TIM-family proteins inhibit HIV-1 release

Minghua Li1, Sherimay D. Ablan2, Chunhui Miao3, Yi-Min Zheng4, Matthew S. Fuller3, Paul D. Rennert6, Wendy Maury5, Marc C. Johnson3, Eric O. Freed3, and Shan-Lu Liu1,7

1Department of Molecular Microbiology and Immunology, Bond Life Sciences Center, University of Missouri, Columbia, MO 65211; 2Virus-Cell Interaction Section, HIV Drug Resistance Program, National Cancer Institute-Frederick, Frederick, MD 21702; 3Department of Immunotherapy, SugarCone Biotech LLC, Holliston, MA 01746; and 4Department of Microbiology, University of Iowa, Iowa City, IA 52245

Edited by Stephen P. Goff, Columbia University College of Physicians and Surgeons, New York, NY, and approved July 23, 2014 (received for review March 18, 2014)

Accumulating evidence indicates that T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins play critical roles in viral infections. Herein, we report that the TIM-family proteins strongly inhibit HIV-1 release, resulting in diminished viral production and replication. Expression of TIM-1 causes HIV-1 Gag and mature viral particles to accumulate on the plasma membrane. Mutation of the phosphatidylserine (PS) binding sites of TIM-1 abolishes its ability to block HIV-1 release. TIM-1, but to a much lesser extent PS-binding deficient mutants, induces PS flipping onto the cell surface; TIM-1 is also found to be incorporated into HIV-1 virions. Importantly, TIM-1 inhibits HIV-1 replication in CD4-positive Jurkat cells, despite its capability of up-regulating CD4 and promoting HIV-1 entry. In addition to TIM-1, TIM-3 and TIM-4 also block the release of HIV-1, as well as that of murine leukemia virus (MLV) and Ebola virus (EBOV); knockdown of TIM-3 in differentiated monocytic-derived macrophages (MDMs) enhances HIV-1 production. The inhibitory effects of TIM-family proteins on virus release are extended to other PS receptors, such as Axl and RAGE. Overall, our study uncovers a novel ability of TIM-family proteins to block the release of HIV-1 and other viruses by interaction with virion- and cell-associated PS. Our work provides new insights into a virus-cell interaction that is mediated by TIMs and PS receptors.

The T-cell immunoglobulin (Ig) and mucin domain (TIM) proteins play essential roles in cellular immunity (1, 2). Certain human pathologies, in particular allergic diseases, are associated with TIM protein dysfunctions and polymorphisms (3–5). Viral infection has recently been linked to TIM proteins, with some TIMs acting as key factors for viral entry. Human TIM-1 was initially discovered as the receptor for hepatitis A virus (HAV), and has been recently shown to function as a receptor or entry cofactor for Ebola virus (EBOV) and Dengue virus (DV) (5–8). TIM-1 polymorphisms have been reported to be associated with severe HAV infection in humans (9). More recent studies revealed that TIM-family proteins promote entry of a wide range of viruses, possibly by interacting with virion-associated phosphatidylserine (PS), highlighting a more general role of TIMs in viral infections (10, 11).

TIM-family proteins are classical type I transmembrane proteins, with the N terminus containing the variable Ig-like (IgV) domain extending from the plasma membrane and the C-terminal tail largely mediating intracellular signaling oriented toward the cytosol (2, 12). Human genes encode three TIM proteins, i.e., TIM-1, TIM-3, and TIM-4, whereas the mouse genome encodes eight TIM members, but only TIM-1, TIM-2, TIM-3 and TIM-4 are expressed. Despite significant sequence variations, the IgV regions of all TIM proteins contain a PS binding site that is absolutely conserved (2). Notably, the functions of TIM-family proteins differ greatly, depending on cell type-specific expression as well as the interactions of these TIMs with other molecules, including TIM-family members (2). Human TIM-1 is predominantly expressed in epithelial and T helper 2 (Th2) cells, and is involved in cell proliferation and apoptotic body uptake, whereas human TIM-3 is expressed in activated T helper cells (Th1), and functions as a negative costimulatory signal, often resulting in immune tolerance and apoptosis (13, 14). Human TIM-4 has been found to be mainly expressed in macrophages and dendritic cells (DCs), and possibly acts as a ligand for TIM-1, thereby facilitating T-cell activation (15, 16).

TIM-1 has been reported to be expressed in activated CD4+ T cells (13, 17), which are the major targets of HIV-1 infection. However, it is currently unknown if TIM-1 plays a role in HIV-1 replication and infection, although reduced TIM-3 expression on NK cells has been reported to be associated with chronic HIV-1 infection (18). Here we report that TIM-1 inhibits HIV-1 release, resulting in decreased virus production. Notably, TIM-1 mutants deficient for PS binding are incapable of blocking HIV-1 release. Similar to human TIM-1, we show that human TIM-3 and TIM-4 also potently inhibit HIV-1 production. The inhibitory effect of TIM-family proteins as well as some PS receptors can be extended to murine leukemia virus (MLV) and EBOV. Our study has revealed a novel and general function of TIMs, and likely other PS receptors, in the release of HIV-1 and other viruses.

Results

TIM-1 Diminishes HIV-1 Production. While using an HIV-1 lentiviral vector to generate a TIM-1–expressing stable cell line for unrelated studies, we serendipitously found that HIV-1 vectors encoding TIM-1 yielded abnormally low transduction levels. We observed that the population of puromycin-resistant colonies in cells transduced by an HIV-1 lentiviral vector encoding TIM-1 (pLenti-puro–TIM-1) was significantly lower than that transduced by the vector control encoding GFP (pLenti-puro-GFP) (Fig. S1). This result provided the first hint that TIM-1 may provide new insights into viral replication and AIDS pathogenesis.

Significance

TIM-family proteins have been recently shown to promote viral entry into host cells. Unexpectedly, we discovered that human TIM-1, along with TIM-3 and TIM-4, potently inhibits HIV-1 release. We showed that TIM-1 is incorporated into HIV-1 virions and retains HIV-1 particles on the plasma membrane via phosphatidylserine (PS), a phospholipid that is exposed on the cellular plasma membrane and the viral envelope. Expression of TIM-1 inhibits HIV-1 replication in CD4+ T cells, and knockdown of TIM-3 in monocytic-derived macrophages enhances HIV-1 production. We extended this function of TIMs to other PS receptors, and demonstrated that they also inhibited release of additional viruses, including murine leukemia virus and Ebola virus. The novel role of TIMs in blocking viral release provides new insights into viral replication and AIDS pathogenesis.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

1To whom correspondence should be addressed. Email: liushan@missouri.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1404851111/-/DCSupplemental.

www.pnas.org/cgi/doi/10.1073/pnas.1404851111

PNAS Early Edition | 1 of 9
impair HIV-1 production. To determine whether the inefficient transduction was caused by the TIM-1 protein, we produced HIV-1 GFP pseudoviral particles bearing different viral envelope glycoproteins in the presence of a TIM-1 mammalian expression plasmid, pCIneo–TIM-1. Again, the resulting viral titers were considerably lower than those produced in the absence of TIM-1, regardless of the pseudotyping envelope used. Ultimately, we were able to establish a stable 293 cell line expressing TIM-1, and confirmed that TIM-1 profoundly diminishes HIV-1 production, resulting in cell supernatants with ∼100-fold reduced viral infectivity (Fig. 1A).

To further investigate the effect of TIM-1 on infectious HIV-1 production, we transiently transfected 293T cells with NL4-3 or LAI proviral DNA, but in the presence of relatively low doses of TIM-1. TIM-1 decreased HIV-1 NL4-3 and LAI particle production in a dose-dependent manner (Fig. 1B and C, Lower). The reverse transcriptase (RT) activity of the harvested viruses in TIM-1-expressing cells was also accordingly reduced, correlating with reduced viral infectivity (Fig. 1D and E). Intriguingly, we observed that the reduced viral release caused by TIM-1 correlated with the increased levels of mature HIV-1 capsid (CA, p24) associated with the viral-producer cells (Fig. 1B and C, compare the p24 changes in Upper and Lower). Altogether, our data revealed that expression of human TIM-1 inhibits cell-free HIV-1 production, which is accompanied by an increase in mature HIV-1 Gag in viral producer cells.

**TIM-1 Blocks HIV-1 Gag Release.** Because expression of TIM-1 led to an accumulation of mature HIV-1 p24 in the cell (Fig. 1B and C), we asked whether the TIM-1 inhibition of HIV-1 production was related to viral protease (PR) activity. To address this issue, we transfected 293T cells with a plasmid encoding the codon-optimized HIV-1 Gag lacking PR, and measured the production of HIV-1 virus-like particles (VLPs) in the presence or absence of TIM-1. As shown in Fig. 1F, the levels of purified VLPs formed by the full-length HIV-1 Gag (Pr55Gag) were also reduced by TIM-1, similar to that of infectious HIV-1. In addition, we treated the full-length HIV-1 proviral DNA-transfected 293T cells with the HIV-1 protease inhibitor Saquinavir; again, HIV-1 release was strongly inhibited by TIM-1 (Fig. 1G).

We next explored what functional domains of Gag might be involved in the TIM-1 inhibition of HIV-1 release. To this end, we cotransfected 293T cells with plasmids encoding TIM-1, along with HIV-1 proviruses encoding Gag lacking matrix (MA) (Fyn10ΔMA) or nucleocapsid (NC) (Fyn10ΔNC) (19–21). As a control, the parental Gag chimera (Fyn10FullMA) was examined in parallel. In all of these constructs, the N-terminal Gag has a 10-aa sequence derived from the Fyn kinase, thus permitting them to associate with the cell membrane in a phospholipid
phosphatidylinositol-(4,5)-bisphosphate [PI(4,5)P2]-independent manner (19, 20). We found that TIM-1 blocked release of all these HIV-1 Gag mutants (Fig. S2A). TIM-1 also inhibited release of three additional Gag–Pol proviral mutants, i.e., HIV-1 PTAP− (defective in budding due to loss of late domain) (22), HIV-1 PR− (defective in maturation due to loss of protease) (22), as well as 29/31KE (known to assemble in intracellular compartments because of mutations in MA) (23, 24) (Fig. S2B). Similar to WT NL4-3, the amount of cell-associated p24 was also increased for the PTAP− and 29/31KE mutants in TIM-1–expressing cells compared with control cells not expressing TIM-1 (Fig. S2B). Collectively, these results show that TIM-1 does not act by binding MA or NC or by disrupting interactions between Gag and ESCRT machinery. The results also suggest that TIM-1 inhibitory activity is not linked to dysregulation of PR activity.

**TIM-1 Retains HIV-1 Virions on the Cell Surface.** To identify the site of Gag accumulation in TIM-1–expressing cells, we performed live-cell imaging and transmission electron microscopy (TEM). In the absence of TIM-1, HIV-1 Gag-GFP was diffusely distributed in the cytosol of 293T cells, with abundant VLPs released into extracellular spaces which showed vigorous Brownian movements (Fig. 2A and Movie S1). By contrast, in TIM-1–expressing cells Gag-GFP was frequently concentrated in cell–cell contact areas, with little movement of VLPs observed (Fig. 2A and Movie S2).

TEM was then carried out to further visualize HIV-1 particle production in TIM-1–expressing 293T cells. We observed markedly increased accumulation of mature HIV-1 particles on the surface of cells expressing TIM-1 (Fig. 2B). This phenotype is reminiscent of that observed with tetherin, an IFN-inducible cellular restriction factor that blocks HIV-1 release (25, 26). No apparent accumulation of HIV-1 particles in intracellular compartments was observed (Fig. S3A and B), a pattern that was different from that observed with the 29/31KE mutant (23, 24).

We then asked whether the block in HIV-1 release induced by TIM-1 could be rescued by treating virus-producer cells with subtilisin A, a serine endoproteinase previously shown to partially rescue the HIV-1 release block imposed by tetherin (25, 27). Subtilisin A substantially increased HIV-1 release from TIM-1–expressing cells, although viral particle release from subtilisin A-treated control cells (not expressing TIM-1) was also increased (Fig. 2C, Left; compare the center two lanes with the right two lanes). No obvious HIV-1 p24 was recovered from the control buffer-treated cells expressing or not expressing TIM-1 (Fig. 2C, Right). Together, these results suggest that the elevated levels of mature p24 associated with TIM-1–expressing virus-producer cells are largely due to the accumulation of mature HIV-1 particles on the cell surface.

**The Phosphatidylserine-Binding Capability of TIM-1 Is Essential for its Inhibition of HIV-1 Release.** TIM-1 is a receptor for PS, a phospholipid that is normally present in the inner leaflet of the plasma membrane; PS can be redistributed to the outer leaflet upon apoptosis or under some pathological conditions, including viral infection (16, 28). Given that PS is important for HIV-1 assembly and has been reported to be enriched in HIV-1 particles (29–32), we first examined if the PS-binding activity of TIM-1 is critical for its inhibition of HIV-1 release. We created three TIM-1 mutants, in which the two critical PS-binding residues of TIM-1, i.e., N114 and L116, were mutated, and we examined if these mutants could rescue the HIV-1 release block imposed by tetherin (Fig. 2C). The phenotypes observed (Fig. 2C, Right) were very similar to those observed with TIM-1, i.e., N114L and L116R mutants, which confirmed the critical role for PS-binding activity of TIM-1.

**Fig. 2.** TIM-1 retains HIV-1 particles on the cell surface. (A) HEK293T cells were cotransfected with plasmids encoding HIV-1 Gag-GFP, with or without TIM-1 expression vector; cells were reseeded onto bottom-top dishes, and live cell images were acquired using a fluorescent microscope (Olympus, 100×). Note that numerous VLPs diffusely are present in “GFP-Gag” control cells compared with cells expressing “GFP-Gag + TIM-1,” where Gag-GFP accumulated at cell–cell contacts. (B) HEK293T cells were transfected with pNL4-3 DNA with or without TIM-1 expression plasmid. Transfected cells were harvested at 24 h posttransfection, fixed in glutaraldehyde, and subjected to TEM. Representative images of thin-sectioned cells are shown. (C) HEK293T cells were transfected with pNL4-3 DNA with or without TIM-1 expression plasmid; viral particles released into supernatants were harvested 24 h after transfection (“untreated”). The cells were then treated with stripping buffer alone (“buffer”) or buffer containing 1 mg/mL subtilisin A for 15 min at 37 °C, followed by adding PMSF to stop the reaction. Cells were washed with PBS, lysed, and subjected to Western blotting (“Cell”). The stripped supernatants (“VLPs”) were concentrated and analyzed by Western blotting. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated.
and D115, were changed to Ala alone or in combination (N114A, D115A, and ND114/115AA). We found that all three TIM-1 mutants exhibited a significantly decreased ability to inhibit HIV-1 release compared with WT TIM-1 in 293T cells (Fig. 3 A and B). The expression of these TIM-1 mutants on the cell surface was confirmed by flow cytometry, with patterns similar to a previous report (Fig. 3C) (8). The inefficient block of HIV-1 release by PS binding-deficient TIM-1 mutants was further confirmed by TEM, showing a phenotype that is indistinguishable from that of control cells not expressing TIM-1 (Fig. S3 C–E).

We next evaluated if TIM-1 is incorporated into HIV-1 virions by taking advantage of these PS-binding deficient mutants that do not efficiently inhibit HIV-1 release. We attempted to immunoprecipitate HIV-1 virions using an antibody against TIM-1, and were able to detect HIV-1 gp41 in the anti–TIM-1 pull-down product for cells expressing both TIM-1 N114A and NL4-3 (Fig. 3D, Top, last lane). A faint gp41 band was also observed in purified virions derived from cells expressing NL4-3 alone, likely due to pulldown of the low endogenous TIM-1 in 293T cells (Fig. 3D, Top, lane 6). Interestingly, we found that the TIM-1 expression level in 293T cells cotransfected with NL4-3 was always higher than that in 293T cells transfected with TIM-1 alone, resulting in enhanced TIM-1 incorporation in purified virions, the signal of which was significantly higher than the background (Fig. 3D, Middle, compare the last four lanes).

Fig. 3. Mutation of the PS-binding sites of TIM-1 diminishes its ability to block HIV-1 release. (A) HEK293T cells were transfected with HIV-1 NL4-3 proviral DNA, along with plasmids encoding WT TIM-1 or its PS-binding mutants. Western blotting was performed to determine HIV-1 Gag expression in transfected 293T cells and purified viral particles. Positions of the Gag precursor Pr55Gag (Pr55) and the mature CA protein (p24) are indicated. (B) The RT activity and infectivity of HIV-1 harvested in A was determined by infection of HeLa-TZM cells. (C) Flow cytometric analysis of TIM-1 expression on the surface of transfected 293T cells using an anti-hTIM-1 antibody. (D) Incorporation of TIM-1 into HIV-1 virions. HEK293T cells were transfected with pNL4-3 proviral DNA in the presence or absence of TIM-1 N114A plasmid. Released virions were purified and coimmunoprecipitated with an anti–TIM-1 antibody at 4 °C overnight. The bound virions, along with cell lysates were resolved by SDS/PAGE, followed by Western blotting using anti–HIV-1 gp41 or anti–TIM-1 antibodies. (E) Effect of EGTA on the TIM-1–mediated inhibition of HIV-1 release. The fold inhibitions between mock (“untreated”) and EGTA-treated cells were indicated. We set cells not expressing TIM-1 and untreated with EGTA to 1.0 for easy comparison. (F) Effect of anti–TIM-1 antibody, ARD5 (against IgV domain) on HIV-1 release. The fold differences in HIV-1 RT activity between mock (“untreated”) and antibody-treated cells are indicated. We set cells not expressing TIM-1 and untreated with anti–TIM-1 antibody to 1.0 for easy comparison. (G) HEK293T cells were transfected with plasmids encoding wild-type TIM-1 or its PS-binding mutants, and their ability to induce PS flipping to the outer leaflets of the plasma membrane was assessed by flow cytometry using Annexin V and Propidium iodide (PI) binding kit (Roche).
Collectively, these results demonstrate that TIM-1 is incorporated into HIV-1 virions.

As divalent calcium (Ca$^{2+}$) is required for binding of TIM-1 to PS (28), we asked whether inhibition of the association between TIM-1 and PS by EGTA could rescue the TIM-1–induced block in HIV-1 release. Indeed, we observed that addition of EGTA to 293T cells expressing TIM-1 markedly rescued HIV-1 production, as measured by RT activity (Fig. 3E). In addition, ARD5, a monoclonal antibody against the IgV domain of TIM-1 (33), also partially overcame the TIM-1–mediated impairment of HIV-1 production (Fig. 3F).

Viral infection has been shown to induce PS flipping to the outer leaflet of the plasma membrane, accounting for, at least in part, PS exposure on the surface of viral particles (34–36). We thus examined if expression of TIM-1 induces PS redistribution on the cell membrane, and if so, whether or not this would be important for the TIM-1 inhibition of HIV-1 release. We observed that TIM-1 induced apparent PS flipping to the cell surface, as evidenced by Annexin V binding (Fig. 3G). Of note, all three PS mutants, which were inefficient at inhibiting HIV-1 release, showed a background level of PS flipping (Fig. 3G). This result indicates that expression of TIM-1 induces PS redistribution to the outer leaflet of the cell membrane.

Despite Enhanced Entry, TIM-1 Inhibits HIV-1 Replication in CD4$^+$ T Cells. To investigate the role of TIM-1 in HIV-1 replication in CD4$^+$ T-cell lines, we transfected Jurkat or Jurkat cells stably expressing TIM-1 with proviral NL4-3 DNA and measured HIV-1 replication kinetics. TIM-1 substantially inhibited HIV-1 replication in Jurkat cells, as evidenced by decreased RT activity (Fig. 4A). The effect of TIM-1 in Jurkat cells was not as pronounced as was observed in 293T cells, possibly due to the relatively low level of TIM-1 expression in Jurkat cells (Fig. 4B). As TIM-1 has been shown to promote entry of a wide range of viruses (8, 10, 11), we tested whether TIM-1 also enhances HIV-1 entry by transducing Jurkat cells with HIV-1 lentiviral vectors bearing NL4-3 Env. Indeed, we found that TIM-1 enhanced entry mediated by HIV-1 Env, but not by G glycoprotein of vesicular stomatitis virus (VSV-G), into Jurkat cells by approximately threefold (Fig. 4C). Interestingly, we observed that the CD4 level in TIM-1 expressing Jurkat cells was consistently higher than that of parental Jurkat cells (Fig. 4D). Hence, the relatively low level of TIM-1 expression, the up-regulation of CD4 expression, as well as enhanced HIV-1 entry all likely contributed to the relatively modest inhibitory effect of TIM-1 on HIV-1 replication in Jurkat cells.

To determine the role of endogenous TIM-1 in HIV-1 replication, we screened a panel of human cell lines, including T-cell and monocytoid lines, for TIM-1 expression. Unfortunately, none of these cell lines, except the human lung epithelial A549 cell line, expressed a significant level of TIM-1 as determined by flow cytometry (Fig. S4A and B). We treated 293, A549, and several other cell lines with IFN-$\alpha_2$b, but did not observe a significant increase in TIM-1 expression (Fig. S4C), suggesting that TIM-1 is not IFN inducible.

TIM-3, TIM-4, and Additional PS Receptors Inhibit Release of HIV-1, MLV, and EBOV. Human TIM-family proteins consist of TIM-1, TIM-3, and TIM-4. We observed that, similar to TIM-1, human TIM-3 and TIM-4 also strongly inhibited HIV-1 release, as shown by the profound reduction of virion-associated Gag, which inversely correlated with increased cell-associated p24 (Fig. 5A). Consistently, the RT activity and viral infectivity was significantly reduced by TIM-3 and TIM-4 (Fig. 5B).

Fig. 4. Effect of TIM-1 on HIV-1 replication and entry. (A) Jurkat or Jurkat cells expressing TIM-1 were transfected with pNL4-3 proviral DNA, and viral replication kinetics were determined by measuring RT activities. (B) Comparison of TIM-1 expression in Jurkat/TIM-1 and 293T/TIM-1 cells by flow cytometry. (C) Expression of TIM-1 increases HIV-1 Env (NL4-3)–mediated but not VSV-G–mediated entry into Jurkat cells. Jurkat or Jurkat/TIM-1 cells were transduced by lentiviral vector (pLenti-GFP-puro) bearing NL4-3 Env or VSV-G and GFP-positive cells were scored by flow cytometry. (D) TIM-1 up-regulates CD4 expression in Jurkat cells. The levels of CD4 expression on the cell surface were determined by flow cytometry using an anti-CD4 antibody.
Interestingly, we observed that monocyte-derived macrophages (MDMs) express an abundant level of TIM-3 (Fig. 5C). We thus tried to knock down the endogenous TIM-3 in MDMs of two healthy donors using lentiviral shRNA (Fig. 5C), followed by infection with NL4-3 pseudotypes (pNL4-3-KFS construct containing a frameshift mutation in Env; ref. 37) bearing VSV-G. The use of VSV-G-pseudotyped HIV-1 increased infection efficiency and bypassed the TIM-1–induced up-regulation of CD4, which would otherwise complicate the analysis. Despite the relatively low TIM-3 knockdown efficiency (∼30% based on means and geometric means of the fluorescence intensity) (Fig. 5C), we consistently observed an enhanced HIV-1 production in MDMs transduced by TIM-3 shRNA compared with that of shRNA control (Fig. 5D).

We next evaluated whether TIM-family proteins affect other viruses. We first expressed human TIM-1 in 293T cells transfected with plasmids encoding Moloney murine leukemia virus (MoMLV) Gag-Pol or EBOV VP40 fused with GFP. TIM-1 indeed inhibited the production of both MoMLV Gag and EBOV GFP-VP40, in a TIM-1 dose-dependent manner (Fig. 5E and F). Similar effects were also found for human TIM-3 and TIM-4, although the effect of TIM-3 appeared to be less on MoMLV (Fig. 5G and H). Interestingly, TIM expression also increased levels of cell-associated CA (p30) for MoMLV as it does for HIV-1 (Fig. 5A, E, and G) suggesting that HIV-1 and MoMLV are inhibited by TIMs via a similar mechanism.

We then tested other PS receptors, i.e., Axl and RAGE, and found that their effect on viral release depended on the tested viruses. Although RAGE inhibited the release of both HIV-1 and EBOV, it did not significantly reduce MoMLV release, although some accumulation of cell-associated MoMLV CA (p30) was observed (Fig. S5B). By contrast, Axl strongly blocked EBOV production, but it showed no obvious inhibition of HIV-1 and MoMLV release (Fig. S5). These differential effects of RAGE and Axl on HIV-1, MoMLV, and EBOV could be due to their binding capabilities to PS present in individual viruses (Discussion). Taken together, these results indicate that TIMs, as well as some other PS receptors, can inhibit viral release.

Discussion

There is an increased appreciation that the PS-binding proteins or PS receptors, such as the TIMs, or the Gas6/TAM complex, play important roles in innate sensing and viral infections (8, 10, 11, 38–40). These proteins, either secreted into culture media (Gas6) or expressed on the cell surface (TIMs and TAMs), can interact with PS present on the surface of infectious virions thus enhancing viral entry (8, 10, 11, 39). Here, we have documented a novel function of TIM-family proteins, which inhibits the...
release of HIV-1, MLV and EBOV from viral producer cells. TIM-1 appears to accomplish this function by accumulating viral particles on the cell surface through associating with virion-associated PS. Consistent with this hypothesis, TIM-1 mutants deficient for PS binding are unable to efficiently block HIV-1 release, and EGTA, which depletes Ca\(^{2+}\) that is required for the association of TIM-1 and PS, overcomes the TIM-1-mediated inhibition of HIV-1 release. In addition, we showed that TIM-1 is incorporated into HIV-1 virions, further supporting the role of TIM-1 and PS interaction in blocking HIV-1 release. Consistently, other PS receptors, including TIM-3, TIM-4, RAGE, and Axl, share with TIM-1 the ability to inhibit the release of HIV-1, MLV, and EBOV, although the degree of inhibition is in some cases virus-dependent. Cumulatively, our study provides new insights into the roles of TIM-family proteins in virus production.

One striking observation in this study is that expression of TIM-1 diminishes HIV-1 production, but increases cell-associated p24 in viral-producer cells. This accumulation of cell-associated CA is also seen with MoMLV. We excluded the possibility that the increased mature Gag in the cell is due to excessively or prematurely activated HIV-1 protease activity, because release of an HIV-1 PR mutant was similarly blocked by TIM-1. In addition, the PR inhibitor, Saquinavir, failed to rescue the TIM-1 phenotype. Our TEM data convincingly demonstrated that mature HIV-1 particles accumulate on the surface of viral producer cells, which can be stripped off by treatment of cells with the protease, subtilisin A. Thus, the biochemical and TEM phenotypes of TIM-1 on HIV-1 release are similar to those of tetherin, which blocks the release of HIV-1 and other viruses from the plasma membrane (41). However, given that TIM-1 is not significantly detected in HEK293, AS49, Jurkat, and peripheral blood mononuclear cells (PBMCs) treated with IFN-α2b (Fig. S4C), we thus suggest that, unlike tetherin, TIM-1 is not a classical IFN-stimulated gene (ISG). TIM-1 is also distinct in terms of IFN inducibility from viperin, another ISG that inhibits HIV-1 release in a virus strain-specific manner (42).

The mechanisms by which TIM-1 and tetherin inhibit HIV-1 release are fundamentally different. Tetherin achieves this function by adopting a unique membrane topology and possibly an axonal configuration, in which pairs of the N-terminal transmembrane domains or pairs of C-terminal GPI anchors are arrayed around assembling virion particles, whereas the remaining pairs of transmembrane domains are inserted into the virus-producing cell membrane (43, 44). By contrast, we propose that TIM-1 accomplishes this task by association of its PS-binding domain located in the IgV domain with the PS exposed on the envelopes of the budding virions. Consistent with this model, PS has been shown to be expressed on the surface of HIV-1 as well as some other viruses (34–36). Furthermore, we show here that TIM-1 mutants, which are deficient in PS binding, cannot effectively block HIV-1 release, do not induce PS flapping, yet are incorporated into HIV-1 virions. Hence, TIM-1 and PS present on the surface of viral producer cells and infectious virions may form a network of interactions, resulting in chains of aggregates of HIV-1 particles on the cell surface (Fig. S6). Exactly how the expression of TIM-1 induces PS flapping at the plasma membrane and how TIM-1 and PS in the virion–cell complex interact to block HIV-1 release remain to be elucidated.

TIM-1 is mainly expressed in T cells and various epithelial cells, and is involved in immune activation, tissue injury, and engulfment of PS-containing apoptotic cells (2). Although we have observed that TIM-1 induces PS flapping, we did not find apparent cell death in TIM-1–expressing cells, suggesting that cell death, if any, should not significantly contribute to the marked reduction of viral production. In Jurkat cells stably expressing a relatively low level of TIM-1, we observed that, despite its ability to promote HIV-1 entry and up-regulate CD4 expression, TIM-1 inhibits HIV-1 replication. These results argue that, at least in Jurkat cells, the inhibition of HIV-1 release by TIM-1 dominates over its enhancement of viral entry, resulting in diminished viral replication.

Consistent with this model, shRNA knockdown of TIM-3 in MDMs enhances HIV-1 release. Future study should focus on determining the role of TIMs in vivo using relevant animal models. Despite apparent differences in molecular structure and immune regulation, all three human TIMs (i.e., TIM-1, TIM-3, and TIM-4) potently inhibit HIV-1 release. This may not be so surprising, given that all of these TIMs contain an IgV domain, in which the PS binding pocket, known as the metal ion-dependent ligand-binding site (MILIBS), is absolutely conserved (2). In the published mouse TIM crystal structures, it has been demonstrated that the Asn and Asp residues (equivalent to human TIM-1 N114 and D115) within MILIBS are crucial for a series of intra- and intermolecular interactions and are involved in metal ion-dependent binding to PS (28, 45, 46). In this work, we demonstrated that mutations of human TIM-1 at positions N114 and D115 resulted in a remarkable loss of inhibition of HIV-1 release. Also, we found that mouse TIM-4 exhibited an even stronger phenotype than human TIM-1 in inhibiting HIV-1 release, the mechanism of which will be explored in future studies. Interestingly, Axl and RAGE, which are PS receptors not belonging to the TIM-family, also block virus release, yet in a virus-dependent manner. It is therefore reasonable to speculate that the differential inhibition of virus release by Axl and RAGE is associated with their capabilities to bind PS present in different viruses. Similarly, the effects of TIMs and PS receptor on viral release can also be cell-type dependent, a phenomenon that has been noted for TIM-mediated promotion of viral entry (11).

Our discovery that human TIMs, as well as Axl and RAGE, function as inhibitory factors to block virus release adds another layer of complexity to the increasingly appreciated role of TIMs in viral replication, innate sensing, and viral infection (2, 12). It will be important to examine the functions of different primate TIMs in the replication of other lentiviruses, and explore their potential roles in retrovirus–host coevolution. Given the strong positive selection that has been shown for TIM-1 (47), and possibly other TIM proteins and PS receptors, primate TIMs would have conserved yet distinct antiviral effects on divergent primate lentiviruses. It might be informative to analyze the enrichment of PS in divergent lentiviruses that are produced from different primate cells to include macaques and dendritic cells, and investigate their possible relationships to the antiviral activities of TIMs and PS receptors. By extension, the polymorphisms of TIMs in the human population, which have been shown to be associated with asthma and some viral diseases (9, 48, 49), could hold important links to AIDS pathogenesis and disease progression.

Materials and Methods

Plasmids. The human TIM-1 gene was cloned into pcDNA3 vector with a FLAG tag at the N terminus (pcDNA3–F–TIM-1), or cloned into pLenti-GFP-Puro vector (Addgene) or pQCKIP retroviral vector (Clontech) with a FLAG tag at the C terminus. The TIM-1 mutants (N114A, D115A, N114/D115AA) were generated by using PCR-based mutagenesis based on pcDNA3–F–TIM-1. The plasmids encoding human TIM-3, TIM-4, Axl, and RAGE have been described (10). The HIV-1 NL-4, HIV-1 Gag-GFP, and HIV-1 Gag constructs were obtained from the National Institutes of Health (NIH) AIDS Reagent Program. The HIV-1 molecular clone pLAI was a gift from Michael Emerman (Fred Hutchinson Cancer Research Center, Seattle). The HIV-1 Fyn-10 constructs (20, 21, 23), MLV Gag-Pol (50), pNL4-3 29/31KE (23, 24), pNL4-3 SPTAP (51), and pNL-3 PR (51) have been described. The EBOV VP40-GFP construct was kindly provided by Kartik Chandran (Albert Einstein College of Medicine, Bronx, NY). The plasmids encoding TIM-3 shRNA, as well as scramble shRNA, were purchased from Sigma.

Cells and Reagents. HEK293, 293T, HTX (a subclone of HT1080), A549, and the HeLaTJMZ indicator cells were grown in Dulbecco’s modified Eagle’s (DMEM) medium, supplemented with 0.5% penicillin/streptomycin and 10% (vol/vol) FBS. SupT1, THP-1, CEM-T4, JLTRG, MT-2, and Jurkat cells were obtained from the NIH AIDS Reagent Program and maintained in RPMI and 10% FBS.
PBMCs were isolated from healthy human donors, and maintained in RPMI medium containing 10% FBS and supplemented with phorbolethoglutinin (PHA-P) and interleukin-2 (IL-2). PBMCs were differentiated from PBMCs and maintained in RPMI medium containing 10% FBS. HEK293 and Jurkat cells stably expressing TIM-1 were established by transducing these cells with HIV-1 or MLV vector expressing TIM-1. The antibodies against HIV-1 Gag, HIV-1 gp41 and CD4 were also from the NIH AIDS Reagent Program. The anti–TIM-1 and TIM-3 antibody was purchased from R&D Systems. The antibody against MLV Gag was produced from hybridoma (R187) at ATCC. The anti–GFP antibody was purchased from Santa Cruz. The secondary anti-mouse IgG proteins conjugated to FITC or HRP were purchased from Sigma. Annexin V apoptosis detection kit was purchased from Santa Cruz. Subtilisin A was purchased from Sigma.

**Virus Production and infection.** For infectious or pseudotyped virus production, 293T cells were transfected with HIV-1 proviral DNAs encoding NL4-3 or LAI, plus plasmids encoding genes of interest (TIM-1, TIM-3, etc.). Alternatively, 293T cells were transfected with HIV-1 lentiviral or MLV vectors, HIV-1 or MLV Gag-Pol, and viral glycoproteins (VSV-G, Env, etc.). For virus-like particle (VLP) production, 293T cells were transfected with plasmids encoding HIV-1 Gag, MLV Gag-Pol, or EBOV VP40-GFP in the presence or absence of TIMs. In all cases, 24 h post-transfection, the supernatants were harvested and passed through a 0.2-μm filter or by centrifugation to remove the cell debris. Viruses were used to infect target HeLa-TZM-bl cells in the presence of polybrene (8 μg/mL). For Jurkat cells, spinoculation at 1,500 × g for 1 h at 4 °C was applied. At 48 h posttransfection, the luciferase activity was measured by following the manufacturer’s instructions. If applicable, viral supernatants were concentrated by ultracentrifugation at 32,000 rpm (Sorvall, Discovery 100SE) for 2 h at 4 °C. The purified virus was dissolved in PBS and detected by Western blotting.

**Subtilisin A Protease Stripping.** HEK293T cells were transfected with HIV-1 proviral pNL4-3 DNA with or without TIM expression plasmid. Twenty-four hours posttransfection, viral particles released into supernatants were harvested and concentrated. The cells were treated either with buffer alone (50 mM Tris-HCl, 150 mM NaCl, 5 mM MgCl₂) or buffer containing 1 mg/mL subtilisin A for 15 min at 37 °C. Subsequently, DMEM containing 5 mM PMSF was added and incubated at 37 °C for 15 min. Following a wash with PBS, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS), and the supernatants were collected, concentrated using sucrose cushion purification, and analyzed by Western blotting.

**HIV-1 Replication and RT Measurement.** For HIV-1 replication, parental Jurkat or Jurkat cells stably expressing TIM-1 were transfected with HIV-1 proviral pNL4-3 DNA using Lipofectamine 2000 (Invitrogen). Forty-eight hours posttransfection, the supernatants were harvested every 2 d, and the HIV-1 replication was determined by measuring HIV-1 RT activity at different time points. Briefly, 10 μL of clarified supernatants containing virions were incubated with 40 μL of reaction mixture at 37 °C for 3 h. The mixture contains 50 mM Tris-HCl pH 7.9, 5 mM MgCl₂, 0.5 mM EDTA, 0.05% Triton X-100, 2% (vol/vol) ethylene glycol, 150 mM KCl, 0.3 mM DTT, 0.3 mM GSH (reduced glutathione) and 0.1% (vol/vol) 14 HPTP (Perkin-Elmer). The reaction was stopped by adding 10% (vol/vol) cold TCA at 4 °C for 30 min, and the mixture was transferred to Millipore MultiScreen Glass Fiber FC plate. After being washed twice with cold 10% (vol/vol) TCA and cold ethanol, the membranes were inserted into Beta Gamma vials and read in Microbeta counter.

**HIV-1 Infection of MDMs.** MDMs were transfected from PBMCs and maintained in RPMI medium containing 10% FBS. MDMs were transiently transfected by lentiviral vectors bearing VSV-G and encoding TIM-3 sRNA or scramble sRNA. Twenty-four hours posttransduction, MDMs were infected with HIV-1 NL4-3/KIFS pseudotypes (defective for Env expression) bearing VSV-G. Six hours after infection, cells were washed three times with PBS, and the viral release was monitored by RT activity 24 h postinfection.

**TEM and Live Cell Imaging.** TEM was performed as described (52). The NL4-3 29/31KE MA mutant, which redirects virus assembly to a late endosomal compartment (24) was used as a control for viewing HIV-1 intracellular assembly. For live cell imaging, 293 cells were cotransfected with HIV-1 Gag-GFP, along with plasmids encoding TIM-1. Twenty-four hours posttransfection, cells were seeded to glass-bottom Microwell dishes (MatTek) and imaged using a fluorescence microscope (Olympus).

**Western Blotting.** The experiments were performed as described (53, 54). Briefly, 293T cells were transfected with plasmids encoding HIV-1 proviral DNAs or HIV-1 Gag, along with plasmids expressing TIMs or P5 receptors. Forty-eight hours posttransfection, cells were collected, washed once with PBS, and lysed in RIPA buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS). The supernatants from culture were harvested and the virus was purified by ultracentrifugation. The SDS lysates and purified viral particles were dissolved in sample buffer, separated on 10% (wt/vol) SDS/PAGE, and detected by using anti-HIV-1 p24, anti-MLV p30 (ATCC, R187), anti–TIM-1, anti-gp41, or anti-GFP (for VP40) antibodies.

**Flow Cytometry.** Transfected or normal cells, including MDMs, were washed twice with cold PBS plus 2% (vol/vol) FCS and incubated with anti–TIM-1, TIM-3, or anti–CD4 for 1 h. For Annexin V and PI staining, transfected cells were incubated with FITC-conjugated Annexin V or PI, and analyzed by flow cytometry.

**ACKNOWLEDGMENTS.** We thank Kunio Nagashima and Ferri Soheilian at the Leidos Electron Microscopy Laboratory for their expert assistance with the TEM analysis and Kaatik Chandran for providing the VP40-GFP construct. This work was supported by National Institutes of Health (N.I.H) Grants R01 AI112381, R21 AI099464, R21 AI105584, R56 AI107095, and U54 AI057160 (to S.-L.L.), R01 GM110776 (to M.C.J.), and R01 AI1077519 (to W.M.). Research in the E.O.F. Laboratory is supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH, and by the Intramural AIDS Targeted Antiviral Program.


