Neuropilin-1, a myeloid cell-specific protein, is an inhibitor of HIV-1 infectivity

Shumei Wang a,b,1, Li Zhao a,b,1, Xiaowei Zhang a,b, Jingjing Zhang c, Hong Shang a,b,d, and Guoxin Liang a,b,c,d,2

aKey Laboratory of AIDS Immunology of Ministry of Health, Department of Laboratory Medicine, The First Affiliated Hospital, China Medical University, Shenyang 110122, China; bNational Clinical Research Center for Laboratory Medicine, The First Affiliated Hospital, China Medical University, Shenyang 110122, China; cResearch Institute for Cancer Therapy, The First Affiliated Hospital, China Medical University, Shenyang 110122, China; and dKey Laboratory of AIDS Immunology, Chinese Academy of Medical Sciences, Shenyang 110001, China

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Myeloid lineage cells such as macrophages and dendritic cells (DCs), targeted by HIV-1, are important vehicles for virus dissemination through the body and as viral reservoirs. Compared to activated lymphocytes, myeloid cells are collectively more resistant to HIV-1 infection. Here we report that NRP-1, encoding transmembrane protein neuropilin-1, is highly expressed in macrophages and DCs but not CD4+ T cells, serving as an anti-HIV factor to inhibit the infectivity of HIV-1 progeny virions. Silencing NRP-1 enhanced the transmission of HIV-1 in macrophages and DCs significantly and increased the infectivity of the virions produced by these cells. We further demonstrated that NRP-1 was packaged into the progeny virions to inhibit their ability to attach to target cells, thus reducing the infectivity of the virions. These data indicate that NRP-1 is a newly identified antiviral protein highly produced in both macrophages and DCs that inhibit HIV-1 infectivity; thus, NRP-1 may be a novel therapeutic strategy for the treatment of HIV-1 infection.

HIV-1 primarily targets two groups of cells in vivo, CD4+ T lymphocytes and myeloid lineage cells such as macrophages and dendritic cells (DCs) (1). Monocytes and granulocytes are collectively called myeloid cells, which originate from hematopoietic stem cells in the bone marrow and are continuously supplied to all tissues via the circulation (2–4). Monocytes mature into macrophages in various tissues, such as the Kupffer cells in the liver and alveolar macrophages in the lungs. Monocytes can also differentiate into DCs in lymphoid organs and Langerhans cells in the skin, functioning as professional antigen-presenting cells.

Primary circulating blood monocytes, along with differentiated macrophages and DCs, are critical in the immune responses to HIV-1 infection. Macrophages and DCs are important targets for HIV-1 replication in vivo; they both serve as vehicles for virus dissemination through the body and viral reservoirs (1, 5, 6). While myeloid cells can support the persistent replication of HIV-1, they do not support the high productivity of HIV-1, in contrast to infected T cells. This resistance varies according to the differentiation status of the myeloid cells, the presence or absence of stimulation, and the different steps in the viral life cycle. For example, a major impairment of HIV-1 occurs during the early phases of infection, such as the inhibition of HIV reverse transcription by SAMHD1 in DCs (7, 8). Nevertheless, the blockage of HIV-1 infection in the later infection phase in these cells remains elusive.

In this study, we investigated whether myeloid cells had factors that restricted HIV-1 infection in the late phases. We conducted experiments to identify and compare the cellular protein produced by macrophages and activated CD4+ T lymphocytes that were incorporated into virions. As a result, we uncovered a transmembrane protein, neuropilin-1 (NRP-1) (9–14), that was only present in the virions produced in macrophages but not CD4+ T cells. Here, we report that the gene encoding NRP-1, a newly identified antiviral factor, is highly expressed in macrophages and DCs. Also, NRP-1 is packaged into virions to restrict HIV-1 infectivity by blocking their attachment to target cells. These findings suggest that NRP-1 can be a novel anti-HIV therapeutic target.

Results

NRP-1 Is Found inside Viral Particles Only in Macrophages. We aimed to identify the factors in myeloid lineage cells that inhibited the late phase of HIV-1 infection. Thus, we investigated the cellular factors packaged in the progeny virions produced in primary macrophages and compare them to those produced in stimulated CD4+ T lymphocytes (Fig. 1L). We compared the virions produced by primary macrophages to those in CD4+ T cells by infecting monocyte-derived macrophages (MDMs) and CD4+ T cells with dual-trophic HIV-1 89.6, and allowing HIV to spread through these cells for 10 d. The media obtained from primary MDMs and CD4+ T cells containing the same number of HIV-1 particles were subsequently layered on a 20% sucrose solution for ultracentrifugation. Then, the virion pellets were purified using anti-CD44 microbeads and a magnetic-based method and subsequently analyzed using liquid chromatography and mass spectrometry (MS) to identify the cellular proteins incorporated into the virions. NRP-1, originally identified in stimulated CD4+ T lymphocytes, was not detected in CD4+ T cells, whereas NRP-1 was purified from the virion pellets of primary MDMs. This result strongly suggests that NRP-1 is a newly identified antiviral factor that inhibits HIV-1 replication in vivo.

Significance

Macrophages and dendritic cells represent an important target for HIV-1 replication in vivo as they serve both as a vehicle for virus dissemination throughout the body and a viral reservoir. However, myeloid cells can support persistent replication of HIV-1 and, in contrast to infected T cells, demonstrate lower productivity. Using proteomics, we discovered that NRP-1 is a host restriction factor that inhibits HIV-1 from infecting macrophages and dendritic cells. NRP-1 is incorporated into the HIV-1 virion particle to inhibit its ability to attach to target cells in a viral envelope glycoprotein-independent manner. Overall, these results provide insights into the ability of myeloid lineage cells to utilize NRP-1 to interfere with HIV-1 infection.

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1S.W. and L.Z. contributed equally to this work.

2To whom correspondence may be addressed. Email: gxliang@cmu.edu.cn.

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NRP-1 is a myeloid cell-specific protein, an inhibitor of HIV-1 infectivity. First, we investigated the anti-HIV-1 activity of NRP-1 in target cells. As results, we did not observe any effect of NRP-1 on the HIV-1 infection of target cells (SI Appendix, Fig. S3A), suggesting that NRP-1 does not inhibit HIV-1 replication in the early phase of replication. Next, we explored the antiviral activity of NRP-1 in producer cells by transfecting HIV-1NL4-3-Luc reporter vectors combined with the expression of VSV-G or various HIV-1 CXCR4- or CCR5-tropic Env proteins into 293T cells in the presence or absence of NRP-1. We did not observe that NRP-1 affected HIV-1 production. Nevertheless, when we used the same amount of the produced virion to infect 293T cells that expressed the HIV-1 receptor/coreceptors, CD4, CCR5, and CXCR4, we found that NRP-1 obviously inhibited HIV-1 infectivity (SI Appendix, Fig. S3B), and the levels of HIV-1 late reverse transcription products (Late RT) were also reduced in the presence of NRP-1 (SI Appendix, Fig. S3C). These results suggested that NRP1 may restrict HIV-1 entry into target cells.

Next, we examined NRP-1 activity with replication-competent proviral vectors in the producer cells. After we measured the viral progeny production, we consistently found that the NRP-1 expression vectors at different dosages did not affect the production of different isolates of HIV-1 (NL4-3, BaL, AD8, and 89.6) or simian immunodeficiency virus (SIV) (SI Appendix, Fig. S4A). On the other hand, the presence of NRP-1 significantly inhibited the infectivity of these HIV-1 isolates in a dose-dependent manner (Fig. 2A, Upper), whereas it mildly affected the infectivity of SIV isolates (Fig. 2B) when we used the same titers of HIV-1 or SIV to infect TZM-bl reporter cells. The highest dosages of NRP-1 could significantly reduce HIV-1 infectivity, such as that of HIV-1NL4-3 by 261-fold, HIV-1BaL by 143-fold reduction, HIV-1Bal by 583-fold, and HIV-1AD8 by 134-fold reduction. In contrast, NRP-1 can reduce SIV infectivity by nearly twofold reduction at the highest dosage. Meanwhile, we also observed that the level of gp120 in the virions in the same number of HIV-1 particles (Fig. 2A, Lower) and found that NRP-1 did not affect the gp120 levels. One possibility is that NRP-1 might be incorporated into HIV-1 particles, thereby inhibiting their infectivity. We also titrated input virions to infect TZM-bl reporter cells in the presence or absence of efavirenz to validate the inhibitory effect of NRP-1 on HIV-1. As results, the infectivity of different input viruses from NRP-1-expressing cells as a neuronal and endothelial cell receptor required for embryonic development, axon guidance, and vasculature formation (15–17), was found in HIV-1 particles produced from macrophages and not from CD4+ T cells (SI Appendix, Table S1) (9–14). To validate NRP-1’s incorporation into viral particles, we infected primary MDMs with or without HIV-1 (SI Appendix, Fig. S1A) and lysed the purified virions to prepare them for Western blotting. As a result, NRP-1 was detected inside virion particles by its specific antibody only in the presence of HIV-1 infection (SI Appendix, Fig. S1B), suggesting that NRP-1 is packaged with HIV-1 virions. We also examined whether overexpressed NRP-1 is packaged into HIV-1 virions and observed that only NRP-1, but not green fluorescent protein (GFP), was detected in the purified virions (SI Appendix, Fig. S1C). Importantly, both virion-associated envelope (Env) and capsid (CA) proteins were intact in the presence of NRP-1. NRP-1 obviously inhibited HIV-1 infectivity (SI Appendix, Fig. S1C), indicating that NRP-1 did not affect viral progeny assembly and maturation.

NRP-1 is also expressed by several types of immune cells in which it participates in critical immune functions (18). Interestingly, in addition to being induced in infiltrating mouse CD8+ T cells involved in cancer immunotherapy, NRP-1 enhances human T cell leukemia virus type-1 (HTLV-1), murine cytomegalovirus (MCMV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection of target cells (9–12). Therefore, we wanted to examine the expression profile of NRP-1 in various target cells of HIV-1. NRP-1 was found to be highly expressed in macrophages, DCs, and PMA-stimulated THP-1 cells and mildly expressed in 293T, HeLa cells; however, its expression is minimal in stimulated or resting CD4+ T cells or Jurkat cells (Fig. 1B and C). Since NRP-1 is expressed at a low level in CD4+ T cells, it is not surprising that NRP-1 was not detected inside virions produced from CD4+ T cells.

Moreover, we found that interferon (IFN) treatment did not up-regulate NRP-1 expression in macrophages or DCs (SI Appendix, Figs. S2A and B). In addition, except for IFN-γ, IFN-α and IFN-β could not induce NRP-1 expression in stimulated CD4+ T cells (SI Appendix, Fig. S1C–E). These results suggest that NRP-1 is not involved in IFN-mediated antiviral activities either in macrophages or DCs. Although IFN-γ slightly up-regulates NRP-1 expression in stimulated CD4+ T cells, the resultant amount of NRP-1 might be insufficient for involvement in IFN-γ mediated antiviral activities.

NRP-1 is also a transmembrane protein (9–17). Thus, we first analyzed its cellular localization in 293T cells using the fusion protein NRP-1-GFP. NRP-1-GFP was confirmed to emit a clear fluorescent membrane signal in 293T cells (Fig. 1D). Afterward, we examined the localization of NRP-1 in primary macrophages or DCs. We observed that NRP-1 was consistently located on the cell membrane in a manner similar to Caveolin-1, which is an integrated plasma membrane protein (Fig. 1E). These data suggest that NRP-1 is synthesized in macrophages and DCs and packaged into HIV-1 particles.
was consistently reduced compared to that of the control viruses (SI Appendix, Fig. S4 B and C).

Interestingly, there are two forms of neuropilins, NRP-1 and NRP-2 (see Fig. 4A), and their respective genes exhibit distinct expression patterns. However, NRP-1 and NRP-2 share a common domain organization with and 44% amino acid sequence identity in humans (19). They are composed of five extracellular structural domains—a1 at the N terminus, followed by a2, b1, b2, c, and a single transmembrane helix and a short cytoplasmic tail (20–24). Domains a1 and a2 belong to the structural family of CUB domains, while domains b1 and b2 share structural homology to coagulation factor V/VIII domains (21, 22, 24, 25). Therefore, we also investigated the effect of NRP-2 on HIV-1 infectivity. We did not observe any effect of NRP-2 on the infectivity of HIV-1NL4-3 despite NRP-2’s sharing 44% amino acid identity with NRP-1 (SI Appendix, Fig. S4D). These results suggest NRP-1 is the only member of its family with anti–HIV-1 activity.

Moreover, we explored the inhibitory effect of NRP-1 on viruses by overexpressing NRP-1 with HIV-2 (C) and murine leukemia virus (MLV) (D) proviral vectors. Two days after transfection, the same titers of HIV-2 or SIV were used to infect TZM-bl reporter cells to measure viral infectivity. Mouse NIH 3T3 cells were infected with the same titers of MLV, and their late reverse-transcript products were measured by qPCR. **P < 0.01; *P < 0.05; ns, not significant. (E) The 293T cells were cotransfected with the construct encoding FLAG-tagged NRP-1 or a mock expression construct at different doses, along with a pNL4-3.GFP.R+E reporter vector combined with various expression constructs of HIV-1 X4, R5-trophic, or dual-trophic Env or VSV-G as indicated. Two days after transfection, 100 ng of the produced HIV-1 virions in the supernatants were used to infect the TZM-bl cells to measure viral infectivity. **P < 0.01; *P < 0.05.
gene or various HIV-1 CXCR4-, CCR5-, or dual-tropic Env to infect TZM-bl cells (Fig. 2E). In contrast to HIV-1 Env, when combined with VSV-G, NRP-1 exhibited a mild inhibitory effect on infectivity. Overall, NRP-1 is likely a novel anti–HIV-1 factor of HIV particle infectivity.

NRP-1 Restricts HIV-1 Spread in Macrophages and DCs. We next investigated the anti-HIV activity of NRP-1 in macrophages because NRP-1 is highly expressed in myeloid lineage cells such as primary macrophages, DCs, and stimulated THP-1 cells. First, we transduced lentiviral short hairpin RNA (shRNA) vectors into THP-1 cells to deplete endogenous NRP-1 and then transfected NL-43 proviral vectors into these THP-1 cells. Afterward, we used the same numbers of the produced virions from these THP-1 cells to infect TZM-bl reporter cells. NRP-1 depletion increased HIV-1 progeny infectivity in THP-1 cells by nearly eightfold (SI Appendix, Fig. S5A and B), suggesting that endogenous NRP-1 could act against HIV-1 infectivity in myeloid cells.

Then, we transduced shRNA lentiviral vectors targeting NRP-1 into primary MDMs and further infected these cells with different dosages of replication-competent HIV-1AD8 for 18 d (Fig. 3A and B). The silencing of NRP-1 could noticeably enhance the spread of HIV-1 during the 18-d infection of the macrophages. Moreover, when we applied a lentiviral shRNA targeting the 3′ untranslated region (3′-UTR) of the NRP-1 transcript, the inhibition of HIV-1 spread was rescued in the endogenous NRP-1–depleted primary MDMs transduced with an exogenous NRP-1 expression lentiviral vector during HIV-1 infection of macrophages (Fig. 3C and D). The exogenous NRP-1 expression was not affected by this shRNA because it did not affect the expression of NRP-1 derived from its

Fig. 3. NRP-1 inhibits HIV-1 infection in macrophages. (A and B) Lentiviral shRNA-transduced MDMs were infected with 10 (low-dose) or 100 (high-dose) ng of HIV-1AD8 for 18 d. Viral production was measured using p24 ELISA at the indicated time points (A). The aliquoted cells were lysed for Western blotting to assess the levels of NRP-1 and GAPDH (B). (C and D) MDMs were transduced with lentiviral shRNA targeting the 3′-UTR of the NRP-1 or control transcript, and the shNRP-1–depleted MDMs were then transduced with or without a lentiviral expression vector encoding an untagged NRP-1. Next, the MDMs were infected with 10 (low-dose) or 100 (high-dose) ng HIV-1AD8 for 18 d, and viral production was measured by p24 ELISA at the indicated time points (C). The shRNA-transduced MDMs were lysed for Western blotting to assess the levels of NRP-1 and GAPDH (D). (E) The MDMs transduced with siRNA targeting NRP-1 or the control were infected with HIV-1AD8 (multiplicity of infection = 1) and washed twice with PBS to remove the input viruses 6 h after infection. At 3 d after infection, 20 ng of the produced HIV-1 virions in the supernatants were used to infect TZM-bl indicator cells to measure viral progeny infectivity. Total RNA was extracted from the MDMs for qPCR to measure NRP-1 transcript levels normalized to GAPDH levels. **P < 0.01.
expression vector, into which the NRP-1 open reading frame was cloned.

We then validated these results from the shRNA-mediated knockdown of NRP-1 by employing the small interfering RNA (siRNA)-mediated knockdown of NRP-1 in primary macrophages. The depletion of NRP-1 using siRNA consistently increased HIV-1AD8 infectivity (Fig. 3E), suggesting that NRP restricted HIV-1 infection in macrophages. In addition, we noticed that the cell membrane protein Serinc5, a Nef-counteracted host restriction factor, was encapsidated into the Nef-defective HIV-1 virosomes to inhibit their infectivity. Although endogenous NRP-1 displayed an inhibitory effect on the transmission of wild-type HIV-1 in macrophages, it remained unclear whether Nef could compromise the NRP-1–mediated suppression of HIV-1 infectivity to some extent. Thus, we employed RNA interference (RNAi) to deplete NRP-1 or Serinc5 in the primary MDMs (SI Appendix, Fig. S6A) to transfect with wild-type or Nef-defective HIV-1 proviral vectors. The depletion of NRP-1 similarly increased the infectivity of the wild-type and Nef-defective virosomes, whereas depletion of Serinc5 enhanced the infectivity of the Nef-defective but not the wild-type virosomes (SI Appendix, Fig. S6B). Therefore, the presence of Nef compromises the antiviral activity of Serinc5 but not NRP-1 in macrophages.

Afterward, we also investigated the anti-HIV activity of NRP-1 in monocyte-derived dendritic cells (MDDCs). We treated shRNA-transduced MDDCs with VLP-Vpx to enhance HIV-1 infection and further infected these primary cells with HIV-1ΔΔ3Δ. The silencing of NRP-1 also increased the spread of HIV-1 in the MDDCs from two independent healthy donors for 18 d (SI Appendix, Fig. S7A and B). These data indicate that NRP-1 is an inhibitor of the late phase of HIV-1 infection both in macrophages and DCs.

**NRP-1 is Incorporated into Virion to Block Its Attachment to Target Cells.** Since we observed that NRP-1 was incorporated into HIV-1 particles, we hypothesized that the antiviral activity of NRP-1 could be attributed to its effect on virosomes. We generated NRP-1 mutants (Δ450–550 and Δ810–923) (Fig. 4A) according to recent findings (19, 26) and transfected the expression vectors encoding the wild-type or mutant NRP-1 into 293T cells. The absence of the C terminus (Δ810–923, mut-2) but not that of b2 domain (Δ450–550, mut-1) abolished the inhibitory effects of NRP-1 on HIV-1 infectivity (Fig. 4B), suggesting that domain b2 was not important in the antiviral activity of NRP-1. We then examined the NRP-1 mutants’ ability to incorporate into virosome particles and found that NRP-1-mut-2 without the transmembrane domain was not packaged into viral particles (Fig. 4C). In contrast, NRP-1-mut-1 exhibiting inhibitory effect on HIV-1 infectivity was incorporated into viral particles. Therefore, the antiviral activity of NRP-1 relies on its incorporation into virosome particles.

Furthermore, we analyzed the entry efficiency of HIV-1 particles into target cells in the presence of NRP-1 using a β-lactamase (Blam)-fused Vpr entry assay. By this system, we can examine the entry of the virosomes carrying the BlaM-Vpr fusion protein by measuring the activity of the BlaM delivered to target cells. The HIV-1ΔΔ4–3, Luc+R+E reporter virus was prepared in 293T cells in the presence or absence of NRP-1; then, these viruses were used to infect primary stimulated CD4+ T cells. The entry efficiency of VSV-G–pseudotyped HIV-1 prepared from NRP-1–overexpressing cells was markedly reduced compared to that of the control viruses (Fig. 5A), and this result was also observed in five independent experiments (Fig. 5B). These data indicated that NRP-1-incorporated HIV-1 particles were severely impaired in their ability to enter target cells and that NRP-1 inhibited HIV-1 virosomes entry.

Next, we performed HIV-1 virosome attachment assays and observed that the ability of the wild-type viropexis HIV-1ΔΔ4–3 to attach to target cells, such as TZM-bl or stimulated CD4+ T cells (Fig. 5C), was consistently impaired in the presence of NRP-1 in the produced cells. In addition, HIV-1ΔΔ4–3,CXCR4-, CCR5-, or dual-tropic Env from NRP-1–expressing cells were also significantly impaired in their ability to attach to susceptible target cells (SI Appendix, Fig. S8). Moreover, the NRP-1 C terminus mutant (Δ810–923, mut-2), which is not incorporated into HIV-1 particles, could not inhibit virosome attachment to target cells (SI Appendix, Fig. S9A and B). However, the NRP-1 mutant (Δ450–550, mut-1), which is incorporated into virosomes, retained the inhibitory effect on virosome attachment to target cells, thereby suggesting that the presence of NRP-1 in virus particles hinders virosome interaction with target cells. Furthermore, we employed RNAi to silence NRP-1 in primary macrophages (Fig. 5D and E) or MDDCs (Fig. 5F and G) to test whether the NRP-1 of these cells could impair HIV-1 attachment to target cells. The loss of endogenous NRP-1 in MDMs or MDDCs consistently increased the attachment of HIV-1 particles to the target cells. These data demonstrate that NRP-1 is incorporated into virosomes to inhibit their ability to attach to target cells, thereby reducing the infectivity of the progeny virosomes.
Discussion

In this study, we have demonstrated that neuropilin family member NRP-1, but not NRP-2, can inhibit the infectivity of HIV-1 progeny virions in macrophages and DCs. NRP-1 is highly expressed in myeloid lineage cells, such as macrophages and DCs, but not in stimulated or resting CD4 T lymphocytes. Moreover, NRP-1 is not induced in IFN-stimulated macrophages, DCs, or CD4 T cells, suggesting that NRP-1 is not a downstream molecule of IFN against virus infections in host cells. NRP-1 is a transmembrane protein that regulates pleiotropic biological processes, including axon guidance, angiogenesis, and vascular permeability (15–17). It is also a host factor enhancing HTLV, MCMV, and SARS-CoV-2 infection (9–12). However, NRP-1 cannot enhance HIV-1 infection of target cells. In contrast, NRP-1 expression is strongly inhibitory to the infectivity of various wild-type HIV-1 strains, including NL4-3, 89.6, Bal, and AD8, whereas NRP-1 does not affect HIV-1 production in producer cells. In addition to restricting HIV-1 virion infectivity, NRP-1 expression in the virus-producer cells can also interfere with the infectivity of HIV-2 and mildly interfere with the infectivity of SIV, whereas it does not affect MLV. The mild inhibitory effect of NRP-1 on HIV-1 infectivity is probably due to insufficient incorporation of NRP-1 into viral particles or the poorly organized structure of packaged NRP-1 in SIV particles. This hypothesis requires further investigation in the future.

Furthermore, we have demonstrated that while NRP-1 is present in intact virion particles, it has no effect on the virion-associated CA and Env proteins. We investigated the molecular mechanisms underlying the antiviral activity of NRP-1 with mutagenesis assays and found that NRP-1 was required to be packaged into viral particles to inhibit their infectivity. Moreover, we demonstrated that NRP-1 inhibited HIV-1 infectivity by interfering with virion attachment to the target cells using virion attachment assays. These data indicate that NRP-1 on the virion surface may sterically disrupt the binding of virions to target cells. In addition, NRP-1 has a highly extended structure with a heavily glycosylated extracellular domain, and its intrinsic structural features may reduce nonspecific binding between the virions and target cells.

Notably, silencing NRP-1 can promote the transmission of HIV-1 infection in primary macrophages or DCs. On the other hand, ectopic expression of NRP-1 in endogenously NRP-1-deficient macrophages can rescue the inhibition of HIV-1 transmission. Therefore, we propose that NRP-1 is a macrophage and DC-specific factor located on the cell membrane to inhibit HIV-1 infectivity. Given the critical role of NRP-1 in hindering virion binding to target cells, we also tested whether Nef could compromise the restriction of HIV-1 infectivity by NRP-1. However, we observed that the silencing of NRP-1 did not preferentially increase the infectivity of Nef-defective HIV-1 compared with wild-type HIV-1 in macrophages. In contrast, silencing Serinc5 increases the infectivity of Nef-defective HIV-1 compared with wild-type HIV-1 in macrophages. Therefore, we propose that NRP-1 is a macrophage and DC-specific factor located on the cell membrane to inhibit HIV-1 infectivity. Given the critical role of NRP-1 in hindering virion binding to target cells, we also tested whether Nef could compromise the restriction of HIV-1 infectivity by NRP-1. However, we observed that the silencing of NRP-1 did not preferentially increase the infectivity of Nef-defective HIV-1 compared with wild-type HIV-1 in macrophages. In contrast, silencing Serinc5 increases the infectivity of Nef-defective but not Nef-positive HIV-1, indicating that Nef counteracts the restriction of Serinc5 to promote HIV-1 infection in primary macrophages. Particularly, NRP-1 also displays an inhibitory effect on the transmission of HIV-1 in DCs.

Myeloid cells generally resist HIV-1 infection compared to CD4 T cells, probably due to the different genes encoding anti–HIV-1 factors in myeloid cells. Thus, the expression of NRP-1 in myeloid lineage cells can partially contribute to their resistance to the spread of HIV-1. Since cell–cell transmission of HIV-1 is a major in vivo event, it is necessary to investigate whether NRP-1 restricts the direct transmission of HIV-1 from macrophages or DCs to CD4 T cells in future research. Notably, NRP-1 is reported to be required within endothelial cells for angiogenesis via vascular endothelial growth factor (VEGF).
Neuropilin-1, a myeloid cell-specific protein, is an inhibitor of HIV-1 infectivity. Neuropilin-1’s interaction with HIV-1 virions may help develop therapeutic strategies that target viral infections.

**Methods**

**Ethics Statement.** This study was approved by the Research and Ethics Committee of The First Affiliated Hospital of China Medical University. All the blood samples were obtained from the healthy donors following the National Health and Medical Research Council guidelines. In addition, informed consent was obtained from each healthy donor before the study. Moreover, the study protocol and informed consent forms were approved by the Institutional Review Board approved of China Medical University.

**Cells and Cell Culture Reagents.** NIH 3T3 (mouse), 293T (human), and TZM-bl cells were cultured in Dulbecco’s modified Eagle’s medium ( Gibco). THP-1 and Jurkat (human) cells were cultured in RPMI-1640 medium (Gibco). Both media were supplemented with 10% fetal bovine serum (FBS; Gibco), 100 μM penicillin, and 100 mg/mL streptomycin. Plasmids were transfected into these 293T cells using Lipofectamine 2000 (Invitrogen). Peripheral blood mononuclear cells (PBMCs) obtained from healthy blood donors were purified by ficoll-Hypaque density gradient centrifugation. CD4+ T cells or monocytes were isolated from the PBMCs via negative selection with human CD4+ T cells or CD14-positive enrichment mixture (STEM-CELL Technologies). The CD4+ T cells were stimulated by adding CD3/CD28 activator magnetic beads (Invitrogen) to the culture medium for 2 d with 50 μM interleukin (IL-2) (Biomol). The isolation and culturing of monocytes, MDMs, and MDDCs were performed (28, 29). MDMs were generated by stimulating monocytes with 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D) and 50 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; R&D) for 7 d. MDDCs were generated by incubating CD14-purified monocytes in IMDM medium (Gibco) supplemented with 10% FBS, 2 mM l-glutamine, 100 μM penicillin, 100 μg/mL streptomycin, 10 mM Hepes, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 ng/mL GM-CSF, and 50 ng/mL IL-4 (Miltenyi Biotech). On day 4, two-thirds of the culture medium was replaced with a fresh medium containing GM-CSF and IL-4. Immature MDDCs were harvested and used for experiments on day 6. Lipofectamine 3000 (Thermo Fisher) was used to deliver siRNA into MDMs or MDDCs.

**Plasmids.** The open reading frames of the expression vectors encoding NRP-1 (Origene) were cloned into pCMV3-Tag2A (Addgene). Also, HIV-1 reporter vectors of NLA-3.Luc.R-E, NLA-3-GFP.R-E’, and NLA-3-GFP.R-E were constructed using CD4+ T cells or CD14-positive enrichment mixture (STEM-CELL Technologies). The CD4+ T cells were stimulated by adding CD3/CD28 activator magnetic beads (Invitrogen) to the culture medium for 2 d with 50 μM interleukin (IL-2) (Biomol). The isolation and culturing of monocytes, MDMs, and MDDCs were performed (28, 29). MDMs were generated by stimulating monocytes with 10 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D) and 50 ng/mL recombinant human macrophage colony-stimulating factor (M-CSF; R&D) for 7 d. MDDCs were generated by incubating CD14-purified monocytes in IMDM medium (Gibco) supplemented with 10% FBS, 2 mM l-glutamine, 100 μM penicillin, 100 μg/mL streptomycin, 10 mM Hepes, 1% nonessential amino acids, 1 mM sodium pyruvate, 10 ng/mL GM-CSF, and 50 ng/mL IL-4 (Miltenyi Biotech). On day 4, two-thirds of the culture medium was replaced with a fresh medium containing GM-CSF and IL-4. Immature MDDCs were harvested and used for experiments on day 6. Lipofectamine 3000 (Thermo Fisher) was used to deliver siRNA into MDMs or MDDCs.

**RNAi in THP-1 Cells, MDMs, or MDDCs.** The shRNA-mediated silencing of NRP-1 (RHS4430-200289197 or RHS4430-200215106 targeting open reading frame; RHS4430-200223232 targeting 3’UTR) or control (catalog no. RHS4346) was achieved by introducing a microRNA-adapted shRNA lentivirus into the THP-1 cells, MDMs, and MDDCs (28, 29). In summary, the freshly isolated monocytes were treated with Vpx and transduced with an shRNA lentivirus. After puromycin selection, the cells were infected with replication-competent CCR5-trophic HIV-1 producer 293T cells and viral supernatants were subsequently reduced in 20 mM dithiothreitol (Sigma) at 95οC. The obtained virion stocks were treated for 1 h at 37οC with DNaese (Takara). DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). qPCR was performed to quantify HIV-1 reverse transcriptase product using qPCR with specific primers for HIV-1 5’-CGTACGGCGGCTGTTCCCG-S and 3’ -CTTGGCAGGGGTCCTCCC-G (29, 31).

**Reverse Transcription Assay.** HIV-2 or MLV particles were purified, and the level of virion-associated reverse transcriptase was measured using the Reverse Transcriptase (RT) Assay (colorimetric, Roche).

**Measurement of Late RT Products of MLV.** Mouse NIH 3T3 cells were infected with MLV for 24 h, and genomic DNA was extracted from the cells to quantify late reverse-transcript product using qPCR with specific primers for MLV 5’-CGTACGGCGGCTGTTCCC-S and 3’-CTTGGCAGGGGTCCTCCCG-G (29, 31).

**Quantification of Early RT Products of HIV-1.** HIV-1 producer 293T cells were washed twice with PBS to remove the transfected HIV-1 plasmid before infection. The obtained viral stocks were treated for 1 h at 37οC with DNaese (Takara). DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen). qPCR was performed to quantify late HIV-1 RT with the following primers, as described previously (32): late RT forward: 5’-AGGAGAAGACTAAGTACCC-S and late RT reverse: 5’-TTGTCATTGTCAGAAGTC-G.

**Identification of NRP-1 Incorporated into HIV-1 Virions from Macrophages.** MDMs generated from the monocytoids isolated from five healthy donors were pretreated with VLP-Vpx and infected with 89.6. At 6 h after infection, the cells were washed with PBS twice to remove the input virions and cultivated for 8 d. Meanwhile, CD4+ T cells isolated from the same healthy donors were treated with Vpx and transduced with an shRNA lentivirus. After puromycin selection, the cells were infected with replication-competent CCR5-trophic HIV-1 particles were centrifuged for 5 min at 1,200 x g. The supernatants were passed through a 0.45-μm filter (Thermo Fisher) and layered on 20% sucrose for a 2-h ultracentrifugation at 25,000 x g at 4οC. The obtained virion pellets were resuspended in 200 μL of PBS with 50 μL of anti-CD44 microbeads for 30 min at room temperature, and the viable and infectious HIV-1 particles were further isolated by a magnetic-based method according to the manufacturer’s instructions (MACS VivaSor HIV Isolation Kit; Miltenyi Biotech). The isolated HIV-1 virions were subsequently reduced in 20 mM dithiothreitol (Sigma) at 95οC for 5 min and alkylated in 50 mM iodoacetamide (Sigma) for 30 min in the dark at room temperature. After alkylation, the samples were transferred to a 10-kDa centrifugal spin filter (Millipore) and washed with 200 μL of 8 M urea three times and 200 μL of 50 mM ammonium bicarbonate twice via centrifugation at 14,000 x g. Next, tryptic digestion was performed using trypsin (Promega) at a ratio of 1:50 (enzyme/substrate, mM) to 200 μL of 50 mM ammonium bicarbonate at 37οC for 16 h. The peptides were recovered by transferring the filter to a new collection tube and centrifuging at 14,000 x g. The peptide yield was increased by washing the filter with 100 μL of 50 mM NaHCO3 twice. The peptides were desalted using a StageTip.

MS experiments were performed on a nanoscale UHPLC system (EASY- LC/100, from Proxeon Biosystems) connected to an Orbitrap Q-Exactive mass spectrometer. A 100 μL sample taken from each NRP-1-binding HIV virion was digested with trypsin (Promega) at a ratio of 1:50 (enzyme/substrate, mM) to 200 μL of 50 mM ammonium bicarbonate at 37οC for 16 h. The peptides were recovered by transferring the filter to a new collection tube and centrifuging at 14,000 x g. The peptide yield was increased by washing the filter with 100 μL of 50 mM NaHCO3 twice. The peptides were desalted using a StageTip. The peptides were dissolved in 0.1% FA with 5% CH3CN and separated on an RP-HPLC analytical column (75 μm x 15 cm) packed with 2-m C18 beads (Thermo Fisher) using a 2-h gradient from 5% to 40% acetonitrile in 0.5% formic acid at a flow rate of 250 nL/min. The spray voltage was set to 2.5 kV, and the temperature of the ion transfer capillary was 275° C. A full MS/MS cycle consisted of one full MS scan (resolution at 70,000; automatic gain control [AGC] value, 1e5; maximum injection time, 100 ms). Moreover, the dynamic exclusion window was set to 40 s, and one microscan was acquired for each MS and MS/MS scan. Subsequently, the unassigned ions and those with a charge of 1+ and 2+ were rejected for MS/MS, and a lock mass correction using a background ion (m/z 445.12003) was applied (33). The raw data were processed using Proteome Discoverer (PD, version 2.1), and the MS/MS spectra were used to search the reviewed Uniprot human protein database. All the searches were performed with a precursor mass tolerance of 7 ppm and a fragment mass tolerance of 20 millimass units, with oxidation (M, +15.9949 Da) and deamidation (N, +0.9840 Da) as variable modifications, carbamidomethylation (+57.0215 Da) as the fixed modification, and allowance for two trypsin-missed degradations. Only the peptides of at least six amino acids were considered. The peptide and protein...
identifications were filtered by PD to control the false discovery rate at <1%. At least one unique peptide was required for protein identification.

**Isolation of Membrane-Associated Proteins.** The isolation of membrane-associated proteins was performed using the Mem-PERPlus Membrane Protein Extraction Kit (Invitrogen) (28). In summary, cells were harvested from suspension cell cultures by centrifugation at 300 × g for 5 min. The obtained cell pellets were washed with 0.5 mL of Cell Wash Solution and centrifuged at 300 × g for 5 min. After supernatants were removed, the cell pellets were resuspended with 1.5 mL of Cell Wash Solution, transferred to a new tube, and centrifuged at 300 × g for 5 min to discard the supernatants. The resultant cell pellets were mixed with 0.75 mL of permeabilization buffer to obtain a homogeneous cell suspension and incubated for 10 min at 4 °C. The permeabilized cells were centrifuged at 16,000 × g for 5 min, and the resultant supernatants containing cell lysates were removed and transferred to a new tube for detection. Next, the pellets were resuspended with 0.5 mL of solubilization buffer, and the mixtures were incubated at 4°C with constant mixing for 30 min. The resultant solution in the tubes was centrifuged at 16,000 × g at 4°C for 15 min. The supernatants containing the solubilized membrane and membrane-associated proteins were moved to a new tube for analysis; the proteins were subjected with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and detected with various antibodies during Western blotting.

**HIV-1 Enrichment Assay.** The cell culture media containing HIV-1 particles were centrifuged at 1,200 × g for 5 min. The supernatants were passed through a 0.45-μm filter (Thermo Fisher) and layered on 20% sucrose for a 2-h ultracentrifugation at 25,000 × g at 4°C. The virion pellets were subsequently isolated using anti-CD44 microbeads with a magnetic-based method according to the manufacturers’ instructions (μMACS VitalVirus HIV Isolation Kit; Miltenyi Biotec). The purified virions were lysed with RIPA buffer (Thermo Fisher) and subjected to SDS-PAGE for Western blotting.

**HIV-1 Attachment Assay.** The attachment of the viral particles was assayed (34, 35). In summary, 3 × 10⁷ T2M-bl cells or 2 × 10⁷ stimulated CD4⁺ T cells were spinoculated with HIV-1 at 1,200 × g at 25°C for 2 h. Then, the infected cells were washed with cold media five times to remove the unbound viral particles. Next, the cells were lysed with 0.5% Triton X-100 to quantify the cell-associated p24agag molecules using ELISA. Virion equivalents were determined by assuming an average of 1,500 p24agag molecules per HIV-1 particle, in other words, 15,800 viral particles per picogram of p24agag.

**The BlaM-Vpr-Based Viral Entry Assay.** HIV-1 particles incorporating a fusion protein between Vpr and BlaM reporter protein were produced by cotransfection of NLA-3-Luc-RI-E with an expression vector encoding BlaM-Vpr. The viruses that were produced were quantified by p24 ELISA, and target cells were incubated with 100 ng of p24 viruses at 37°C for 4 h to allow viral entry. After washing three times with Hank’s balanced salt solution (HBSS) (Thermo Fisher), cells were resuspended and loaded with 1 μM CCF2-AM dye (Thermo Fisher), a fluorescent substrate for BlaM, in HBSS containing 1 mg/mL Pluronic F-127 surfactant (Thermo Fisher) and 0.001% acetic acid for 1 h at room temperature and then washed twice with HBSS. The BlaM reaction, which corresponds to the cleavage of intracellular CCF2 dye by BlaM-Vpr, was developed for 14 h at room temperature in HBSS supplemented with 10% FBS. Cells were washed three times with PBS and fixed in a 1:2.5% solution of parafomaldehyde. The fluorescence was monitored at 520 nm and 447 nm by means of flow cytometry using Sony ID7000.

**Western Blotting and Antibodies.** Standard Western blotting was performed to detect cellular proteins using various antibodies, including monoclonal rabbit anti-NR1-1 (catalog no. ab81321, 1:1,000 dilution; Abcam), polyclonal goat anti-gp120 (catalog no. ARP-288, 1:20,000; NIH AIDS Reagent Program), rabbit anti-GAPDH (catalog no. PA1-987, 1:1,000; Thermo Fisher), mouse monoclonal anti-FLAG (catalog no. F18B, 1:1,000; Sigma), rabbit polyclonal anti-p24 (catalog no. ab63913, 1:1,000; Abcam), and mouse IgG-HRP (catalog no. 6789, 1:5,000; Abcam).

**Microscopy.** The cells were photographed using the Thermo Fisher EVOS FL Imaging System.

**qPCR.** Total RNA was extracted from the cells using TRIzol (Invitrogen). Then, the obtained RNA was dissolved in 100 μL of DPEC-H₂O, and 1 μg of the purified RNA was treated with DNase I (amplification grade, Invitrogen) for 10 to 15 min at room temperature. Next, the RNA was immediately primed with oligo-dT and reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen). Afterward, real-time PCR analysis was performed using the ΔΔCT method with various primer sets (SI Appendix, Table S2). Finally, the results were normalized to the amplification results of the internal control, GAPDH.

**Statistical Analysis.** Statistical analysis was performed using Prism 6.0 (GraphPad Software). Unpaired two-tailed Student’s t tests were used for statistical comparison between groups, unless otherwise specifically mentioned. Each experiment was performed three times independently, and the data were presented as the mean ± SEM.

**Data Availability.** All study data are included in the article and/or SI Appendix.

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