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Urokinase plasminogen activator surface receptor restricts HIV-1 replication by blocking virion release from the cell membrane

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The urokinase-type plasminogen activator (uPA) system consists of the proteinase uPA, its receptor (PLAUR/uPAR). Under physiological conditions, uPA and PLAUR are predominantly expressed by blood cells, including neutrophils, monocytes, and macrophages, and play important roles in cell activation, adhesion, migration, and extravasation. Here, we report that PLAUR, which is highly expressed in macrophages and dendritic cells (DCs) but hardly expressed in CD4$^+$ T cells, inhibits the release of HIV-1 progeny virions from the cell membrane. Silencing *PLAUR* markedly enhanced the transmission of HIV-1 in macrophages and DCs. We further demonstrated that PLAUR is localized at the cell membrane to block the release of HIV-1 virions. Interestingly, we found that uPA compromises the PLAUR-mediated inhibition to slightly enhance HIV-1 production in primary macrophages and DCs. In the absence of PLAUR, this enhanced effect induced by uPA is abrogated. In conclusion, PLAUR is a new anti-HIV-1 protein produced in both macrophages and DCs where it inhibits HIV-1 transmission. This discovery may provide a novel therapeutic target for combating HIV.

HIV-1 | PLAUR | uPA

Significance

Macrophages and DCs play numerous roles in HIV-1 pathogenesis. They are generally less permissive to HIV-1 infection when compared to stimulated CD4$^+$T lymphocytes, a phenomenon particularly acute during the early phases of infection. The identification of cellular factors contributing to the resistance to HIV-1 infection is important for understanding virus–host interactions. In this study, we have discovered PLAUR as a new antiviral factor in macrophages and DCs, which blocks the release of HIV-1 progeny from the cell membrane. Notably, the ligand uPA slightly promotes HIV-1 production in macrophages by interfering with the antiviral activity of PLAUR. Overall, these results provide insights into the molecular mechanisms of how the uPA–PLAUR system interferes with HIV-1 infection in myeloid lineage cells.

Macrophages and dendritic cells (DCs) represent important in vivo targets for the replication of HIV-1 because they serve both as a vehicle for disseminating the virus throughout the body and as a viral reservoir (1–6). However, despite being able to support the persistent replication of the virus, the myeloid lineage macrophages and DCs are collectively less permissive to HIV-1 infection compared with activated lymphocytes. The urokinase-type plasminogen activator (uPA) system consists of proteinase uPA, its transmembrane receptor (PLAUR/uPAR or CD87), and its inhibitors plasminogen activator-1 (PAI-1) and plasminogen activator-2 (PAI-2) (7, 8). uPA is synthesized as an inactive precursor which gets proteolytically activated. It binds to a specific receptor, PLAUR, localized at the cell surface (9). uPA and PLAUR are expressed by inflammatory cells, including neutrophils, monocytes, macrophages, and activated T lymphocytes (9). The uPA–PLAUR system is involved in pericellular proteolysis, cell migration, signal transduction, and chemokine-like activities (10–13). Interestingly, during HIV-1 infection, the cell surface expression of PLAUR is enhanced in monocytes and T lymphocytes (14, 15), and the uPA–PLAUR interaction has been shown to interfere with HIV replication in monocyte-derived macrophages (MDMs) (16). In addition, uPA has been shown to cleave the HIV-1 gp120 protein in its hypervariable V3 loop, a region involved in determining cell tropism and chemokine coreceptor usage (17). The serum levels of soluble PLAUR in a cohort of HIV-1-infected individuals, measured before initiating anti-retroviral therapy, also highly correlated with the severity of disease progression (18).

These observations suggest that the uPA–PLAUR interaction may play an important role in the pathogenesis of HIV-1 infection and its progression toward AIDS. Granulocytes and monocytes have the highest expression of PLAUR among blood cells, but the granulocytes of HIV-infected individuals express lower amounts of PLAUR. Moreover, its levels were significantly correlated with the number of CD4$^+$ T lymphocytes in these individuals (19). However, the signaling pathway triggered by the uPA–PLAUR interaction leading to this antiviral effect remains elusive. The study aimed to investigate the effect of PLAUR and the uPA–PLAUR interaction on HIV-1 infection of myeloid lineage cells. In this study, we show that PLAUR acts as a new antiviral factor to block HIV-1 virion release from the cell membrane. We further demonstrate that uPA compromises this PLAUR-mediated inhibition to slightly promote HIV-1 production in myeloid cells. These findings present the uPA–PLAUR interaction as a novel anti-HIV therapeutic target.

Results

PLAUR Is Mainly Expressed in Macrophages and DCs. We first determined the expression profile of PLAUR in various host cells. We found it to be highly expressed in THP-1 cells, myeloid lineage primary macrophages, DCs, and monocytes (Fig. 1 *A* and *B*), while it was

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The authors declare no competing interest.

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barely expressed in stimulated or resting CD4$^+$ T lymphocytes, 293T, Jurkat, and HeLa cells. Further, we examined whether interferon (IFN)-α, β, or γ could induce PLAUR expression in CD4$^+$ T cells and macrophages. None of these IFNs could upregulate *PLAUR* expression although the IFN-responsive *ISG-15, CXCL10*, and *BST-2* were upregulated (*SI Appendix*, Fig. S1 *A* and *B*). Therefore, PLAUR is likely not involved in the IFN-mediated antiviral activities either in the CD4$^+$ T cells or macrophages. Thereafter, we stained PLAUR-expressing viable cells using a PLAUR-specific monoclonal antibody to examine the cell membrane localization of PLAUR in 293T and stimulated CD4$^+$ T cells. As a result, in contrast to the isotype control antibody IgG, either the C terminus of GFP-tagged or non-tagged PLAUR appeared as a distinct membrane-localized pattern in

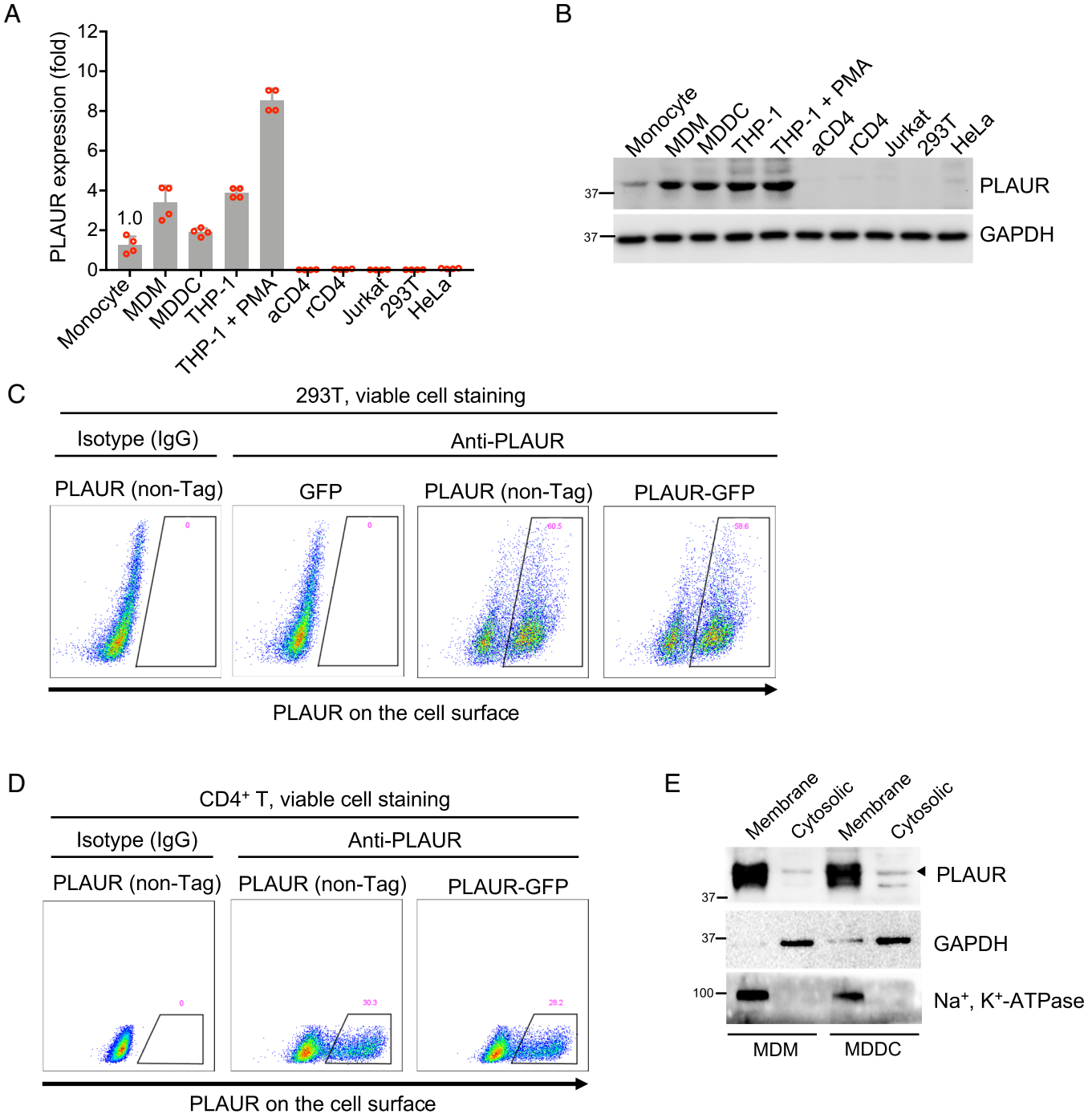

Fig. 1. PLAUR Is Highly Expressed in Myeloid Cells and Localized at the Cell Membrane. (*A* and *B*) Total RNA was extracted from primary monocytes, MDMs, MDDCs, THP-1, PMA-treated THP-1, stimulated or resting CD4$^+$ T cells, established cell lines 293T, HeLa, and Jurkat cells. *PLAUR* transcript levels were measured using quantitative PCR and then normalized to *GAPDH* levels (*A*), and data are plotted as mean ± SEM of three independent experiments. Meanwhile, western blotting was conducted to assess the level of PLAUR and GAPDH (*B*). (*C* and *D*). 293T cells (*C*) and stimulated CD4$^+$ T cells (*D*) exogenously expressed vectors encoding GFP-tagged, non-tagged PLAUR, or GFP. At 48 h after transfection in 293T cells or 96 h after lentiviral vector transduction in CD4$^+$ T cells, viable cells were harvested and stained with mouse anti-PLAUR or isotype (IgG) antibodies to measure the levels of the C-terminal GFP-tagged PLAUR and non-tagged PLAUR at the cell surface using a PE-conjugated goat anti-mouse antibody (*E*). MDMs or MDDCs were lysed, and membrane-bound and cytosolic proteins were separated to assess the levels of PLAUR, Na^+/K^+-ATPase, and GAPDH using western blotting with specific antibodies. All micrographs or blotting data are representative of three independent experiments.

293T or stimulated CD4$^+$ T cells (Fig. 1 *C* and *D*). Moreover, we also determined the subcellular localization of PLAUR in primary macrophages or DCs by purifying the membrane-bound proteins. PLAUR was consistently found on the cell membrane just like Na^+/K^+-ATPase, an integrated plasma membrane protein (Fig. 1*E*). Overall, we confirmed that PLAUR is a myeloid-specific transmembrane protein.

PLAUR Inhibits HIV-1, Simian Immunodeficiency Virus (SIV), HIV-2, and Murine Leukemia Virus (MLV) Titers in Producer Cells. Furthermore, we set out to examine the potential roles of PLAUR involved in HIV-1 replication. At first, we did not observe any effect of PLAUR on the HIV-1 infection of target cells (*SI Appendix*, Fig. S2*A*), suggesting that PLAUR does not inhibit HIV-1 replication in its early phase. Next, we explored the antiviral activity of PLAUR in producer cells by transfecting HIV-$1_{NL4\text{-}3.Luc.R^-.E^-}$ reporter vectors into 293T cells in the presence or absence of PLAUR at different doses (Fig. 2*A*). We found that PLAUR did not affect the long terminal repeats promoter-driven luciferase reporter expression. Nevertheless, it decreased the HIV-1 titer in the culture supernatants from 3.7-fold to 11.1-fold in a dose-dependent manner. However, PLAUR did not influence the expressions of HIV-1 Gag and Env proteins in the cell lysate (Fig. 2*B*). Therefore, we hypothesized that PLAUR may block the release of the HIV-1 progeny from the cell. We confirmed these results with different HIV-1 isolates in different host cells. Consistently, PLAUR inhibited the production of CXCR4-, CCR5-, or dual-tropic replication-competent HIV-1 (NL4-3, BaL, AD8, 89.6) either in HeLa or 293T cells (*SI Appendix*, Fig. S2*B* and Fig. 2*C*). Moreover, we also examined if the GFP-tagged and non-tagged PLAUR exhibited similar anti-HIV activity indicating the effect of C-terminal GFP fusion on the antiviral activity of PLAUR against HIV-1. As shown in *SI Appendix*, Fig. S2 *C* and *D*, C-terminal GFP fusion had no significant effect on the antiviral activity of PLAUR. Thereafter, we investigated the antiviral effect of PLAUR against other viruses and observed that PLAUR had no effect on the cytomegalovirus (CMV) promoter-driven production of hepatitis B virus (HBV) but it inhibited the titers of SIV isolates SIV_{mac293} and SIV_{agm}, HIV-2_{Rod}, and MLV in 293T cells (Fig. 2 *D*–*G*). Therefore, PLAUR may exhibit a broad antiviral activity against retroviruses.

PLAUR Inhibits HIV-1 Spread in CD4$^+$ T Cells. Although PLAUR is barely expressed endogenously in CD4$^+$ T cells, we were curious whether expressing PLAUR exogenously in these cells could inhibit the spread of HIV-1 infection. First, we transduced lentiviral vectors expressing PLAUR-GFP or non-tagged PLAUR into Jurkat cells and confirmed that both of the expressions did not influence cell proliferation (Fig. 3 *A* and *B*). Next, we challenged these cells with replication-competent HIV-1 and observed that the spread of the virus was attenuated in PLAUR or PLAUR-GFP-expressing cells during the 8-d infection (Fig. 3*C*). Moreover, we also transduced lentiviral vectors expressing PLAUR-GFP or non-tagged PLAUR into primary stimulated CD4$^+$ T cells isolated from independent healthy donors and observed the same results (Fig. 3 *D*–*F* and *SI Appendix*, Fig. S2 *E*–*G*). Therefore, PLAUR could inhibit HIV-1 infection in T cells as well.

PLAUR Inhibits HIV-1 Transmission in Macrophages and DCs. We next explored the anti-HIV activity of PLAUR in primary macrophages since it is highly expressed in myeloid lineage and THP-1 cells. First, we depleted the endogenous expression using a lentiviral short hairpin RNA (shRNA) vector targeting *PLAUR*; then, we infected these cells with vesicular stomatitis virus G-glycoprotein (VSV-G)-pseudotyped HIV-1NL4-3.Luc.R$^-$.E$^-$ (*SI Appendix*, Fig. S3 *A* and *B*). Data showed that PLAUR depletion increased the production of HIV-1 progeny by nearly sixfold during a single-round infection in THP-1 cells, suggesting that endogenous PLAUR could limit HIV-1 production in myeloid cells.

Furthermore, we explored the potential anti-HIV-1 activity of endogenous PLAUR in primary MDMs and found that the silencing of PLAUR noticeably enhanced the spread of the CCR5-tropic replication-competent HIV-1_{AD8} during the 15-d infection period in MDMs (Fig. 4 *A* and *B*). In addition, a similar effect was observed when PLAUR was depleted using an shRNA targeting the 3′-untranslated region (UTR) of the *PLAUR* transcript. Moreover, viral inhibition was rescued in these cells by supplementing an exogenous PLAUR-GFP lentiviral vector (Fig. 4 *C* and *D*). The 3′-UTR-targeting shRNA did not deplete the expression of PLAUR-GFP because only the open reading frame (ORF) of PLAUR was cloned into the expression vector. Overall, these data indicate that endogenous PLAUR could inhibit HIV-1 transmission in primary macrophages.

We also investigated the anti-HIV activity of PLAUR in monocyte-derived DCs (MDDCs). The shRNA-mediated depletion of PLAUR enhanced the spread of HIV-1 over the 15-d infection period in MDDCs, which were made more permissible to the virus by pre-treating them with virus-like particle (VLP)-Vpx to deplete the SAMHD1 protein (Fig. 4 *E* and *F*). Taken together, these data indicate that PLAUR inhibits HIV-1 spread both in macrophages and DCs.

PLAUR Inhibits HIV-1 Progeny Release from the Cell Membrane. To investigate the molecular mechanisms underlying the anti-HIV behavior of PLAUR, we generated three GFP-tagged PLAUR mutants: Δ1–22, Δ23–44, and Δ89–110 (*SI Appendix*, Fig. S4*A*). These were individually transfected into 293T cells along with HIV-$1_{NL4\text{-}3}$ proviral vectors. As shown in *SI Appendix*, Fig. S4*B*, wild-type PLAUR, Δ23–44, and Δ89–110 PLAUR mutants inhibited HIV-1. We observed membrane-localized signals corresponding to the wild-type and mutant PLAUR using an anti-PLAUR specific antibody to stain the viable cells (*SI Appendix*, Fig. S4*B*). However, the loss of signal peptide (Δ1–22 mutant) resulted in the loss of anti-HIV activity with no membrane-localized signal observed for this mutant (*SI Appendix*, Fig. S4*C*). Thus, targeting to the cell membrane is critical for the anti-HIV-1 activity of PLAUR. Next, as expected, we found out that PLAUR was colocalized with HIV-1 Gag protein at the cell membrane (*SI Appendix*, Fig. S4 *D* and *E*). Moreover, as PLAUR was found to colocalize with Gag protein at the cell membrane, we examined whether PLAUR was incorporated into HIV-1 virions by purifying them from the supernatants of infected 293T cells and subsequently detecting proteins using western blotting. NPR-1, an HIV-1 virion-packaged host restriction factor, was consistently detected in the virions (20) but PLAUR was not (*SI Appendix*, Fig. S5*A*), indicating that PLAUR was not incorporated into the viral particles and instead colocalized with HIV-1 Gag at the cell membrane.

Since the level of cellular HIV-1 Gag was not affected in the presence of PLAUR when trypsin was used to harvest adherent 293T cells, and PLAUR inhibited HIV-1 titers in culture supernatants, we hypothesized that it may block the release of HIV-1 progeny from the cell membrane. To this end, we employed electron microscopy to examine the membrane-associated HIV-1 particles. As shown in *SI Appendix*, Fig. S5 *B* and *C*, HIV-1 virions remained tethered to the cell surface in the presence of PLAUR but not in its absence. Therefore, PLAUR may inhibit the viral

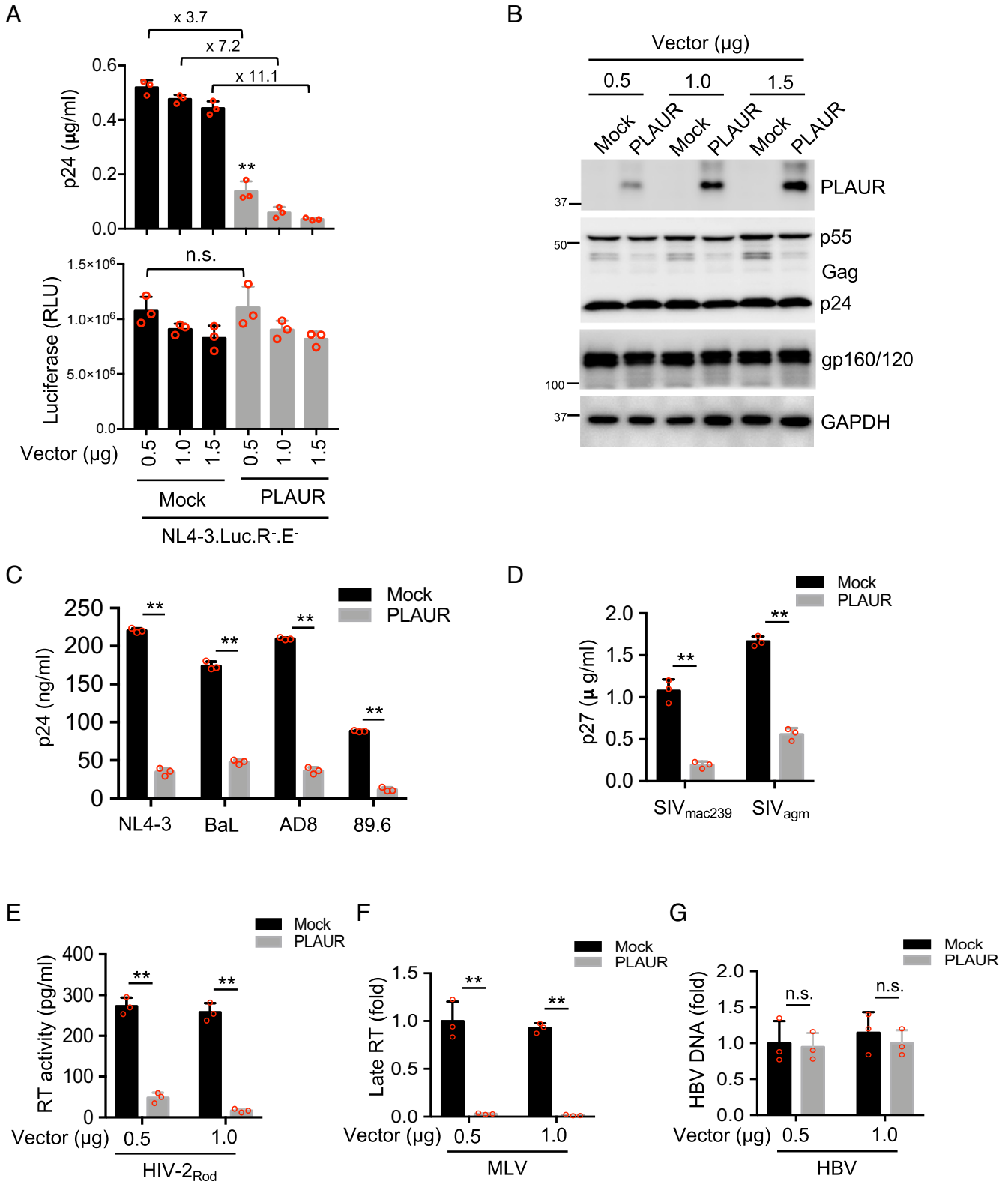

Fig. 2. PLAUR Restricts HIV-1 Production in Producer Cells. (*A* and *B*) 293T cells were transfected with a construct encoding FLAG-tagged PLAUR or a mock expression construct along with HIV-$1_{NL4\text{-}3.Luc.R^-.E^-}$ reporter vectors at the indicated doses. At 48 h after transfection, cells were treated with trypsin and collected by quick centrifugation and lysed to measure the luciferase activity and the HIV-1 capsids in culture were measured using p24 ELISA (*A*). Western blotting was conducted to assess PLAUR, Gag, and GAPDH expression (*B*). $^{**}P < 0.01$ (two-tailed unpaired Student's *t* test), data are plotted as mean ± SEM of three independent experiments. (*C*–*G*) 293T cells were transfected with a construct encoding FLAG-tagged PLAUR or a mock expression construct along with proviral vectors of HIV-1 (*C*), SIV (*D*), HIV-2 (*E*), MLV (*F*), or HBV (*G*, a CMV promoter) as indicated. At 48 h after transfection, the HIV-1 or SIV capsids in culture were measured using p24 or p27 ELISA, respectively. HIV-2 capsids in culture were measured by HIV-2 RT activity. Mouse NIH3T3 cells were infected with the produced MLV, and their late reverse-transcript products were measured by qPCR. The secreted HBV virion-associated DNA genome was measured by qPCR. $^{**}P < 0.01$ (two-tailed, unpaired Student's *t* test), data are plotted as mean ± SEM of three independent experiments. All blotting data are representative of three independent experiments.

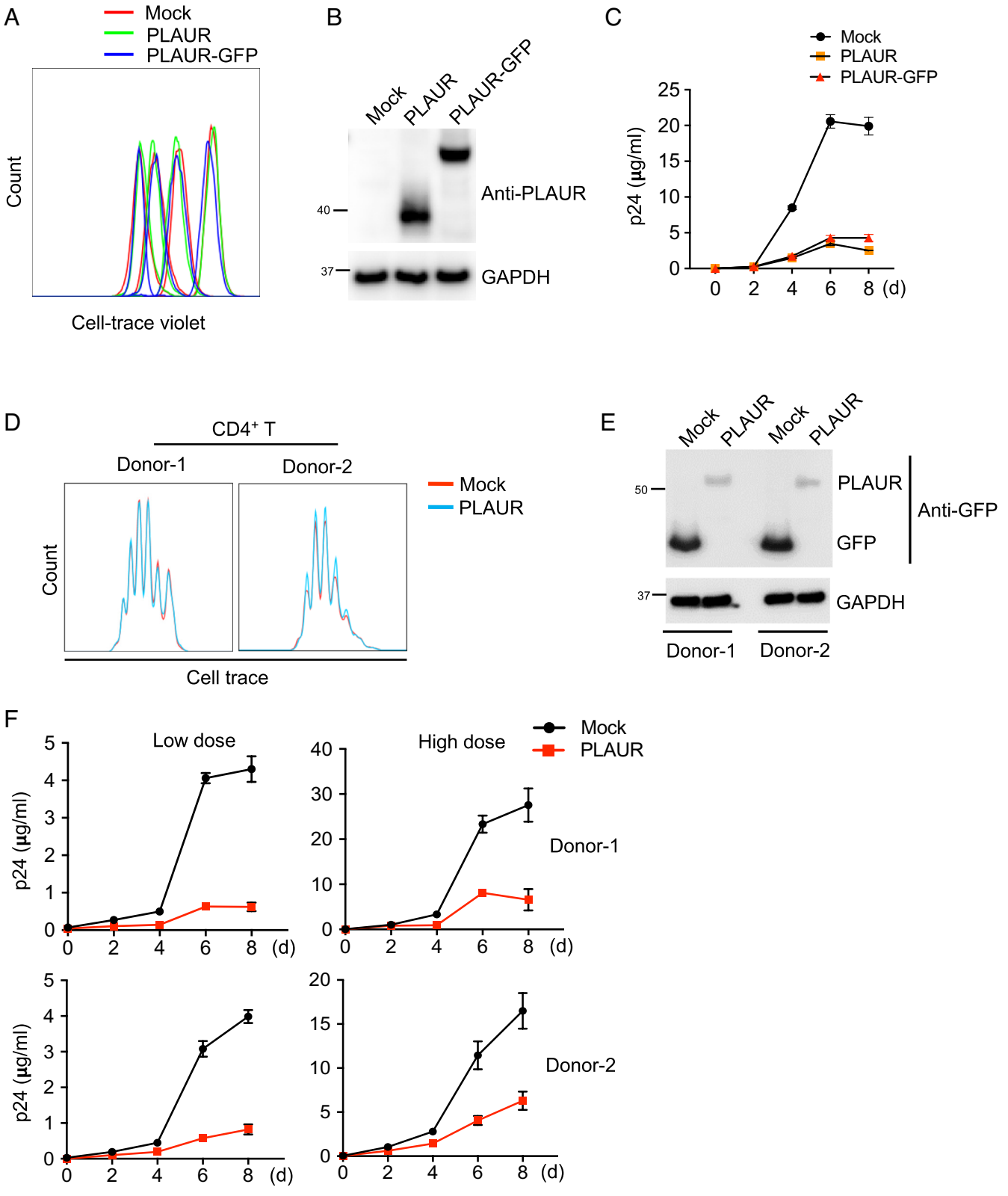

Fig. 3. Overexpression of PLAUR Restricts HIV-1 Spread in T Lymphocytes. (*A*–*C*) Lentiviral transduction of C-terminal GFP-tagged PLAUR, non-Tagged PLAUR, or mock expression vectors in Jurkat cells infected with 10 ng of HIV-1_{NL4-3} for 8 d. Aliquoted cells were stained with CellTrace Violet to assess cell proliferation at days 2, 4, 6, and 8 (*A*). Cells were lysed for western blotting to assess PLAUR-GFP, PLAUR, and GAPDH expression (*B*). The viral production in culture was measured by p24 ELISA at the indicated time points (*C*). Data are plotted as mean ± SD of triplicates and representative of three independent experiments. (*D* and *E*) Lentiviral GFP-tagged PLAUR or Mock (GFP) expression vectors-transduced stimulated $CD4^+$ T cells were infected with 5 (low dose) or 50 (high dose) ng of HIV-1_{NL4-3} for 8 d, and the aliquoted cells were stained by CellTrace Violet to assess cell proliferation (*D*). Cells were lysed for western blotting to assess PLAUR and GAPDH expression (*E*). The viral production in culture was measured by p24 ELISA at the indicated time points (*F*). Data are plotted as mean ± SD of triplicates.

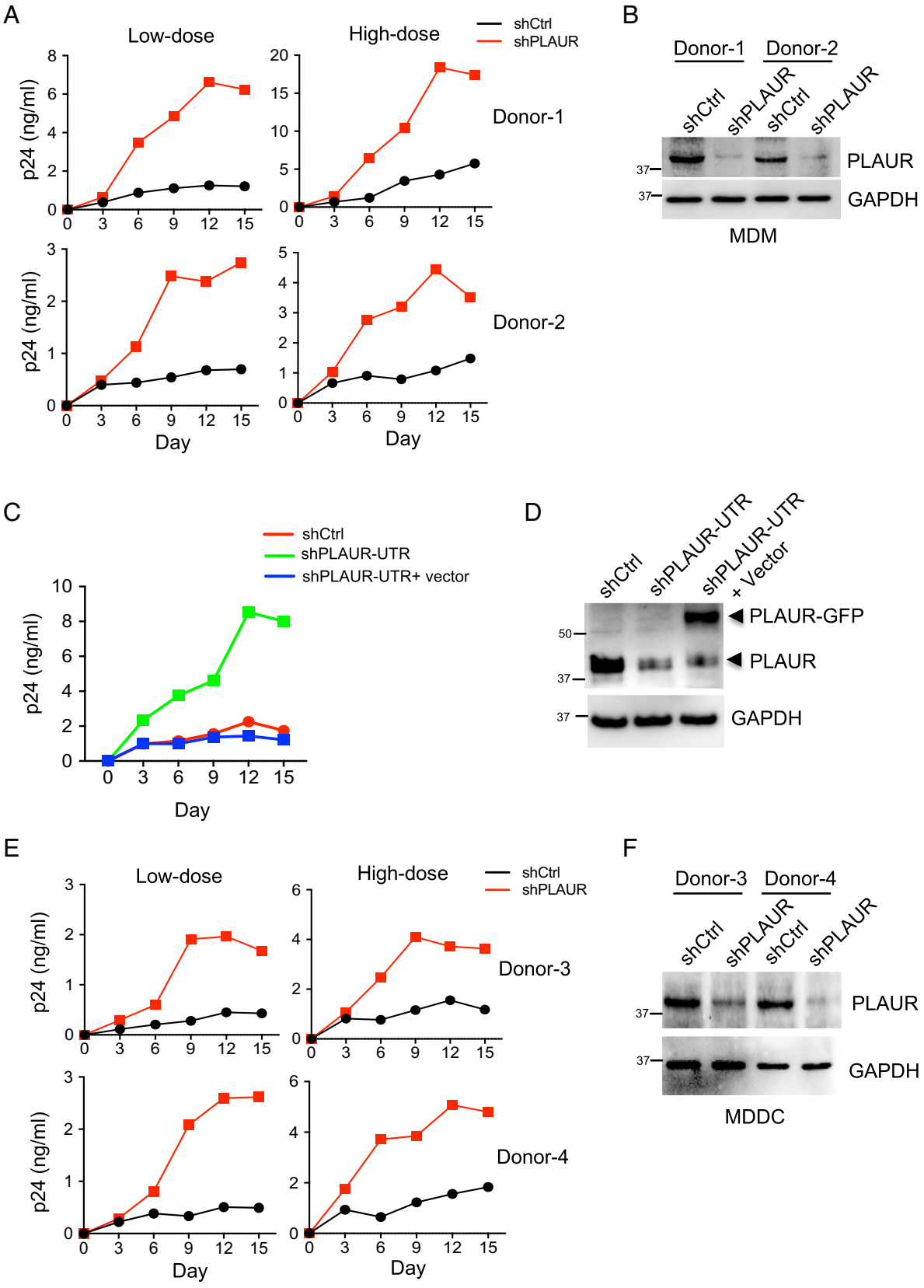

Fig. 4. PLAUR Restricts HIV-1 Transmission in Primary Macrophages and DCs. (*A* and *B*) Lentiviral shRNA-transduced MDMs were infected with 10 or 100 ng of HIV-1_{AD8} for 15 d. Viral production was measured by p24 ELISA at the indicated time points (*A*). Before infection, the aliquoted MDMs were lysed for western blotting to assess PLAUR and GAPDH expression (*B*). (*C* and *D*) MDMs were transduced with lentiviral shRNA targeting the 3′-UTR of the *PLAUR* or control transcript, and the *shPLAUR*-depleted MDMs were then transduced with or without a lentiviral expression vector encoding a GFP-tagged PLAUR. Next, the MDMs were infected with 50 ng HIV-1_{AD8} for 15 d, and viral production was measured by p24 ELISA at the indicated time points (*C*). Before infection, the aliquoted shRNA-transduced MDMs were lysed for western blotting to assess the levels of PLAUR and GAPDH (*D*). (*E* and *F*) Lentiviral shRNA-transduced MDDCs were pretreated with VLP-Vpx and further infected with 10 or 100 ng of HIV-1_{AD8} for 15 d. Viral production was measured by p24 ELISA at the indicated time points (*E*). Before infection, the aliquoted MDDCs were lysed for western blotting to assess PLAUR and GAPDH expression (*F*).

replication cycle by blocking the release of HIV-1 particles from the cell membrane. Notably, the viral protein Vpu counteracts the IFN-induced host restriction factor tetherin (BST2), which blocks HIV-1 release from the cell surface (21). Thereafter, we examined whether Vpu could counteract the inhibitory effect of PLAUR. Data showed that neither Vpu nor Nef could alleviate the PLAUR-mediated restrictive effect on HIV-1 (*SI Appendix*, Fig. S6 *A* and *B*). Therefore, these findings collectively indicate that PLAUR blocks HIV-1 virion release from the cell membrane to inhibit HIV-1 replication.

Notably, the newly formed virions have been shown to accumulate in highly interconnected intracellular compartments in HIV-1 infected macrophages (22–24), which are observed as invaginations of the macrophage plasma membrane (25, 26). As HIV-1 virions bud into these intracellular compartments containing the tetraspanin CD81 (26, 27), we used CD81 to visualize these virus-containing intracellular compartments (VCCs) to investigate the role of PLAUR in HIV-1 accumulation in the VCCs of macrophages. We directly transfected proviral HIV-1 vectors into MDMs to ensure that the virions were produced from the transfected viral vectors. Thereafter, we used a specific anti-p24 antibody to label the produced HIV-1 virions and hence, avoided the staining of incoming virions by using HIV-1 to infect the macrophages. We also stained the macrophages with anti-p24 and anti-CD81 antibodies to visualize produced virions in VCCs in the presence or absence of PLAUR. We observed that the knockdown of PLAUR consistently increased levels of produced HIV-1 virions in the culture (Fig. 5*A*). Similarly, p24CA levels in the virions were increased in the absence of PLAUR (Fig. 5*B*). However, p24CA levels in cell lysates decreased, while levels of pr55-Gag in cell lysates remained unchanged when PLAUR was depleted, thereby indicating that PLAUR restricts HIV-1 release from macrophages. In parallel experiments, puncta of p24CA staining were observed in VCCs and colocalized with CD81 in the presence of PLAUR (Fig. 5 *C* and *D*). However, the p24CA staining was not evidently observed in VCCs and colocalization of p24CA and CD81 was significantly reduced in the absence of PLAUR. Additionally, overall levels of CD81 were unaffected by the presence or absence of PLAUR, suggesting that the depletion of PLAUR has no effect on the formation of VCCs in macrophages. Therefore, the inhibitory effect of PLAUR on HIV-1 replication may result in the accumulation of HIV-1 virions within the VCCs of macrophages.

The Ligand uPA Compromises the PLAUR-Mediated Inhibition of HIV-1 Production. uPA has been shown to inhibit or increase HIV-1 replication in myeloid cells by different mechanisms (16, 17, 28). The inconsistency in results may arise from different culture conditions used for myeloid cells or different timings of uPA treatment. These previous findings prompted us to evaluate the potential contribution of uPA to the antiviral activity of PLAUR. Since uPA has been suggested to affect the HIV-1 envelope (17), we investigated its effect on viral production after infecting MDMs with a VSV-G-pseudotyped reporter HIV-$1_{NL4\text{-}3.Luc.R^-.E^-}$ to sustain a single-round infection. We observed no inhibitory effect of uPA on HIV-1 production, but the same dose slightly increased viral production in primary macrophages and stimulated U937 cells (*SI Appendix*, Fig. S7 *A* and *B*). We also found that uPA at a dose of 6.4 ng/mL resulted in apparent myeloid cell death and deformations within 6 d of treatment (*SI Appendix*, Fig. S7*C*). Probably, these high-dose uPA-induced deformed cells or cell death may indirectly disturb HIV-1 replication, resulting in the inhibition of HIV-1 production in myeloid cells. Meanwhile, we also found that uPA did not affect PLAUR protein levels in macrophages and U937 cells at the indicated dose (*SI Appendix*, Fig. S7 *D–F*). In addition, we obtained similar results for the uPA-treated primary DCs (*SI Appendix*, Fig. S7 *G–I*). Therefore, in our experiments, uPA treatment could slightly promote HIV-1 production within 6 d of infection at the doses we used in this study.

Afterward, we investigated whether this mild increase in HIV-1 production involved uPA interfering with the antiviral activity of PLAUR. Results revealed that PLAUR depletion in primary macrophages consistently promoted HIV-1 production in the absence of uPA (*SI Appendix*, Fig. S8 *A* and *B*). In contrast, PLAUR depletion compromised the uPA-mediated enhancement of HIV-1 production, suggesting that uPA in myeloid cells interferes with PLAUR antiviral activity. Overall, the uPA–PLAUR interaction seems to negate the PLAUR anti-HIV activity to some extent.

Discussion

Myeloid lineage macrophages and DCs can support persistent replication but not high titers of HIV-1, compared with infected T lymphocytes, probably due to different genes encoding anti-HIV-1 factors in myeloid cells. Notably, the uPA–PLAUR interaction has been shown to inhibit HIV replication in myeloid cells (16, 28) and plays an important role in the progression of AIDS (18); however, the antiviral mechanisms involved remain elusive. In the present study, we discovered that PLAUR, mainly expressed on the surface of myeloid cells, acts as a new anti-HIV-1 factor in macrophages and DCs. PLAUR was not upregulated in the presence of IFNs, suggesting that it is not a part of the IFN-mediated antiviral response. PLAUR interfered with the production of different tropic HIV-1 strains, including the CXCR4-tropic NL4-3, the CCR5-tropic BaL, AD8, and the dual-tropic 89.6. In addition, it inhibited HIV-2, SIV, and MLV, but had no effect on HBV production, indicating that it may possess a potentially broad anti-retrovirus activity. HIV-1 spread in $CD4^+$ T cells was also consistently inhibited by overexpressing PLAUR. In contrast, silencing PLAUR resulted in enhanced HIV-1 transmission in primary macrophages and DCs. These data together suggest that PLAUR is a new anti-HIV-1 factor mainly expressed in myeloid cells.

To identify the stage of the HIV-1 life cycle that is inhibited by PLAUR, we observed PLAUR localized at the cell membrane along with HIV-1 Gag protein. Although PLAUR inhibits HIV-1 production, viral proteins, like Gag and Env, are intact in its presence, suggesting that PLAUR may not interrupt HIV-1 progeny assembly, but it may block virion release from the cell membrane. Electron microscopy revealed that HIV-1 virions remained tethered to cell surfaces in the presence of PLAUR. As PLAUR inhibits the production of HIV-1, SIV, and MLV in producer cells in a manner similar to tetherin (21, 29), PLAUR may also act non-specifically to inhibit retrovirus release. As PLAUR is a GPI-modified membrane protein, it may partition into the lipid raft cholesterol-rich domains in the plasma membrane where the assembly and budding of enveloped retroviruses occurs. Thus, PLAUR appears to have a particular propensity for localizing at the sites of viral budding and may be present at the assembly sites of HIV-1 or other enveloped virus particles which have a similar lipid composition. Further studies are required to elucidate the molecular mechanisms underlying the contribution of PLAUR in virion attachment to the cell membrane. Interestingly, unlike tetherin, whose inhibition of HIV-1 release is counteracted by viral Vpu, neither Vpu nor Nef rescued the inhibitory effect of PLAUR on HIV-1 production, indicating that PLAUR interferes with

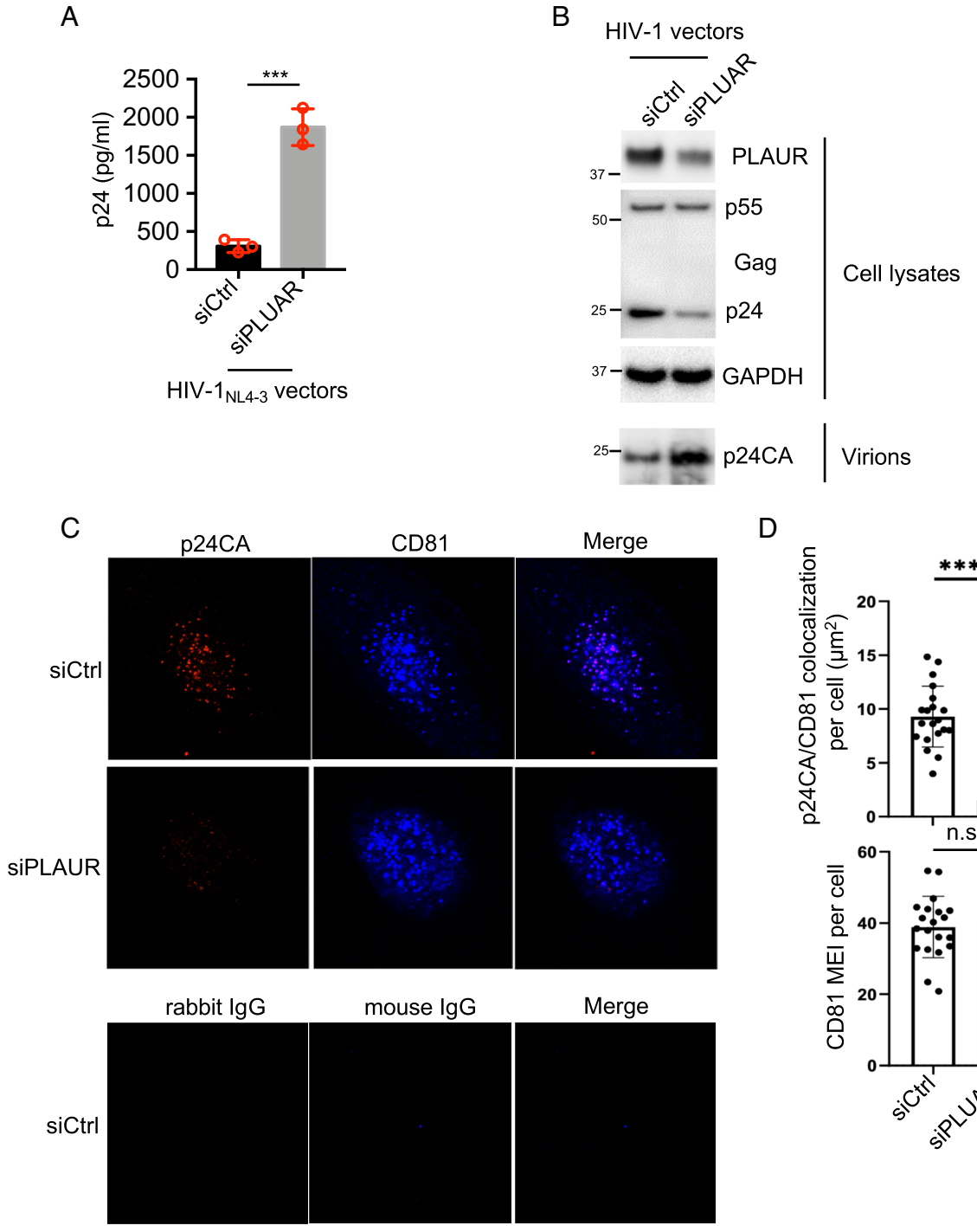

Fig. 5. Depletion of PLAUR Promotes the Release of HIV-1 Virions from Macrophages. (*A*) MDMs were co-transfected with siRNA against PLAUR or control in addition to proviral vectors of HIV-1_{NL4-3}. After 72 h of co-transfection, viral production was measured by p24 ELISA (*A*). ***$P < 0.001$ (two-tailed, unpaired Student's *t* test). Data are plotted as the mean ± SD of triplicates and are representative of three independent experiments. (*B*) Cells were lysed for western blotting to measure PLAUR and GAPDH protein levels using specific antibodies. Virion particles in the supernatants were enriched by sucrose cushion ultracentrifugation and subsequently lysed for western blotting to measure p24CA levels. (*C*) Aliquoted cells from (*B*) were fixed and immunostained with rabbit anti-p24 antibodies, mouse anti-CD81 antibodies, or non-specific rabbit or mouse IgG and again probed with goat anti-rabbit or anti-mouse antibodies conjugated to Alexa Fluor 555 or 647. (*D*) Colocalization between p24CA and CD81 puncta and the mean fluorescence intensity (MFI) of CD81 were calculated from the analysis of 20 cells using ZEN software (ZEN 3.3, Carl Zeiss Microscopy GmbH). Error bars represent the SEM. ****$P < 0.0001$; n.s., not significant (two-tailed, unpaired Student's *t* test). All micrograph data are representative of three independent experiments.

HIV-1 virion release probably by a mechanism different from that of tetherin. In HIV-1-infected macrophages, newly synthesized virions are sequestered into subcellular VCCs which are intracellular plasma membrane-connected compartments (22–26). Similar to the plasma membrane, VCCs also contain cholesterol and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) (26, 30). We did not observe the accumulation of HIV-1 virions in the VCCs of macrophages in the absence of PLAUR; however, the increase in the release of HIV-1 virions indicated that the inhibitory effect of PLAUR may contribute to the accumulation of newly synthesized HIV-1 virions in the subcellular VCCs of macrophages.

In contrast to previous findings (16, 28), the doses of uPA used in this study did not inhibit HIV-1 replication in myeloid cells in a short infection period. Instead, we found that uPA slightly enhanced HIV-1 production within 6 d in primary macrophages and stimulated U937 cells. In these experiments, we used a reporter instead of replication-competent HIV-1 to examine the effect of uPA in a single-round infection because uPA has been shown to affect HIV-1 envelopes and influence viral infection. Since PLAUR blocks HIV-1 release at the late phase of viral replication, the single-round infection system would be more accurate to study the effect of uPA on PLAUR antiviral activity. Unlike previous studies wherein myeloid cells were pretreated with uPA (16, 28), we treated myeloid cells with uPA after HIV-1 infection. In addition, we infected primary macrophages with HIV-1 for 6 d, which is a shorter period compared with the previous studies (16, 28). Collectively, uPA could enhance HIV-1 production in primary macrophages under the experimental conditions used in this study. Importantly, upon silencing PLAUR, uPA no longer enhanced HIV-1 production, suggesting that the uPA-mediated slight upregulation of HIV-1 titers was due to the inhibition of PLAUR antiviral activity in myeloid cells. However, this study did not reveal the mechanism by which uPA compromises restrictive activity of PLAUR to enhance HIV-1 titers in myeloid cells. Accordingly, the effect of uPA binding on the antiviral activity of PLAUR should be clarified in future studies. Interestingly, the amino-terminal fragment (ATF) of uPA, is catalytically inactive but suppresses the release of HIV-1 particles from HIV-1-infected myeloid cell lines via binding to PLAUR (31). Theoretically, ATF may bind to PLAUR and promote conformational changes that increase the antiviral activity of PLAUR, which is also required an investigation in the future.

The uPA–PLAUR system has multiple and bidirectional interactions with chemokines and their receptors; therefore, understanding how uPA compromises the inhibitory effect of PLAUR is important. The uPA–PLAUR interaction might also change the configuration of the receptor, thereby modulating its anti-HIV-1 activity. Since cell–cell transmission of HIV-1 is a major in vivo event, future work also needs to answer whether PLAUR can inhibit the direct transmission of HIV-1 from macrophages or DCs to $CD4^+$ T cells. Overall, our study provides insights into the ability of myeloid lineage cells to interfere with HIV-1 replication through PLAUR. Further elucidation of PLAUR's interaction with HIV-1, HIV-2, and other viruses 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 Hospital of China Medical University. All the blood samples were obtained from healthy donors following the National Health and Medical Research Council guidelines. In addition, informed consent was obtained from each donor before the study. The study protocol and informed consent forms were approved by the Institutional Review Board of China Medical University.

Cells and Cell Culture Reagents. NIH3T3 (mouse), 293T, and TZM-bl cells were cultured in Dulbecco's modified Eagle's edium (Gibco). THP-1 and Jurkat (human) cells were cultured in Roswell Park Memorial Institute-1640 medium (Gibco). Both media were supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin, and 100 mg/mL streptomycin. Plasmids were transfected into the 293T cells using Lipofectamine 2000 (Invitrogen). Peripheral blood mononuclear cells (PBMCs), obtained from healthy blood donors, were purified via Ficoll-Hypaque density gradient centrifugation. $CD4^+$ T cells or monocytes were isolated from the PBMCs via negative selection with human $CD4^+$ T cells or a CD14-positive enrichment cocktail (StemCell Technologies). The $CD4^+$ T cells were stimulated by adding CD3/CD28 activator magnetic beads (Invitrogen) to the culture medium for 2 d with 50 U/mL interleukin-2 (IL-2, Biomol). The isolation and culturing of monocytes, MDMs, and MDDCs were performed as previously reported (32, 33). 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 Iscove's modified Dulbecco's medium (Gibco) supplemented with 10% FBS, 2 mM L-glutamine, 100 IU/mL penicillin, 100 mg/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 Biotec). 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 small interfering RNA into MDMs or MDDCs. uPA was purchased from R&D (Cat.1310-SE-010) and used according to the manufacturer's instructions.

Plasmids. The PLAUR expression vectors were purchased from OriGene or their ORFs were de novo cloned into the pCMV-3Tag-2A vector (Addgene). HIV-1 reporter vectors of NL4-3.Luc.R-E-, and proviral vectors of NL4-3, 89.6, SIV_{mac239}, SIV_{agm}, $HIV\text{-}2_{Rod}$, and MLV were obtained from the NIH AIDS Program. The proviral vector of $HIV\text{-}1_{AD8}$ was gifted by Dr. E. Freed and the MLV-luc reporter vector was gifted by Dr. G. Gao.

RNA Interference in THP-1 Cells, MDMs, MDDCs, or $CD4^+$ T Cells. To achieve shRNA-mediated silencing of *PLAUR* (RHS4430-200306322: 5′-AACCCACACA-CAACCTCGG-3′ targeting ORF; RHS-3′-UTR: 5′-GGAGAGCTCTTGTTATTAT-3′ targeting 3′-UTR) or control (Catalog #RHS4346), a microRNA-adapted shRNA lentivirus was introduced into THP-1 cells, MDMs, and MDDCs, as previously described (32, 33). Briefly, the freshly isolated monocytes were treated with VLP-Vpx and transduced with shRNA lentivirus particles. After puromycin selection, the cells were infected with replication-competent CCR5-tropic HIV-1 and washed twice with cold phosphate-buffered saline (PBS) to remove the input virus 6 h after infection.

Enzyme-Linked Immunosorbent Assay (ELISA) for Virions. p24 or p27 levels in culture supernatants were measured via ELISA according to the manufacturer's instructions (ABL Corporation).

Reverse Transcriptase Assay. HIV-2 virions were purified and the level of virion-associated reverse transcriptase was measured according to the manufacturer's instructions (Reverse Transcriptase Assay, colorimetric, Roche).

Measurement of Late Reverse-Transcript Products of MLV. Mouse NIH3T3 cells were infected with MLV for 24 h, and genomic DNA was extracted for real-time PCR (qPCR) to measure the late reverse-transcript product using specific primers: MLV

5′-CGTCAGCGGGGGTCTTTC-3′ and 5′-CTGGGCAGGGGTCTCCCG-3′, as previously described (33, 34).

Luciferase Detection Assay. Luciferase activity in the cell lysates was quantified as relative luminescence units according to the manufacturer's instructions (Promega).

Isolation of Membrane-Associated Proteins. The isolation of membrane-associated proteins was performed using the Mem-PER™Plus Membrane Protein Extraction Kit (Invitrogen) (32). In summary, cells were harvested from suspension cell cultures by centrifugation at 300× g for 5 min. The obtained cell pellets were washed with 3 mL Cell Wash Solution and centrifuged at 300× g for 5 min. After the supernatants were removed, the cell pellets were resuspended in 1.5 mL Cell Wash Solution, transferred to a new tube, and centrifuged at 300× g for 5 min to discard the supernatants. The resulting cell pellets were mixed with 0.75 mL 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 15 min, and the resultant supernatants containing cytosolic proteins were removed and transferred to a new tube for detection. The pellets were resuspended in 0.5 mL 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 to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected with various antibodies during western blotting.

HIV-1 Virion 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 (ThermoFisher) and layered onto 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 (ThermoFisher) and subjected to SDS-PAGE for western blotting as previously described (20).

HBV Nucleocapsid-Associated DNA Purification. Briefly, the secreted HBV particles were purified from culture supernatants by using PEG8000 (30% in NaCl) precipitation. The purified relaxed circular DNA from the nucleocapsid-associated DNA was obtained after the following treatments: DNase I digestion to eliminate transfected plasmids, proteinase K and SDS digestion, phenol–chloroform extraction, and isopropanol precipitation. HBV DNA was quantified using qPCR analysis with the primer set 5′-GAATTGATGACTCTAGCTACCTG-3′ and 5′-GAAACCACAATAGTTGCCTGATC-3′, as described previously (35).

Western Blotting and Antibodies. Western blotting was performed using the standard method to detect cellular proteins. The antibodies used in this study were as follows: monoclonal mouse anti-PLAUR (Santa Cruz, Cat.sc-376118, 1:1,000), polyclonal goat anti-gp120 (NIH AIDS Reagent Program, Cat.ARP-288, 1:20,000), rabbit anti-GAPDH (Thermo, Cat.PA1-987, 1:1,000), mouse monoclonal anti-FLAG (SIGMA, Cat.F1804, 1:1,000), rabbit polyclonal anti-p24 (Abcam, Cat.ab63913, 1:1,000), mouse Igs-HRP (Abcam, Cat.6789, 1:5,000), rabbit and mouse IgG Trueblot (eBioscience, Cat.18-8816-33,1:1,000; Cat.18-8817-33, 1:1,000), and rabbit or mouse IgG isotype control (Abcam, Cat.18413; Cat.ab18413).

Microscopy. Cells and virions were imaged using a Zeiss LSM 980 microscope with an Airyscan 2 Imaging System and a JEM-1400 Flash electron microscope. MDMs were fixed and stained with rabbit anti-p24 antibodies, mouse anti-CD81 antibodies, or non-specific rabbit or mouse IgG and then visualized with Alexa Fluor 555- or Alexa Fluor 647-conjugated goat anti-rabbit or anti-mouse secondary antibodies according to the manufacturer's instructions (Imag-iT Fixation/Permeabilization kits, Invitrogen). Continuous z-axis images were obtained using a confocal laser-scanning microscopy (LSM 980, Carl Zeiss Microscopy).

qPCR. Total RNA was extracted from cells using TRIzol (Invitrogen), according to the manufacturer's instructions. The obtained RNA was dissolved in 100 μL of diethyl pyrocarbonate-treated water, and 1 μg of the purified RNA was treated with DNase I (amplification grade, Invitrogen) for 10 to 15 min at room temperature, according to the manufacturer's instructions. The RNA was immediately primed with oligo-dT and reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen). qPCR analysis was performed using the ΔΔCT method. The results were normalized against the amplification results for the internal control (GAPDH). The primers used in this study are shown in *SI Appendix*, Table S1.

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 mentioned. Each experiment was performed three times independently, and the data were presented as the mean ± SEM.

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

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