearing during the time course of the experiment (18).

Rhodamine and mCherry were excited with a 561-nm laser and detected with a 615-nm emission wheel. DiO, FITC, EGFP, and Alexa Fluor 488 were excited with a 488-nm laser and detected with a 525-nm emission wheel. ECFP was excited with a 440-nm laser and detected with a 485-nm emission wheel. Alexa Fluor 647 and iRFP were excited with a 640-nm laser and detected with a 705-nm emission wheel. Cell nuclei were stained with Hoechst 33324, which was excited with a 405-nm laser and detected with a 445-nm emission wheel. The images were captured with an EM-CCD (Hamamatsu C9100-14). The imaging data were analyzed with Volocity 6.3.0 and Fiji.

Virus p24 assays. To analyze the effects of different drugs (Cyto D, Lat A, SU6656, LY294002, TGF- β , VEGF, PDGF, and maraviroc) and siRNA target oligonucleotides (FMNL1 or vinculin) on HIV-1 entry into hMDMs, the virus was used to infect hMDMs (1×10^5) pretreated with the CCR5 inhibitor (0.1, 0.5, 1, or 5 μ M

maraviroc), podosome or actin inhibitors (10 μ M SU6656, 10 μ M LY294002, 2 μ g/ml Cyto D, or 2 μ M Lat A), or angiogenic factors (5 ng/ml TGF- β , 30 ng/ml VEGF, or 20 ng/ml PDGF). Then the cells were washed, and cell media were replaced with new fresh media. HIV-1_{AD8} (100 ng p24) was used to infect 1×10^5 target cells that had been pretreated with or without different drugs for 1 h or target oligonucleotides for 72 h. The cells were then treated with trypsin (Gibco) for 5 min at 37°C to remove uninternalized viruses and washed twice with PBS before lysis. The viral internalization at 3 h postinfection or virion release (measured as p24 in the supernatant) at 1 to 7 days was quantified with a p24 ELISA kit. The cells were washed, and the cell media were replaced with new fresh media at 3 h postinfection for virion release assay. The cytotoxicity by treatment of different inhibitors in hMDMs was determined using the LIVE/DEAD viability/cytotoxicity kit for mammalian cells according to the manufacturer's instructions. The cytotoxicities of these inhibitors were compared with that of cells in the positive cytotoxic group, which were pretreated with 0.01 μ M H₂O₂ for 1 h. The fluorescent images were monitored with the UltraView VoX spinning-disk confocal system (PerkinElmer) using a 10 $\times$, 0.3-NA air-immersion lens objective.

Statistical analysis. All data in the text and figures are expressed as means $\pm$ standard deviation (SD). The statistical analysis was performed with the Origin 8.0 software. Unpaired *t* tests were used for only two independent groups. The one-way analysis of variance (ANOVA) with Tukey's multiple-comparison test was performed to determine significant differences among multiple comparisons in different macrophage donors. A *P* value of less than 0.05 was considered a statistically significant difference.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 2, AVI file, 2.8 MB.

SUPPLEMENTAL FILE 3, AVI file, 0.3 MB.

SUPPLEMENTAL FILE 4, AVI file, 1.5 MB.

SUPPLEMENTAL FILE 5, AVI file, 0.2 MB.

SUPPLEMENTAL FILE 6, AVI file, 0.5 MB.

SUPPLEMENTAL FILE 7, AVI file, 1.4 MB.

SUPPLEMENTAL FILE 8, AVI file, 1.8 MB.

SUPPLEMENTAL FILE 9, AVI file, 0.4 MB.

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Z.C. conceived and designed the project. W.L., J.L., and Y.L. performed the experiments. W.Y., Q.L., K.W., and X.Z. contributed materials/reagents. W.L., Z.Z., and X.-E.Z. analyzed the data. W.L. and Z.C. wrote the paper.

We declare no competing financial interests.

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