Interferon-inducible LY6E Protein Promotes HIV-1 Infection

Jingyou Yu1*, Chen Liang2*, and Shan-Lu Liu3,4,5

From the 1Center for Retrovirus Research, 2,5Center for Microbial Interface Biology, and 3Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio 43210, the 4McGill AIDS Centre, Lady Davis Institute, Montreal, Quebec H3T 1E2, Canada, and the 5Department of Microbiology and Immunology, McGill University, Montreal, Quebec H3A 2B4, Canada

Interferon-mediated innate immunity is crucial for elimination of microbial infection. Upon viral infection, IFN is induced, which activates hundreds of effectors, collectively known as interferon-stimulated genes (ISGs), to combat pathogen invasion (1, 2). IFN has been shown to be a potent HIV inhibitor in cell culture systems and can transiently decrease HIV viral load in AIDS patients (3, 4). However, in chronically infected individuals, HIV-1 viral load positively correlates with the expression of type I IFN and ISGs (4). The mechanisms underlying these distinct effects of IFNs and ISGs in HIV infection and AIDS patients are currently poorly understood.

Lymphocyte antigen 6 complex, locus E (LY6E), also known as thymic shared antigen 1 (TSA-1), stem cell antigen 2 (SCA-2), and retinoic acid-induced gene E (RIG-E), is an IFN-inducible protein belonging to the Ly-6/uPAR family, whose members share structural and functional similarities. The C terminus of this family protein contains a glycosylphosphatidylinositol (GPI) anchor, which allows them to attach to the lipid raft microdomain of the plasma membrane, critical for their biological functions. LY6E was initially characterized as a thymocyte marker that discriminates between immature and mature T cell subsets because its expression inversely correlates with T cell receptor (TCR/CD3) (5). Up to now, LY6E has been shown to actively modulate TCR-mediated signaling, T cell activation, and development, possibly through its interaction with the TCR/CD3ε chain (6–9). Other physiological roles of LY6E include cellular adhesion, migration, and cytokine production (10, 11). Clinically, systemic lupus erythematosus patients have been shown to express a higher level of LY6E in peripheral blood mononuclear cells (PBMCs) compared with healthy individuals (12–14). In cell culture systems, LY6E down-regulates the CD4/TLR4 signaling pathway, which partly explains its regulatory function in the immune response to pathogen infection (15).

The role of LY6E in viral infection has not been well studied. One early report showed that chicken LY6E specifically interacts with Marek’s disease virus US10 protein and confers chicken resistance to Marek’s disease virus infection (16). LY6E has also been shown to restrict VSV replication in HEK293T cells (17). However, other studies demonstrated that the susceptibility of mice to mouse adenovirus type 1 is partially determined by one or more members of the LY6 family (18, 19), indicating that LY6 could play a positive role in viral infection. In addition, LY6E has been reported to promote the replication of yellow fever virus in STAT1−/− fibroblasts and, to a lesser extent, in Huh-7 cells (20, 21). Knocking down LY6E in HeLa cells reduces susceptibility to West Nile virus infection (22). The role of LY6E in HIV-1 infection has remained murky. A whole-genome analysis identified a SNP, rs2572886, in the LY6E/uPAR family that is associated with susceptibility to HIV-1, possibly because of increased LY6E expression (23).

In this study, we show that HIV-1 infection in human PBMCs induces LY6E expression, which, in turn, promotes HIV-1 replication. Mechanistically, we find that LY6E enhances HIV-1 entry and gene expression. Our work reveals a positive role of

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† To whom correspondence should be addressed: Center for Retrovirus Research, Center for Microbial Interface Biology, Dept. of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210. Tel.: 614-292-8690; E-mail: liu.6244@osu.edu.

2 The abbreviations used are: ISG, interferon-stimulated gene; GPI, glycosylphosphatidylinositol; TCR, T cell receptor; PBMC, peripheral blood mononuclear cell; VSV, vesicular stomatitis virus; HIV-1, HIV, type 1; PI-PLC, phosphoinositide phospholipase C; JSRV, Jaagsiekte sheep retrovirus; CPZ, chlorpromazine; Luc, luciferase; PMMA, phorbol 12-myristate 13-acetate; TRITC, tetramethylrhodamine isothiocyanate; Ab, antibody; IFITM, interferon-inducible transmembrane protein.

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LY6E in HIV-1 infection, which could have implications for AIDS pathogenesis.

**Results**

*HIV-1 Infection Induces LY6E Expression*—Viral infection often triggers the host innate immune response, leading to the production of type I IFN and downstream effectors. We examined whether HIV-1 infection in human PBMCs can induce type I IFN production and LY6E expression. We infected phytohemagglutinin/IL2-activated PBMCs with HIV-1 NL4.3 bearing VSV-G for a period of 48 h and quantified the mRNA levels of LY6E and representative ISGs by qRT-PCR. We observed that HIV-1 infection up-regulated the expression of IFN-β, LY6E, and ISG15 after 24 h of infection and continued to increase at 48 h (Fig. 1, A–C). Of note, the CXCL10 level, which is normally induced by IFN-γ, did not show apparent change following HIV-1 infection (Fig. 1D), similar to a previous report (24). We noted that the up-regulation of IFN-β, LY6E, and ISG15 correlated with the expression of HIV-1 Gag in infected cells (Fig. 1E), suggesting that HIV infection likely triggers a type I response and, therefore, induces LY6E expression in activated human PBMCs. To confirm this latter effect, we treated PBMCs with interferon α receptor-neutralizing antibody (α-IFNRA1) for 24 h (10 μg/ml) and observed that the expression of IFN-β, ISG15, and LY6E induced by HIV-1 infection was completely abolished (data not shown), suggesting that LY6E induction following HIV-1 infection is indeed IFN signaling-dependent.

*Knockdown of Endogenous LY6E Impairs HIV-1 Infection*—We next examined the role of endogenous LY6E in HIV-1 infection. To this end, we transduced activated human PBMCs

FIGURE 1. HIV-1 infection induces LY6E expression in human PBMCs. A–E, HIV-1 NL4.3-bearing VSV-G was used to infect activated human PBMCs (pooled from two healthy donors) for different periods of time, and IFN-β (A), LY6E (B), ISG15 (C), CXCL10 (D), and HIV-1 GAG (E) mRNA expression levels were examined by qRT-PCR. PBMCs not infected with HIV-1 were set as a negative control. The mRNA copy number of each gene at time point 0 was set to 1 for comparison. Results are the means ± S.D. of three independent experiments.
with lentiviral vectors expressing shRNA against LY6E or control shRNA, either in the presence or absence of IFN-α, and infected these cells with HIV-1 NL4.3-bearing VSV-G (to increase the infection efficiency of the first round) for a total of 55 h; intracellular p24, HIV-1 production, as well as the infectivity of newly produced virions were measured. No deleterious effect on cell proliferation was observed in LY6E shRNA-transduced cells compared with control shRNA (data not shown). The knockdown efficiency of LY6E was confirmed by flow cytometry using an anti-LY6E antibody, showing that both shRNA clones (LY6E-C1 and C2) reduced LY6E expression in the cell (Fig. 2A) (mean florescence intensity in the absence of IFN: 3950 for control shRNA, 1477 for shRNA LY6E-C1, and 1518 for shRNA LY6E-C2). Interestingly, we noticed that treat-
ment of cells with IFN-α2b modestly up-regulated LY6E expression in PBMCs (∼20% increase based on geometric mean measurements) (Fig. 2A, see mean fluorescence intensity in the legend), possibly because of the mixed target cell populations treated and other experimental conditions. In the absence of IFN-α, knockdown of LY6E decreased the intracellular p24 level of HIV-1 by 30–50%; similar extents of decrease of HIV-1 infection were noticed in IFN-α-treated LY6E knockdown cells (Fig. 2, B and C). Release of newly produced HIV-1 virions was measured by an RT assay, showing that knockdown of LY6E decreased HIV-1 production by 20–50% compared with cells expressing control shRNA, regardless of IFN-α treatment (Fig. 2D). The same virus stocks with equivalent amounts were also used to infect indicator HeLa-TZM cells, which showed similar extents of inhibition in shRNA LY6E knockdown cells, suggesting that LY6E does not appear to change the HIV-1 infectivity, at least in human PBMCs (Fig. 2E). Collectively, these data imply that endogenous LY6E supports HIV-1 infection in primary human PBMCs.

**HIV-1 Replication Is Diminished in CD4⁺ T Lymphoid Cells Expressing LY6E shRNA**—To determine whether LY6E plays a role in prolonged HIV-1 replication or cell-cell spread, we generated two SupT1 stable cell lines expressing shRNA LY6E (LY6E shRNA-C1 and C2, respectively). We first confirmed the knockdown efficiency of LY6E in these cells by qRT-PCR, showing that LY6E expression in both cell lines was reduced, i.e. ∼20% and ∼60% for C1 and C2, respectively (Fig. 3A). Flow cytometric analysis showed that LY6E protein on the cell surface was also decreased by shRNA (Fig. 4E without PI-PLC treatment). B, for the short-term replication assay, HIV-1 NL4.3 bearing VSV-G was used for infection for 6 h, and viral replication was determined after an additional 48 h of infection in the presence or absence of IFN-α. Relative data are shown in A and B by setting the values of shRNA control, either with or without IFN-α, to 1.0, and the results are means ± S.D. of five independent experiments. *, p < 0.05; **, p < 0.01. C, for the long-term replication assay, 5 ng of NL4.3 was used for infection, and cell supernatants were collected every 2 days, with viral production monitored by measuring the absolute RT activity. Note that one representative experiment is shown for the long-term replication.

**LY6E Does Not Affect HIV-1 Binding to Target Cells**—We interrogated the possible steps of HIV-1 infection that are affected by LY6E. We primarily focused on viral entry because LY6E is a GPI-anchored protein and is expressed on the plasma membrane. We infected the stable SupT1 cell lines expressing
FIGURE 4. Depletion of LY6E in SupT1 cells decreases HIV-1 entry. A, entry of infectious HIV-1 bearing NL4.3 Env or VSV-G was determined by using the BlaM-Vpr assay. B, entry of HIV-1 lentiviral pseudotypes bearing HIV-1 Env or VSV-G was determined by using the pLenti-puro-GFP vector. In both cases, SupT1 cells stably expressing shRNA control or LY6E were used as target cells for infection. C and D, representative flow cytometric profiles of pLenti-puro-GFP infection. E and F, the effect of PI-PLC on the expression of LY6E on the cell surface was determined by flow cytometry. No Ab., without primary and secondary antibodies; 2nd Ab., second antibody alone. G, the effect of PI-PLC on entry of pLenti-puro-GFP bearing HIV-1 Env or VSV-G was determined by measuring the GFP signals of infected cells using flow cytometry 48 h after infection. Relative data are shown by setting the values of shRNA control to 1.0. Results are the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.
shRNA LY6E with HIV-1 BlaM-Vpr virions bearing either NL4.3 Env or VSV-G and determined the viral uptake by flow cytometry. Although viral entry mediated by NL4.3 Env was decreased in LY6E knockdown cell lines, i.e. by ~20% to 50%, VSV-G-mediated HIV-1 entry was unaffected (Fig. 4A). Similar results were also obtained in a single-round HIV-1 infection assay using an HIV-1 lentiviral vector encoding GFP (pLenti-puro-GFP) bearing NL4.3 Env or VSV-G (Fig. 4, B–D). We next treated SupT1 cells with phosphoinositide phospholipase C (PI-PLC), a chemical that is known to cleave GPI-anchored proteins on the plasma membrane (Fig. 4E). We observed that, although PI-PLC generally reduced HIV-1 entry, the inhibitory effect of LY6E knockdown on HIV-1 entry, but not that of VSV, disappeared (Fig. 4, F and G). Altogether, these results indicate that LY6E specifically promotes HIV-1 but not VSV entry and that the surface expression of LY6E is critical for this functional effect.

To dissect in detail the steps of HIV-1 entry possibly affected by LY6E, we next used HIV-iGFP, an infectious NL4.3 clone that contains an internal GFP within Gag (25), and examined HIV-1 binding and trafficking in target cells expressing shRNA LY6E. We incubated the purified HIV-iGFP virions with SupT1 cells at 4 °C for 2 h, and, following extensive washes, flow cytometry was carried out to quantify the GFP fluorescence signal on the cell surface; Env-deficient HIV-iGFP particles served as a negative control. However, we did not observe any significant differences between control and shRNA LY6E-expressing cells in HIV-iGFP virion binding (Fig. 5A). To confirm this result, we infected the SupT1 cells with WT NL4.3

FIGURE 5. Knockdown of LY6E has no effect on HIV-1 binding to CD4 or target cells but reduces Env-mediated membrane fusion. A, virus-binding assay. HIV-iGFP virions were allowed to bind to SupT1 cells expressing shRNA control or shRNA LY6E at 4 °C for 2 h. After extensive washes, GFP-positive cells were quantified by flow cytometry. HIV-iGFP particles containing no Env were used as a negative control. B and C, Env-CD4 binding assay. Cells were infected with adjusted amounts of HIV-1 NL4.3 to achieve an equivalent gp120 expression on the cell surface (C). Soluble CD4-Ig fusion proteins were used as primary antibodies for flow cytometry. D, effect of LY6E knockdown on the cell surface expression of CD4 and CXCR4. Anti-CD4 and anti-CXCR4 antibodies were used as primary antibodies for flow cytometric analysis. E, effect of LY6E knockdown on HIV-1 entry kinetics. SupT1 cells were spinoculated with HIV-iGFP virions at 4 °C for 1 h and switched to 37 °C to initiate infection. At the indicated time points, cells were trypsinized and subjected to flow cytometric analysis. F, effect of LY6E knockdown on cell-cell fusion. HIV-1 Tat and the indicated Env plasmids were co-transfected into 293T cells. These cells were cocultured with HeLa-TZM cells expressing shRNA control or LY6E at a 1:1 ratio for 16 h. Cocultured cells were lysed, and their firefly luciferase activities were measured. G, effect of CPZ treatment on cell-cell fusion affected by LY6E knockdown. In the Env-mediated cell-cell fusion assay, 0.5 mM CPZ was applied to cocultured cells, and cell-cell fusion efficiency was determined. Relative data are shown by setting the values of shRNA control to 1.0. Results are the means ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.
and measured the Env binding to soluble CD4 (CD4-Ig) on the cell surface. Note that the amounts of HIV-1 virions used were adjusted for infection to ensure that the gp120 expression on the cell surface would be comparable between control and shRNA LY6E cells. Again, we found no difference in soluble CD4 binding (Fig. 5, B and C). The expression levels of CD4 and co-receptor CXCR4 on the surfaces of these target cells were examined, showing no apparent differences between shRNA control and shRNA LY6E cells (Fig. 5D). Although Env-CD4 binding does not exactly mimic the virus-cell binding, the result showed that LY6E does not affect the Env-CD4 interaction on the cell surface.

**Knockdown of LY6E Impedes HIV-1 Uptake and Env-mediated Membrane Fusion**—We next examined whether the entry kinetics and/or membrane fusion of HIV-1 are affected by LY6E. Following extensive washes of unbound virions, we shifted the HIV-iGFP-cell complex to 37 °C for various periods of time to allow viral uptake into target cells. At each time point, non-internalized HIV-1 virions on the cell surface were removed by trypsin, and cells were examined for GFP signals of non-internalized viral particle by flow cytometry. As shown in Fig. 5E, HIV-1 uptake appeared to plateau in shRNA control cells after 60 min of the temperature switch, but there was an ~50% reduction in LY6E knockdown cells compared with the control (Fig. 5E).

We then performed a cell-cell fusion assay to determine whether HIV-1 fusion is influenced by LY6E. To this end, we cotransfected the HIV-1 Tat and Env derived from either BH10, NL4.3, or AD8 strains into HEK293T effector cells and cocultured these cells with target HeLa-TZM cells stably expressing shRNA control or shRNA LY6E. VSV-G was shown to be unaffected by LY6E in SupT1 cells (Fig. 4, A and B). Similar to the results shown in Fig. 3B, knockdown of LY6E in SupT1 cells resulted in decreased RT activity (Fig. 6A) in this single-round infection assay, although the extent of the relatively low knockdown efficiency of LY6E in HeLa-TZM cells (around 10–20%) based on qPCR (data not shown), we observed a 2- to 3-fold reduction in Env-mediated cell-cell fusion, particularly for shRNA-C2 HeLa-TZM cells (Fig. 5F).

Note that, in these experiments, we used Jaagsiekte sheep retrovirus (JSRV) Env, which is known not to render fusion in HeLa-TZM cells because of the lack of a functional Hyal2 receptor, as a negative control (Fig. 5F). Taken together, these results indicate that HIV-1 Env-mediated fusion is impaired in LY6E knockdown cells.

**LY6E Enhances HIV-1 Gene Expression**—To determine the possible post-entry steps of HIV-1 infection that are affected by LY6E, we utilized an Env-defective NL4.3-based HIV-1 vector (NL4.3-KFS) (28), which allows measurement of single-round infection and gene expression when pseudotyped with VSV-G. VSV-G was shown to be unaffected by LY6E in SupT1 cells (Fig. 4, A and B). Similar to the results shown in Fig. 3B, knockdown of LY6E in SupT1 cells resulted in decreased RT activity (Fig. 6A) in this single-round infection assay, although the extent

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**FIGURE 4.** Knockdown of endogenous LY6E attenuates LTR-driven gene expression. A and B, effect of LY6E knockdown on HIV-1 gene expression. HIV-1 NL4.3-KFS (Env-deficient) pseudotypes bearing VSV-G were used to infect SupT1 cells expressing shRNA control or shRNA LY6E. A, viral release was determined by measuring the RT activities. B, HIV-1 NL4.3 Gag (top panel) was examined by Western blotting; β-actin served as a loading control (bottom panel). C and D, effect of LY6E knockdown on LTR-driven luciferase gene expression. C, NL4.3-Luc R-E- pseudotypes bearing VSG-G was used to infect cells. D, LTR-driven luciferase gene expression was measured 48 h post-infection. An HIV-LTR-Luc vector was transfected into SupT1 cells together with HIV-1 Tat and pRLTK. Cells were lysed, and firefly luciferase activity was measured 48 h post-transfection. Relative data are shown by setting the values of shRNA control to 1.0. Results are means ± S.D. of five independent experiments. *, p < 0.05; **, p < 0.01.
was less compared with that of multi-round replication. Noticeably, the expression of HIV-1 Gag and Env in SupT1 cells expressing shRNA LY6E, in particular clone 2 (LY6E-C2), was decreased, which correlated with their RT activity and LY6E knockdown efficiency (Fig. 6B). To confirm the effect of LY6E on HIV-1 gene expression, we used another pNL4.3Luc-R’ E- vector (29) in which the Vpr and Env genes are defective and whose Nef gene is fused with a firefly luciferase gene; this vector allows the luciferase activity to be measured as an indicator of LTR-driven gene expression. As shown in Fig. 6C, knockdown of LY6E resulted in a 2- to 3-fold reduction in luciferase activity (Fig. 6C). Similar results were also obtained by using another luciferase-based HIV-1 vector, HIV-LTR-Luc (Fig. 6D). Collectively, these results indicate that LY6E up-regulates HIV-1 gene expression in addition to promoting viral entry, although the detailed mechanism needs to be elucidated.

**LY6E Promotes the Replication of HIV-1 AD8 in THP-1 Cells**—The results presented so far were mostly from human primary PBMCs or CD4-positive T cell lines with CXCR4-using NL4.3 HIV-1. Thus, it would be interesting to determine whether the effects of LY6E in these cells can also be observed in monocytes and/or macrophages, especially with CCR5-using HIV-1. THP-1 is a monocyte cell line that expresses a high level of LY6E (15). We thus applied a lentiviral vector transduction strategy and established two stable THP-1 cell lines expressing shRNA against LY6E. The knockdown efficiency of LY6E in these THP-1 cells was similar to that observed in SupT1 cells (Fig. 3A), again with shRNA-LY6E-C2 exhibiting higher efficiency compared with shRNA-LY6E-C1 (data not shown). We first tested infection of HIV-1 NL4.3 bearing VSV-G in these shRNA LY6E cells, and we observed that the viral production was reduced by ~2-fold (Fig. 7A). We used equivalent amounts to infect HeLa-TZM cells, and we observed similar extents of decrease in viral infectivity in shRNA LY6E cells (Fig. 7B). As a control, we found that triple knockdown of IFITM1, 2, and 3 (shRNA IFITMs) increased the RT activity and infectivity, consistent with our previous report as expected (Fig. 7B) (30).

We next treated THP-1 cells with PMA to differentiate them into macrophages and infected these cells with CCR5-using HIV-1 NL4.3(AD8) (AD8 Env in the backbone of NL4.3 provirus) (31). Similar to HIV-1 NL4.3, the RT activity and viral infectivity of AD8 virus in both shRNA LY6E cell lines were decreased (Fig. 7, C and D). Noticeably, the extent of the inhibitory effect of LY6E knockdown on AD8 infectivity was more pronounced than its effect on viral production, suggesting that LY6E may have additional effects on the infectivity of HIV-1 NL4.3 (AD8) virus. Taken together, these results demonstrate that LY6E actively regulates HIV-1 replication in monocytes as well as in monocyte-derived macrophages.

**Discussion**

In this study, we provide evidence that LY6E is a positive modulator of HIV-1 infection in primary human PBMCs, immortalized CD4+ T lymphoid cells, and macrophages. Our
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results lend support to a recent study showing that up-regulation of LY6E correlates with severe HIV-1 infection and rapid progression to AIDS (15). However, different from the mechanism of this prior study showing that LY6E down-regulates CD14 expression, therefore dampening LPS-stimulated host innate immunity to HIV-1 infection (15), we find in this work that LY6E promotes HIV-1 entry into host cells and up-regulates viral gene expression, therefore increasing viral replication.

The effect of LY6E on HIV-1 entry we have discovered in this study is probably not surprising, given that LY6E is a GPI-anchored protein that is predominantly expressed on the plasma membrane and likely localized in lipid rafts. It is well established that lipid rafts are critical for HIV-1 CD4 receptor- and coreceptor-mediated cell signaling and essential for HIV-1 entry (32–34). Moreover, some GPI-anchored proteins have been shown to promote viral infection. For example, THY-1/CD90 and human Hyal2 enhance infection of human CMV and JSRV by directly associating with viral envelope glycoproteins (35, 36). However, it cannot be inferred that all GPI-anchored proteins would have the same effect as LY6E on HIV-1 infection. It remains to be determined whether LY6E may directly associate with HIV-1 Env, but we have shown in this study that LY6E does not modulate the expression levels of CD4 and CXCR4 on the cell surface, nor does it alter their interactions with HIV-1 Env. Interestingly, we have found that the membrane fusion capability of HIV-1 Env is enhanced by LY6E. Because HIV-1 hemifusion is not affected by LY6E, we speculate that fusion pore expansion of HIV-1 Env could be affected by LY6E. More experiments are needed to determine how exactly LY6E promotes HIV-1 fusion and entry.

IFN-mediated signaling plays critical roles in host antiviral immunity, largely by up-regulating the expression of thousands of ISGs that act on the different steps of viral replication. For instance, IFITM proteins inhibit viral entry and cell-to-cell infection, Tetherin (Bst2) blocks viral release, and MX2 impedes HIV-1 core delivery into the nucleus (30, 37–41). However, accumulating data have shown that IFNs and some ISGs could function as positive regulators to enhance viral infection. One noticeable example is that IFN-α, IFN-γ, and IFN-α efficiently promote infection by human coronavirus HCoV-OC43, and, among the ISGs shown to be associated with this phenotype, IFITM proteins have been demonstrated to promote HCoV-OC43 entry with unknown mechanisms (42). Another example is adenosine deaminases acting on RNA 1 (ADAR1) protein, which has been shown to promote infection by HIV-1, measles virus, VSV, hepatitis D virus, and equine infectious anemia virus (1, 43–45) either by inhibiting the activity of double-stranded RNA-activated protein kinase R or IFN regulatory factor IFR3 or by enhancing viral protein expression through associations with the LTR and Rev response element regions of the lentiviruses.

Indeed, we show in this study that LY6E can also increase HIV-1 gene expression. One possibility is that LY6E, either in the form of RNA or protein, increases the activities of some transcription factors recruited to the HIV-1 LTR promoter region that is important for viral gene expression. Alternatively, LY6E protein could function as a suppressor of intracellular cellular signaling cascades, including the type I interferon signaling pathway that normally suppresses viral infection, therefore enhancing HIV-1 replication. In the latter case, LY6E could negatively regulate host innate immunity and prevent type I IFN-induced inflammation. Indeed, LY6E has been shown recently to dampen the TLR4-mediated pro-inflammatory response by down-regulating CD14, a key molecule involved in the LPS-triggered inflammatory signaling pathway (15). It has been increasingly recognized that the host innate immune response must be finely regulated to maintain normal cellular hemostasis and to minimize viral infection-induced pathogenesis.

Although we show in this study that LY6E promotes HIV-1 infection, the functional roles of LY6E in other viral infections could be more complicated and require further investigation. It is also important to emphasize that the effects of LY6E are sometimes virus strain-dependent and cell type-specific (20). For instance, LY6E can significantly enhance the replication of yellow fever virus in STAT−/− fibroblasts, but its effect in HuH7 cells is modest (20). Hence, it would be important to discover cellular factors that directly associate with LY6E, possibly underlying the cell type-specific effects of LY6E. It would also be interesting to examine whether other LY6/Ly-6/uPAR members, such as LY6A, LY6C, LY6D, and LY6M, could have similar or different functions in HIV and other viral infections. From this perspective, the results from this study should facilitate new avenues of investigation that will lead to a better understanding of the role of LY6E in AIDS pathogenesis and other viral infections.

Materials and Methods

Plasmids and Reagents—LY6E mission shRNA was purchased from Sigma (clone 1, TRCN0000275445; clone 2, TRCN0000155033). CPZ stock was 50 mM and purchased from Sigma. Antibodies against HIV-1 Gag p24 (catalog no. 1513), HIV-1 Env gp120 (catalog nos. 288 and 526), CD4 (catalog no. 724), and CXCR4 (catalog no. 4083) were all obtained from the NIH AIDS Reagents Program. IFN-α was purchased from Invitrogen. IFN-α receptor-neutralizing antibody (catalog no. ab10739) was purchased from Abcam.

Quantitative RT-PCR—For HIV-1-infected PBMCs, cells were collected 0, 12, 24, 36, and 48 h after infection; total RNA was extracted using the RNeasy mini kit (Qiagen). Real-time PCR was conducted by using equal amounts (50 ng) of total cellular RNA using the following primer pairs: LY6E, 5’-CTC-CAGGCAGGACCGCCATC and 5’-CGAGATTCCCAAT-GCCGGCACT; human IFN-β, 5’-AGG ACAGGATGAACT TTGAC and 5’-TGATAGACATAGCCAGG AG; human ISG15, 5’-CGCAGACTCCGCCAAGATGC and 5’-TTC-GTCGGATTTCGATCCA; human CXCL10, 5’-GCGAT- TCAAGAGTACCTC and 5’-TTTGAGCAATGCTCACA-CAG; and HIV-Gag, 5’-GCGAGAAGTTTGGCTGAAG and 5’-CACATTTCCACGGCCCTT. The RT-PCR reactions were performed according to the instructions of the manufacturer of the one-step kit (Power SYBR Green RNA-to-C_T, ABI).

Cells and Viruses—HEK293T and HeLa-TZM cells were maintained in DMEM supplemented with 10% (v/v) FBS. SupT1, THP-1, and their derivative cells were cultured in RPMI
1640 medium with 10% FBS. Lentiviral pseudotypes expressing shRNA control or shRNA LY6E were generated by co-transfecting 293T cells with VSV-G, HIV-1 Gag-pol, and pLenti-based vectors at a ratio of 0.5:1:1. Viruses in the supernatants were collected every 12 h. The collected viruses were used to transduce SupT1 or THP-1 cells, and positive cell populations were selected by using 1 µg/ml puromycin.

Infectious HIV-1 stocks were prepared by transfection of 293T cells with the proviral constructs NL4.3, NL4.3(AD8) (31), or BH10; the VSV-G-encoding plasmid was co-transfected with HIV-1 proviral DNA at a ratio of 1:5 to produce VSV-G-bearing HIV-1 particles, which solely facilitates the first round of viral entry (46, 47). HIV-1 Env-defective HIV-1 stocks were made by co-transfection of 293T cells with pNL4.3 (KFS) (28) or pNL4.3-Luc-R-E- (29) along with VSV-G or HIV-1 Env. pLenti-puro-GFP-based pseudotypes were produced by transfection of 293T cells with plasmids encoding NL4.3 Env or VSV-G together with pLenti-puro-GFP and HIV-1 Gag-PolΔ8.2 at a ratio of 0.5:1:1.

**SupT1 Transfection**—SupT1/shRNA control or SupT1/shRNA LY6E cells (1 x 10^6) were transfected with 500 ng of pSV-Tat, 1 µg of pNL4.3-LTR-Luc, and 1 µg of pRLTK by using Lipofectamine LTX reagent (Invitrogen). 48 h post-transfection, cells were lysed, and firefly and Renilla luciferase activity was determined by using a Dual-Luciferase kit (Promega).

**Cell-Cell Fusion**—Viral envelope (NL4.3, AD8, BH10, JSRV, or VSV) plasmids were cotransfected with pSV-Tat into 293T effector cells. 24 h post-transfection, cells were detached with 5 mM EDTA/PBS for 10–20 min. Target HeLa-TZM cells expressing shRNA control or LY6E were detached with 5 mM EDTA/PBS solution for 20–30 min. Equal numbers of 293T cells and HeLa-TZM cells (2 x 10^5) were mixed thoroughly and seeded onto 24-well plates; the firefly luciferase activity in the cells was measured 16 h after coculture. When needed, DMEM containing 0.5 mM CPZ was applied to mixed cells for 1 min and then switched to normal DMEM.

**Virion-Cell Fusion**—The BlaM-Vpr-based HIV-1 entry assay was used for virion-cell fusion as described previously (48). Briefly, SupT1 cells were spinoculated with HIV-1 at 1680 x g at 4 °C. CCF2 substrate was loaded into cells 1 h after infection, and then cells were washed three times with PBS. Cells were maintained in CO2-independent medium (plus 0.5 mM proline) overnight before being fixed with 3.7% formaldehyde and analyzed by flow cytometry.

**Viral Infection**—PBMCs (kindly provided by Dr. Eric Freed, National Institute of Health) were stimulated with 20 IU/ml human IL-2 and 5 µg/ml phytohemagglutinin of Phaseolus vulgaris (Sigma-Aldrich, Inc.) for 48 h. Activated PBMCs were spinoculated with VSV-G-pseudotyped NL4.3 for 1 h at 4 °C. 6 h after infection, unbound viruses were removed, and newly produced virions were collected at the indicated times. When applicable, lentiviral pseudotypes expressing shRNAs were used to knock down the endogenous LY6E in PBMCs after IL-2 and P. vulgaris phytohemagglutinin stimulation. When needed, 10 µg/ml IFN-α receptor-neutralizing IgG or control IgG was added to PBMCs for 1 h during infection.

For HIV-1 pLenti-puro-GFP-based infection, cells (SupT1, HeLa-TZM, THP-1, etc.) were spinoculated, and 48 h post-infection, GFP-positive cells were analyzed by flow cytometry. When applicable, THP-1 cells were differentiated into macrophage-like cells by 10 nM PMA, and differentiated cells were infected with VSV-pseudotyped NL4.3 (AD8) virus (Env of AD8 in the backbone of NL4.3, a kind gift from Eric Freed, National Institutes of Health). Six hours post-infection, unbound viruses were removed, and cells and produced viruses were analyzed.

**RT Assay**—HIV-1 production was determined by measuring viral RT activity. Briefly, 10 µl of cell supernatants was mixed with 40 µl of reaction mixture (50 mM Tris-HCl (pH 7.9), 5 mM MgCl2, 0.5 mM EGTA, 0.05% Triton X-100, 2% (v/v) ethylene glycol, 150 mM KCl, 5 mM DTT, 0.3 mM GSH (reduced glutathione), 0.5 units/ml poly(rA)-oligo(dT), and 0.1 µCi/µl 3H-labeled dTTP (PerkinElmer Life Sciences) and incubated at 37 °C for 3 h. Samples were then placed on ice for 30 min with 150 µl of prechilled 10% (mass/volume (m/v)) TCA and filtered onto an ELISA plate (Merck Millipore), and the RT activity was measured in a Microbect counter (Beckman Coulter).

**Viral Infectivity Assay**—HIV-1 infectivity was determined by infecting indicator HeLa-TZM cells. Briefly, 36–48 h after infection, cells were washed once with PBS and lysed with lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100). Approximately 10 µl of cell lysates was incubated with 10 µl of firefly luciferase substrates to determine HIV-1 infectivity.

**Long-term HIV-1 Replication Assay**—SupT1 cells were infected with NL4.3 equivalent to 5 ng of viral p24. 6 h later, unbound virus was removed by PBS. Viral particles secreted into the culture medium were collected every 2 days until cells became completely lysed. Harvested cell supernatants were centrifuged at 3500 rpm for 10 min to remove the cell debris, and RT activity was measured.

**Viral Binding and Uptake Assay**—For the virion binding assay, SupT1 cells were incubated with viral particles at 4 °C for 2 h. After three washes with PBS to remove unbound virus, cells were fixed with 3.7% formaldehyde for 10 min and subjected to flow cytometric analysis. For virus uptake, following viral binding, the virion-cell complex was shifted to 37 °C for 0, 5, 10, 30, or 60 min, respectively. Cells were replaced on ice to stop viral uptake before being digested with 0.05% (w/v) trypsin to remove noninternalized particles on the cell surface and fixed for flow cytometric analysis.

**Western Blotting**—Western blotting was performed as described previously (30). Briefly, cells were lysed in prechilled radioimmune precipitation assay buffer (1% Nonidet P-40, 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and protease inhibitor mixture) for 20 min, and protein samples were subjected to 10% SDS-PAGE. After proteins were transferred to a PVDF membrane, primary antibodies of interest were applied, and protein signals were detected using a Fuji Film imaging system.

**Cell Surface or Intracellular Staining**—Cells were nonpermeabilized or permeabilized with a permeabilization buffer (Thermo Fisher Scientific), washed twice with cold PBS plus 2% FBS, detached with PBS containing 5 mM EDTA, and incubated on ice with the appropriate primary antibodies for 1 h. After three washes with PBS plus 2% FBS, cells were further incubated with FITC or TRITC-conjugated secondary antibodies.
for 45 min. After two washes, cells were fixed with 3.7% formaldehyde for 10 min and analyzed by flow cytometry.

Assessment of Cell Proliferation—Cell proliferation was monitored by using the WST-1 assay according to the manual provided by the company (Sigma). Briefly, 5 × 10⁴ Sup-T1 or THP-1 cells were seeded in 96-well plates. 0, 24, and 48 h after cell seeding, 10 µl of WST-1 substrate was applied to each well, followed by 15-min incubation at 37 °C. After gentle shaking at room temperature for 1 min, absorbance was read at 450 nm (with reference at 620 nm).

Statistical Analyses—All statistical analyses were carried out in GraphPad Prism5 with Student’s t tests or one-way analysis of variance unless otherwise noted. Typically, data from at least three to five independent experiments were used for analysis.

Author Contributions—J. Y., C. L., and S. L. L. designed the experiments. J. Y., C. L., and S. L. L. analyzed the data, and all authors contributed to writing the paper.

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