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Mannose Receptor 1 Restricts HIV Particle Release from Infected Macrophages

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SUMMARY

Human mannose receptor 1 (hMRC1) is expressed on the surface of most tissue macrophages, dendritic cells, and select lymphatic or liver endothelial cells. HMRC1 contributes to the binding of HIV-1 to monocyte-derived macrophages (MDMs) and is involved in the endocytic uptake of HIV-1 into these cells. Here, we identify hMRC1 as an antiviral factor that inhibits virus release through a bone marrow stromal antigen 2 (BST-2)-like mechanism. Virions produced in the presence of hMRC1 accumulated in clusters at the cell surface but were fully infectious. HIV-1 counteracted the effect by transcriptional silencing of hMRC1. The effect of hMRC1 was not virus isolate specific. Surprisingly, deletion of the Env protein, which is known to interact with hMRC1, did not relieve the hMRC1 antiviral activity, suggesting the involvement of additional cellular factor(s) in the process. Our data reveal an antiviral mechanism that is active in primary human macrophages and is counteracted by HIV-1 through downregulation of hMRC1.

In Brief

hMRC1 is a surface receptor in macrophages that binds glycoproteins and contributes to innate immunity. Sukegawa et al. report that hMRC1 inhibits detachment of mature and infectious HIV-1 virions from the surface of infected cells. The hMRC1-imposed restriction of virus release is similar to, but independent of, the BST-2/tetherin-imposed restriction.

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SUPPLEMENTAL INFORMATION

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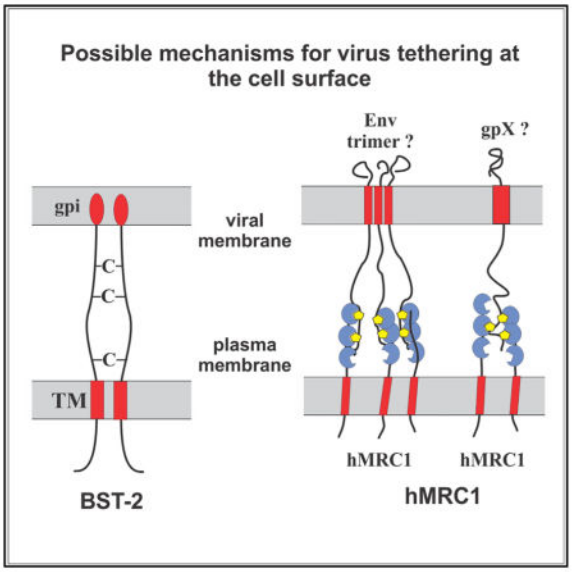
AUTHOR CONTRIBUTIONS

S.S., F.B., and K.S. designed the experiments. S.S and E.M. performed the experiments. H.F. performed evolutionary analyses. S.S. and K.S. wrote the manuscript. All authors discussed results and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.



INTRODUCTION

Human mannose receptor C-type 1 (hMRC1), also known as macrophage mannose receptor or CD206, is a 175-kDa single-pass transmembrane glycoprotein that belongs to the C-type lectin family and is expressed on the surface of most tissue macrophages, dendritic cells (DCs), and some lymphatic or liver endothelial cells (reviewed in Azad et al., 2014). The expression levels of macrophage mannose receptor on macrophages are estimated to reach upward of 100,000 molecules per cell (Stahl and Ezekowitz, 1998). The protein consists of an N-terminal cysteine-rich domain, a fibronectin type II repeat, and eight carbohydrate recognition domains (reviewed in Taylor et al., 2005) and exists in an extended conformation that places domains with different functions at distinct positions with respect to the plasma membrane (Napper et al., 2001). MRC1 was initially identified in rat alveolar macrophages and ascribed a role in the clearance of endogenous mannose-containing glycoproteins (Stahl et al., 1978; Allavena et al., 2004; Lee et al., 2002). Human mannose receptor has also been implicated in the CD4-independent infection of astrocytes (Liu et al., 2004), and interaction of HIV-1 with mannose receptor was found to increase production of matrix metalloproteinases in astrocytes and vaginal epithelial cells, which may contribute to the reduction in type IV collagen and affect the integrity of the blood-brain barrier (López-Herrera et al., 2005). It may also lead to degradation of tight junction proteins (Fanibunda et al., 2011) and increase the risk of sexual transmission of HIV through facilitation of virus transport across the vaginal epithelium (Jadhav et al., 2013).

More recently, MRC1 was reported to serve as entry receptor for invading pathogens, such as bacteria, fungi, viruses (including HIV-1), and other parasites (reviewed in Azad et al., 2014). Interestingly, the hMRC1-mediated uptake of HIV-1 by macrophages does not lead to productive infection (Trujillo et al., 2007; Pontow et al., 1992). Instead, hMRC1-mediated uptake of HIV-1, Dengue virus, hepatitis B virus (HBV), or influenza A virus results in the processing of pathogens in major histocompatibility complex-class II (MHC-II)-containing

compartments for subsequent antigen presentation (Astarie-Dequeker et al., 1999; Ezekowitz et al., 1991; Allavena et al., 2004; Taylor et al., 2005). Thus, MRC1 constitutes part of a cellular innate host defense mechanism. Nevertheless, binding of HIV-1 to the surface of macrophages via the mannose receptor was shown to facilitate virus transmission to T cells, indicating that HIV-1 can also use the interaction with hMRC1 to its own advantage (Nguyen and Hildreth, 2003). Some pathogens, including HIV-1, have evolved to antagonize the innate immune function of MRC1 by downregulating mannose receptor from the cell surface (Ezekowitz et al., 1981; Basu et al., 1991; Shepherd et al., 1997; Koziel et al., 1998). In the case of HIV-1, Nef was reported to induce cell surface down-modulation of hMRC1 without affecting its steady-state levels (Vigerust et al., 2005). In addition, HIV-1 Tat was reported to inhibit transcription from the hMRC1 promoter (Caldwell et al., 2000). However, the precise mechanism of HIV-induced down-modulation of hMRC1 from the surface of productively infected macrophages remains unclear.

Our overall interest in the HIV-induced modulation of cell surface markers such as CD4 or bone marrow stromal antigen 2 (BST-2) and their functional consequences for virus replication (Willey et al., 1992, Miyagi et al., 2009) prompted us to examine the functional correlation between hMRC1 expression on primary human macrophages (monocyte-derived macrophages [MDMs]) and productive HIV-1 replication. Indeed, we found that HIV-1 infection of MDMs induced a progressive loss of hMRC1 at both mRNA and protein levels. Interestingly, we found that silencing of hMRC1 in MDMs resulted in enhanced virus production similar to what was previously observed in response to the HIV-1 Vpu-induced antagonism of BST-2. Conversely, overexpression of hMRC1 following transient transfection of HEK293T cells resulted in significant inhibition of particle release. Despite its phenotypical similarity to BST-2-mediated restriction of virus release, hMRC1-dependent inhibition of virus production was BST-2 independent because: (1) it was observed in HEK293T cells, which are inherently BST-2-negative; and (2) the presence of Vpu had no impact on hMRC1-dependent inhibition of virus release. Surprisingly, deletion of Env did not alleviate the hMRC1-imposed inhibition of virus release, suggesting the involvement of additional cellular factor(s) in the process. Overall, our data suggest that hMRC1 represents an antiviral factor affecting virus release in a BST-2-like but BST-2-independent manner.

RESULTS

HIV Infection of MDMs Induces a Reduction of Endogenous hMRC1 Expression

To verify the expression profile of mannose receptor 1 in human cells (hMRC1), we compared the cell surface expression of hMRC1 in monocytes and MDMs, as well as phorbol 12-myristate 13-acetate (PMA)-stimulated cultures of the monocytoid cell lines THP1 and U937 (Figure 1A). Because of their macrophage-like characteristics, we had previously used differentiated THP1 and U937 cultures for studies involving SAMHD1 (Welbourn et al., 2012). We found that hMRC1 was efficiently expressed on differentiated MDMs, but not on undifferentiated monocytes. Unexpectedly, despite their general macrophage-like properties, PMA-stimulated U937 and THP1 cells did not express detectable levels of hMRC1, thus rendering these cells unsuitable as models to study effects of endogenous hMRC1 on HIV-1 replication.

It was previously shown that hMRC1 contributes to the binding and transmission of HIV-1 by macrophages and is involved in the endocytic uptake of HIV-1 into macrophages (Nguyen and Hildreth, 2003). However, a potential impact of HIV-1 infection on hMRC1 expression in macrophages has not been assessed. To do so, terminally differentiated MDMs were infected with the R5-tropic HIV-1 AD8, and infection was allowed to proceed for 16 days. Uninfected MDMs were cultured in parallel under the same conditions. We found that HIV-1 infection of MDMs resulted in significant down-modulation of cellular hMRC1 (Figure 1B). Significant reductions in hMRC1 levels were also observed when MDMs were infected for 3 or 6 days at high MOI (~1) using vesicular stomatitis virus G protein (VSV-G)-pseudotyped AD8 virus (Figure 1C). Under the conditions used, at least 40% of the MDMs were infected by day 6. Real-time PCR in these cultures revealed that hMRC1 mRNA levels were downregulated in most HIV-infected MDMs when compared with the corresponding mock-infected cultures, both on days 3 and 6 post-infection (Figure 1D), even though there was significant donor variation. Thus, reduction in hMRC1 protein levels following HIV infection of MDM is most likely due to transcriptional inhibition of the hMRC1 gene.

hMRC1 Inhibits HIV-1 Virus Release from Infected Macrophages

The fact that infection of MDM cultures for 3 days resulted in only partial reduction of hMRC1 (see Figure 1C, lane 2) allowed us to assess the impact of hMRC1 on HIV-1 replication by further reducing hMRC1 levels using siRNA-mediated gene silencing (Figure 2). In this experiment, MDMs were transfected prior to infection with Ctrl-siRNA or hMRC1-siRNA twice in 3-day intervals and then infected with VSV-G-pseudotyped Env-positive AD8 wild-type (WT) virus for 3 days as described in the Experimental Procedures. VSV-G-pseudotyped virus was used to maximize the number of infected MDMs. Following 3 days of infection, cultured MDMs were harvested, and a fraction of the cells was processed for immunoblotting to assess the success of the siRNA-mediated knockdown of hMRC1 and to control for comparable levels of HIV infection (Figure 2A). The remaining MDMs were subjected to pulse-chase analysis to quantify virus production and release (Figures 2B and 2C). As expected, HIV infection alone induced a reduction in hMRC1 levels as indicated by the lower protein levels in si-Ctrl-treated cells (Figure 2A, compare lanes 1 and 3). Of note, treatment with hMRC1-specific siRNA further reduced hMRC1 to undetectable levels (Figure 2A, lane 2). Interestingly, pulse-chase analysis revealed an increase in virus release from hMRC1-silenced cells (Figure 2C). The effect was highly reproducible and was statistically significant ($p = 0.02$). These results suggest that endogenous hMRC1 in MDMs may function to inhibit virus release.

Exogenous Expression of hMRC1 Broadly Inhibits the Release of Lentiviruses

The relatively modest effect of hMRC1 on virus release from HIV-1-infected MDMs could be explained by the fact that virus infection itself already caused a significant reduction of hMRC1 expression (see Figure 2A). Thus, the enhanced virus release observed after siRNA treatment in Figures 2B and 2C may represent an additional boost. To test this hypothesis, we examined the effect of hMRC1 on virus production following overexpression in HEK293T cells. These cells lack expression of endogenous hMRC1 [Figure 3A, (−)]. We analyzed the effect of increasing amounts of exogenously expressed hMRC1 on the release

of four different lentiviruses: HIV-1 NL4-3, HIV-1 AD8, pSIVcpzMB897, and HIV-2 ROD10. To that end, HEK293T cells were transfected with increasing amounts of hMRC1 plasmid DNA together with constant amounts of lentivirus vectors pNL4-3, pAD8, pSIVcpzMB897, or pROD10, respectively. As a readout, we compared intracellular viral protein expression (Figure 3A) against amounts of cell-free virus-associated reverse-transcriptase activity (Figure 3B). Note that the levels of exogenously expressed hMRC1 in lanes 3, 6, 9, and 12 correspond well to the levels of endogenous hMRC1 in macrophages when normalized against tubulin (Figure S1). We found that transfection of increasing amounts of hMRC1 had little effect on intracellular Gag expression irrespective of the virus tested (Figure 3A, CA). In contrast, expression of increasing amounts of hMRC1 was paralleled by a dose-dependent decrease in the secretion of virus-associated reverse-transcriptase activity (Figure 3B). The effects observed following overexpression of hMRC1 were significantly stronger (up to 70% reduction in virus-associated reverse-transcriptase activity) than those observed after silencing residual hMRC1 in infected macrophages, consistent with our above-stated hypothesis. It is interesting that even though hMRC1 is a myeloid-specific factor, its effect on virus release was readily reproducible in non-myeloid HEK293T cells and was not limited to R5 tropic virus isolates. Of note, although the effect of hMRC1 on virus release is reminiscent of the restriction of virus release from peripheral blood mononuclear cells (PBMCs) and macrophages antagonized by Vpu (e.g., Schubert et al., 1995), now known to be caused by BST-2 (Neil et al., 2008; Van Damme et al., 2008), the hMRC1-imposed inhibition of virus release is clearly BST-2 independent because HEK293T cells not only lack expression of endogenous hMRC1, but also do not express BST-2. Also, both HIV-1 NL4-3 and AD8 express functional Vpu to antagonize BST-2, and the Env protein of the HIV-2 ROD10 isolate was previously shown to have Vpu-like activity against BST-2 as well (Bour and Strebel, 1996). We therefore conclude that hMRC1 is an antiviral factor that inhibits virus release from HIV-infected macrophages in a BST-2-independent manner. Unlike BST-2 or other restriction factors such as Trim5α, expression of hMRC1 was not responsive to type 1 or type 2 interferon treatment (Figure S2), nor did the HIV-induced down-modulation of hMRC1 involve signaling through the interferon receptor (Figure S3). Finally, evolutionary analyses revealed that the hMRC1 gene is under negative selection (Figure S4), indicating that the gene is highly conserved.

hMRC1 Imposes a BST-2-like Restriction to Virus Release

To further demonstrate the inhibitory effect of hMRC1 on virus release, we performed a series of pulse-chase analyses in HEK293T cells to directly follow the fate of newly synthesized Gag protein. For that purpose, the X4- or R5-tropic HIV-1 clones NL4-3 and AD8, respectively, were transfected into HEK293T cells in the absence or presence of hMRC1 (Figure 4). To ascertain comparable expression of viral Gag proteins, a portion of the transfected cells was used to analyze intracellular protein expression by immunoblotting (Figures 4A and 4D). Indeed, intracellular Gag levels were comparable in the presence or absence of hMRC1 for both viruses. The remaining cells were pulse-labeled for 30 min with [^{35}S]-Expre^{35}S^{35}S-label and chased for up to 2 hr as described in the Experimental Procedures. At each time point, equal aliquots of cells were harvested, and cells and cell-free virus-containing supernatants were collected separately. Each fraction was subjected to immuno-precipitation with an HIV-1-positive patient serum. Immunoprecipitated proteins

were separated by SDS-PAGE and visualized by fluorography (Figures 4B and 4E). Virus release of NL4-3 and AD8 from two independent analyses was quantified by phosphoimage analysis (Figures 4C and 4F). Viral proteins released into the cell-free supernatants were calculated as percentage of the total intra-cellular and extra-cellular Gag protein and plotted as a function of time. Consistent with the results from Figure 3, hMRC1 expression reduced HIV-1 release by 40%–60% relative to hMRC1-negative controls. These results confirm that hMRC1 expression does not affect *de novo* biosynthesis of viral Gag proteins but inhibits their release as cell-free particles.

To further characterize the effects of hMRC1 on virus release, we performed electron microscopy on HEK293T cells transfected with AD8 DNA in the presence or absence of hMRC1 (Figure 5). The experiments were designed to provide a qualitative rather than a quantitative assessment of budding profiles and particle morphology. In the absence of hMRC1, we observed normal budding structures at the cell surface with occasional virions near the plasma membrane (Figure 5, top row). In contrast, co-expression of hMRC1 resulted in the accumulation of viral particles in clusters at the plasma membrane (Figure 5, bottom row). These results provide further evidence that hMRC1 expression in HEK293T cells inhibits virus particle release in a manner reminiscent of BST-2 (Klimkait et al., 1990; Neil et al., 2008).

Virus Particles Retained by hMRC1 Can Be Physically Detached from the Cell Surface and Are Infectious

We and others previously demonstrated that Vpu-defective HIV-1 virions tethered to the surface of virus-producing cells can be released by physical force or by treatment with protease (Neil et al., 2008; Klimkait et al., 1990; Miyagi et al., 2011). To test whether virions retained by hMRC1 can be similarly released by physical force, we performed a vortexing experiment as described in the Experimental Procedures and outlined in cartoon fashion in the top part of Figure 6A. In this experiment, HEK293T cells were transfected with pAD8 wild-type in the presence or absence of hMRC1. Virus-containing supernatants (V1) were collected 24 hr later. The cell pellet was vortexed for 30 s and then washed with culture medium. Cell extracts (C), supernatants (V1), and wash fractions (V2) were analyzed by immunoblotting for the presence of viral Gag proteins and hMRC1 (Figure 6A). We found that vortexing released more viral particles from cells expressing hMRC1 than from cells lacking hMRC1 expression (Figure 6A, compare lanes 5 and 6). The results from three independent experiments are quantified in Figure 6B. The data confirm that hMRC1 expression inhibits the spontaneous release of viral particles, which can then be released by vortexing. Of note, these particles are fully infectious (Figure 6C). These results further support the notion that hMRC1 imposes a BST-2-like restriction on virus release.

The Effect of hMRC1 on Virus Release Does Not Require Env Expression

Based on the fact that hMRC1 is expressed at the cell surface and can interact with the HIV-1 Env glycoprotein, we speculated that the accumulation of virions at the surface of hMRC1-expressing cells is mediated by an interaction of hMRC1 and Env as indicated in Figure 7Aa. In this model, HIV-1 Env present in viral particles interacts with hMRC1 at the surface of the virus-producing cells resulting in the non-covalent retention of virions at the

cell surface. To test this hypothesis, we analyzed the effects of hMRC1 on an Env-deficient variant of AD8. Surprisingly, release of Env-deficient AD8 virions was still sensitive to hMRC1 (Figure 7B). This result does not rule out a contribution of the viral Env on hMRC1-mediated particle retention. However, it suggests that other cell-derived glycoproteins also contribute to this phenomenon (Figure 7Ab). It is interesting to note that capture of HIV-1 particles could be prevented by soluble mannose-binding lectin and mannose-receptor-specific antibodies (Pollicita et al., 2008; Liu et al., 2004). However, our own attempts to neutralize the hMRC1-mediated inhibition of virus release using mannose binding lectin 2 or employing three different hMRC1-specific antibodies were unsuccessful (Figures S5 and S6). This could suggest that capture of extracellular virions and retention of nascent virions by hMRC1 may be mechanistically distinct.

DISCUSSION

Macrophages are professional phagocytes that patrol the bloodstream and surrounding tissues to capture foreign antigens and contribute to host immune defense by killing phagocytosed pathogens, secretion of inflammatory cytokines, and antigen presentation to T cells. The macrophage mannose receptor plays an important role in this process (Stahl, 1990). Indeed, studies performed in rats more than 40 years ago had demonstrated the rapid clearance of glycoproteins containing high-mannose chains from the bloodstream (Stahl et al., 1976), a finding that was subsequently attributed to the presence of mannose receptors on liver Kupffer and endothelial cells (Schle-singer et al., 1976, 1978). Thus, although the role of hMRC1 in capturing pathogens such as HIV-1 is well documented, our study reveals a previously unappreciated function of mannose receptor, which is the retention of newly released particles at the surface of virus-producing cells. As such, the effect of mannose receptor expression resembles that of BST-2, another cell-surface glycoprotein with the ability to tether virions to the cell surface and inhibit release of cell-free virions (Neil et al., 2008; Van Damme et al., 2008). As with BST-2, which is counteracted by the HIV-1 Vpu, HIV-2 Env, as well as SIV Nef proteins (reviewed in Strebel, 2013), HIV-1 attempts to minimize the impact of hMRC1-induced particle retention by reducing its expression following infection of macrophages. This explains the relatively modest effect of hMRC1 on virus release from infected macrophages. Importantly, however, inhibition of virus release by hMRC1 is quite comparable with that observed by BST-2 when virus-induced down-modulation is bypassed (Figure 4). Indeed, the accumulation of HIV-1 particle clusters at the surface of hMRC1-expressing cells as observed by electron microscopy is reminiscent of the particle clusters observed in the absence of Vpu at the surface of BST-2-expressing cells. While BST-2 expression is regulated by HIV-1 at the post-translational level, our data presented in Figure 1D clearly indicate that HIV-induced hMRC1 down-modulation occurs at the transcriptional level. However, the exact mechanism of transcriptional hMRC1 silencing remains unclear and will be the subject of future investigations. Of note, hMRC1 is not expressed in undifferentiated monocytes, but only in differentiated macrophages (Figure 1A). However, unlike other recently identified restriction factors (e.g., Trim5α or BST-2), hMRC1 expression is not interferon responsive, and the HIV-induced hMRC1 down-modulation does not involve type 1 interferon receptor signaling. Also, hMRC1 is not under positive selection. However, the fact that hMRC1 is not evolving as a result of selection

pressure could be explained by the fact that hMRC1 does not recognize protein motifs on its targets but specific sugar moieties (i.e., mannose modifications), which may be evolutionarily conserved.

Our observation that deletion of the *env* gene did not rescue the hMRC1-induced inhibition of virus release was unexpected. However, this finding may not be all that surprising if one takes into consideration the fact that binding of glycoproteins to mannose receptor is not specific to HIV-1 Env but targets any glycoprotein with terminal mannose, fucose, or *N*-acetyl glucosamine moieties (reviewed in Taylor et al., 2005). It also does not rule out that Env, when present, contributes to the particle retention. Of note, the Env protein is not the only particle-associated glycoprotein with the potential to bind mannose receptor; in fact, proteomics studies of macrophage-derived virions identified a large number of cellular proteins, including multiple membrane-associated glycoproteins (Chertova et al., 2006). It is therefore conceivable that the interaction of viral particles with cell-surface mannose receptor is mediated not by one single protein but through the concerted action of multiple mannose-containing glycoproteins. Attempts to identify these factors via immune affinity/mass spectrometry are ongoing. As with particles tethered by BST-2, particles retained by hMRC1 could be stripped off by physical force. Such particles not only contained Env glycoprotein, as evidenced by the fact that they retained full infectivity (Figure 6C), they also appear to contain hMRC1 (Figure 6A), although we cannot rule out that at least some of the extracellular hMRC1 is microvesicle associated. In fact, particles released by vortexing appeared to be enriched in hMRC1 (Figure 6A). It is therefore possible that particle-associated hMRC1 interacts with surface glycoproteins on the host cell and contributes to the retention of virions that way (Figure 7A). Previous studies found that pretreatment of virus particles with mannose binding lectin or with antibodies to hMRC1 prevented the mannose-receptor-mediated retention of these particles (Liu et al., 2004; Pollicita et al., 2008). Our own attempts to neutralize hMRC1 using mannose binding lectin 2 (MBL2) or antibodies to hMRC1 failed (Figures S5 and S6). The reasons are not clear; however, it is possible that the antibodies used may not recognize the epitopes on hMRC1 involved in particle retention. Alternatively, virus-hMRC1 interactions may already have formed by the time the antibodies or MBL2 gain access to the complexes and may not be able to dissolve such pre-existing interactions. Finally, it cannot be ruled out that the hMRC1-mediated uptake of particles and retention of nascent virions are mechanistically distinct.

Irrespective of exactly what proteins are involved in virion retention, the phenomenon in itself is a reflection of the role of macrophages in the immune system: capturing pathogens and removing them from circulation in the bloodstream.

EXPERIMENTAL PROCEDURES

Cells

HEK293T and TZM-bl cells were maintained in DMEM with 4.5 g/L glucose (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a 37°C and 5.0% CO_2 environment. Human monocytes from multiple anonymous normal donors were elutriated as described previously (Gruber et al., 1995). Elutriated monocytes were obtained from the NIH blood bank under

protocol 99-CC-0168: “Collection and Distribution of Blood Components from Healthy Donors for In Vitro Research Use.” Monocytes (2×10^6 cells/well in a 12-well plate) were cultured in 1 mL of complete DMEM supplemented with 10% pooled human serum (Gemini Bio-Products, West Sacramento, CA, USA) for 5–7 days to allow differentiation into macrophages (MDMs).

Plasmids and Viral Vectors

An hMRC1 cDNA clone (NM_002438) in the backbone of pCMV6Entry was purchased from Origene (catalog number [Cat no.] SC303200; Origene Technologies, Rockville, MD, USA). Infectious molecular clones pNL4-3 (Adachi et al., 1986), pAD8 (Theodore et al., 1996), and pROD10 (Clavel et al., 1986; Ryan-Graham and Peden, 1995) have been reported previously. The SIVcpz molecular clone pSIVcpzMB897 (Van Heuverswyn et al., 2007) was a gift of Beatrice Hahn. An Env mutant of pAD8 was created by QuikChange (Stratagene, La Jolla, CA, USA) mutagenesis using the wild-type constructs as template and the following mutagenesis primers Env: 5′-ATGATCTGTAGTGCTGCAGAATAATTGTGGGTCACAGTTT-3′ and 5′-AAACTGTGACCCACAATTATT CTGCAGCACTACAGATCAT-3′. Clones were selected for gain of an additional PstI site, and sequences were confirmed by sequence analysis.

Transient Transfection of HEK293T Cells

For transient transfection of HEK293T cells, 3×10^6 cells were plated in a 25-cm^2 flask and grown overnight. The following day, cells were transfected using Lipofectamine PLUS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total amounts of plasmid DNAs in all samples were adjusted to 6 μg with empty vector DNA as appropriate. After 24 hr, cells were scraped, washed with PBS, suspended in PBS (100 μL/10^6 cells), and mixed with an equal volume of 2× sample buffer. For analysis of virus-associated proteins, virus-containing culture supernatants were harvested and pre-cleared by low-speed centrifugation (5 min, 1,500 rpm). Supernatants were then filtered through a 0.45-μm syringe filter and concentrated by pelleting through a 2-mL 20% sucrose cushion (75 min, 35,000 rpm, 4°C). Pellets were lysed in 200 μL/5 mL input of 2× sample buffer and processed for immunoblot analysis.

Replication in MDMs

Virus stocks for the infection of MDMs were prepared as follows: HEK293T cells (in 75-cm^2 flasks) were transfected with 15 μg of pAD8 DNA using Lipofectamine PLUS (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For the production of VSVg-pseudotyped virus stocks, 1.5 μg of pCMV-VSVg vector DNA was cotransfected with 13.5 μg of pAD8 DNA. Virus-containing supernatants were harvested 2 days later, and cellular debris was removed by centrifugation (5 min, 1,500 rpm), followed by filtration (0.45 μm).

For infection of MDMs, the culture medium was removed from the cells and replaced with 1 mL of virus stock. Virus was allowed to adsorb for 4 hr at 37°C before medium was replaced by 1 mL of complete DMEM supplemented with 10% human serum. Half of the medium (0.5 mL) was replaced by fresh medium every 3 days. For immunoblot analysis, MDMs

were washed once with PBS, suspended in radio immuno-precipitation assay (RIPA) buffer (50 mM Tris [pH 7.2], 1.0% Nonidet P-40 [NP-40], 2.5 mg/mL sodium deoxycholate, 150 mM NaCl, 0.1% SDS, 1.0 mM EDTA), mixed with an equal volume of 2× sample buffer (4% SDS, 125 mM Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 10% glycerol, 0.002% bromophenol blue), and heated at 95°C for 5–10 min.

RNA Isolation and qRT-PCR

Total cellular RNA was extracted using RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed with 20 ng of total RNA and specific primer sets for hMRC1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using iTaq Universal SYBR green One-Step Kit (Bio-Rad, Hercules, CA, USA). Primers for qRT-PCR were as follows: hMRC1 sense: 5′-AAAGCTGCCA ACAACAGAAC GCTGAG-3′; antisense: 5′-ATATAGCCCA GTTTCTGAAC ACATTCC-3′; GAPDH sense: 5′-AAGGTCGGAG TCAACGGATT-3′and GAPDH antisense: 5′-CTCCTG GAAG ATGGTGATGG-3′ (Singh et al., 2011). RT-PCR was carried out using the CFX96 Touch Real-Time PCR detection system (Bio-Rad, Hercules, CA, USA). Relative mRNA levels were determined using the ΔΔCt quantification method (Livak and Schmittgen, 2001).

Fluorescence-Activated Cell Sorting Analysis

For analysis of cell-surface expression of hMRC1, cells were washed twice with ice-cold 20 mM EDTA-PBS, followed by two washes in ice-cold 1% BSA-PBS. Cells were stained in 1% BSA-PBS for 30 min at room temperature in the dark with allophycocyanin (APC)-conjugated anti-human CD206 antibody (α-hMRC1; BioLegend, San Diego, CA, USA) or APC-conjugated Mouse IgG_1 (BD Biosciences Immunocytometry Systems, Mountain View, CA, USA) as isotype control. Cells were then washed twice with ice-cold 1% BSA-PBS and fixed with 1% formaldehyde in PBS. Finally, cells were analyzed on a FACSCalibur (BD Biosciences Immunocytometry Systems, Mountain View, CA, USA). Data analysis was performed using FlowJo (Tree Star, San Carlos, CA, USA).

Immunoblot Analysis

Cells were washed once with PBS, suspended in PBS (100 μL/10^6 cells), and mixed with an equal volume of 2× sample buffer. Samples were heated at 95°C with occasional vortexing until samples were completely dissolved. Samples were subjected to SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes, and reacted with primary antibodies as described in the text. Human MRC1 was identified using a rabbit monoclonal antibody to hMRC1 (clone ab125028; Abcam, Cambridge, MA, USA). HIV-1 Gag was identified using pooled HIV immunoglobulin (Ig; NIH Research and Reference Reagent Program; Cat no. 3957), and tubulin was identified using a mouse monoclonal antibody to alpha-tubulin (T9026; Sigma-Aldrich, St. Louis, MO, USA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (GE Healthcare, Piscataway, NJ, USA), and proteins were visualized by enhanced chemiluminescence (Clarity Western ECL substrate 170-5061; Bio-Rad Laboratories, Hercules, CA, USA).

Metabolic Labeling and Pulse-Chase Analysis

For pulse-chase analysis, transfected HEK293T cells or infected MDMs were harvested by scraping, washed with PBS, and suspended in 5 mL of labeling medium (methionine- and cysteine-free RPMI [MP Biomedical, Solon, OH, USA] containing 5% fetal calf serum [FCS]). Samples were incubated for 20 min at 37°C to deplete the intracellular methionine/cysteine pool. Cells were then labeled for 30 min (HEK293T) or 45 min (MDMs) at 37°C in 200 μL of labeling medium, supplemented with 30 μL (300 μCi) of [^{35}S]-Expre^{35}S^{35}S-label (NEG072; PerkinElmer, Waltham, MA, USA). After the labeling period, unincorporated isotope was removed, and equal aliquots of cells were distributed into tubes containing 1 mL of pre-warmed complete DMEM and chased for the times indicated in the text. Cells and virus-containing supernatants were harvested separately at each time point and stored on dry ice until all samples had been collected. For immunoprecipitation of intracellular and virus-associated Gag proteins, cells and virus-containing supernatants were lysed in 200 μL of Triton X-100-based lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 10% glycerol) and incubated on ice for 5 min. After lysis, the cell extracts were pelleted at 13,000 rpm for 5 min and clarified supernatants were added to 1.0 mL of 0.1% BSA/PBS at 4°C. Cell and virus lysates were immunoprecipitated (1 hr, 4°C on a rotator) with pooled HIV Ig serum immobilized to protein A Sepharose beads (P-3391; Sigma-Aldrich, St. Louis, MO, USA). Precipitated proteins were solubilized by boiling in sample buffer and separated by SDS-PAGE. Gels were fixed and dried. Gels were exposed to Kodak XMR film, and proteins were visualized by fluorography. For protein quantitation, gels were exposed to imaging plates, and analysis of the relevant bands was performed using a FujiFilm FLA-7000 PhosphorImager.

Virus Detachment

For removal of virus particles attached to producer cells, cells were first detached from the flasks by scraping. Cells were then pelleted by low-speed centrifugation (5 min, 1,500 rpm), and cell-free supernatants were removed (fraction V1 in Figure 6A). The cell pellets were then agitated for 30 s on a vortexer at full speed. Vortexed cells were then suspended in an equal volume of complete culture medium and subjected to a second round of low-speed centrifugation. The cell-free supernatants were collected (fraction V2), and the cell pellet was used to prepare whole-cell extracts. Supernatants V1 and V2 were filtered (0.45 μm), concentrated by pelleting through a cushion of 20% sucrose, and processed for immunoblotting. To test the infectivity of the viral particles present in fractions V1 and V2, we used an aliquot of the unconcentrated supernatants to infect TZM-bl indicator cells as described below.

Viral Infectivity Assay

Virus-containing supernatants were pre-cleared by low-speed centrifugation (5 min, 1,500 rpm) and filtered through a 0.45-μm syringe filter. TZM-bl indicator cells ($CD4^+$, $CCR5^+$, $CXCR4^+$) were plated in a 24-well plate (1 mL; 5×10^4 cells/well) and infected with 150 μL of viral supernatant. Typically, infections were performed in triplicate. Cells were lysed in the wells 48 hr later with 200 μL of lysis buffer (25 mM Tris-HCl [pH 7.8], 8.0 mM $MgCl_2$, 1.0 mM DTT, 1.0% Triton X-100, 15% glycerol). Luciferase activity in the lysate was

determined by combining 5 μL of each lysate with 20 μL of luciferase substrate (Steady-Glo; Promega, Madison, WI, USA). Light emission was measured using a Modulus II microplate reader (Turner Biosystems, Sunnyvale, CA, USA). Values were normalized by reverse-transcriptase activity.

Knockdown of hMRC1 in MDMs

Human monocytes were allowed to differentiate into MDM for 4 days. At that point MDMs were transfected twice in 3-day intervals either with non-targeting interference RNA (siRNA) (5′-TGGTTTACATGTCGACTAA-3′; GE Dharmacon, Lafayette, CO, USA) or hMRC1-specific siRNA (5′-GAAGCAAAGTGGATT ACGT-3′) using TransIT-TKO transfection reagent (Mirus Bio, Madison, WI, USA). Three days after the second siRNA transfection, cells were infected with VSV-G-pseudotyped AD8 virus. Four hours after infection, virus input was replaced by fresh medium, and MDMs were transfected once again with siRNA. Three days after infection, cells were harvested and subjected to metabolic labeling and pulse-chase analysis for virus release.

EM Analysis

For electron microscopy (EM) analysis of virus-producing HEK293T cells, 0.2×10^6 cells were plated on Thermanox Plastic coverslip (Thermo Fisher Scientific, Rochester, NY, USA) in a 12-well plate and grown overnight. The following day, cells were transfected directly on the coverslip using Lipofectamine PLUS (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total amounts of plasmids DNAs in all samples were adjusted to 3.0 μg with empty vector DNA as appropriate. After 32 hr, the supernatants were removed and the cells were fixed for 1 hr at room temperature in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4; Electron Microscopy Sciences, Hatfield, PA, USA) and stained with samarium acetate. Epon/araldite resin-embedded samples were cut into 80-nm sections using an EM UC6 ultramicrotome (Leica) and diamond knife (Diatome), placed on hexagonal 100 mesh grids, and post-stained with 2% aqueous samarium acetate and Reynolds lead citrate. Sections were imaged on a Hitachi 7500 transmission electron microscope using an AMT digital camera.

Statistical Analysis

The average values of all of the data are presented with error bars indicating the SD or SEM. Statistical significance was analyzed in Microsoft Excel using Student's t test. A one-tailed (e.g., H_A: $\mu_{\text{hMCRsi}} > \mu_{\text{Ctrl}}$), paired t test was used for statistical analysis of bar graphs. The t test with the assumption of homoscedasticity was used for the analysis of virus release kinetics. Homoscedasticity assumes that the variance associated with the release kinetics is approximately the same regardless of antiviral factor. We utilized Student's t test because the number of data points from the experiments is relatively small. The calculated mean and SD may, by chance, deviate from the population mean and SD. The null hypothesis (i.e., H_0: $\mu_{\text{hMCRsi}} = \mu_{\text{Ctrl}}$) is rejected if the p value is less than the significance levels of $*\alpha = 0.05$, $**\alpha = 0.01$, and $***\alpha = 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Human mannose receptor (hMRC1) is an HIV restriction factor
- hMRC1 functions specifically in macrophages
- hMRC1 inhibits detachment of budding virions from the cell surface
- HIV-1 counteracts hMRC1 by transcriptional silencing of the hMRC1 gene

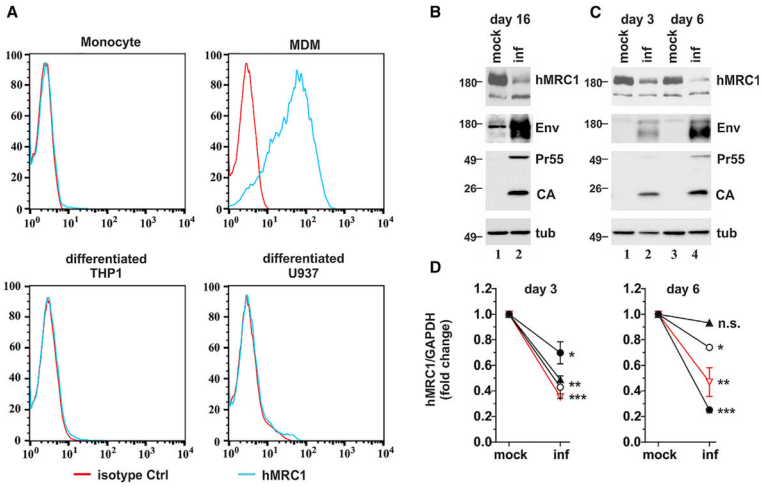

Figure 1. HIV Infection of MDMs Results in Reduced Levels of Endogenous hMRC1

(A) Undifferentiated monocytes, monocyte-derived macrophages (MDMs), as well as PMA-stimulated THP1 and U937 cells were subjected to flow cytometric (fluorescence-activated cell sorting [FACS]) analysis to compare surface expression of hMRC1. For detection of hMRC1, cells were stained with APC-conjugated anti-human CD206 antibody (blue lines). Cells stained with APC-conjugated mouse IgG1 served as isotype controls (red lines).

(B) MDMs were infected with wild-type AD8 virus and cultured for 16 days (inf). Uninfected MDMs were cultured in parallel (mock). After 16 days, cells were harvested and cell extracts were subjected to immunoblot analysis using antibodies to hMRC1, HIV-1 (CA, Pr55, Env), and tubulin (tub). Molecular weight standards are indicated on the left.

(C) MDMs were infected with VSV-G-pseudotyped AD8 virus stocks at an MOI of ~1 (inf). Uninfected MDMs were cultured in parallel (mock). Cells were cultured for either 3 (day 3) or 6 days (day 6). Cell extracts were subjected to immunoblot analysis as in (B). Representative results from one of four independent donors are shown.

(D) Levels of endogenous *hMRC1* mRNA in the cultures shown in (C) were determined by qRT-PCR. GAPDH mRNA served as internal reference. Data are shown for four independent donors. Data shown in red correspond to the donor used for the immunoblot in (C). Error bars represent the SEM calculated from duplicate qRT-PCRs. Statistical significance was determined using a one-tailed, paired Student's t test. The null hypothesis is rejected if the p value is less than the significance levels, i.e., *α = 0.05; **α = 0.01; ***α = 0.001.

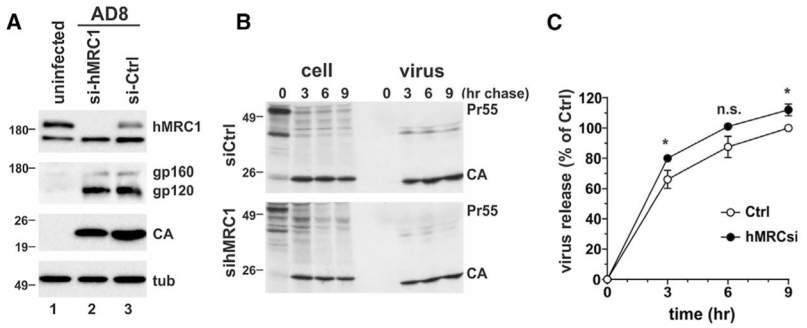

Figure 2. Silencing of hMRC1 Promotes Virus Release from HIV-1-Infected MDMs

(A) MDMs were treated with control siRNA or hMRC1-specific siRNA prior to infection with VSV-G-pseudotyped AD8 virus (MOI ~1). Three days after infection, cells were harvested and subjected to immunoblot analysis as in Figure 1B. Uninfected MDMs were analyzed in parallel.

(B) Three days postinfection, MDMs treated with control siRNA or hMRC1 siRNA were pulse-labeled for 45 min with [^{35}S]-labeling reagent and chased for up to 9 hr. Equal aliquots of cells and virus-containing supernatants were collected at the times indicated and immuno-precipitated with pooled human immunodeficiency virus immunoglobulin (HIV-IG). Immunoprecipitated samples were separated by SDS-PAGE, and proteins were visualized by fluorography. Results from one of three independent experiments are shown.

(C) Gag-specific bands in (B) were quantified by phosphoimage analysis, and virus release was calculated for each time point by determining the percentage of cell-free Gag protein relative to the sum of intra-cellular and extra-cellular Gag proteins. The maximum release observed in the sample treated with control siRNA was defined as 100%. Results shown are derived from two independent experiments using MDMs from two different donors. Error bars reflect the SEM. Statistical significance was determined using the Student's t test with the assumption of homoscedasticity ($p > 0.05$). n.s., not significant.

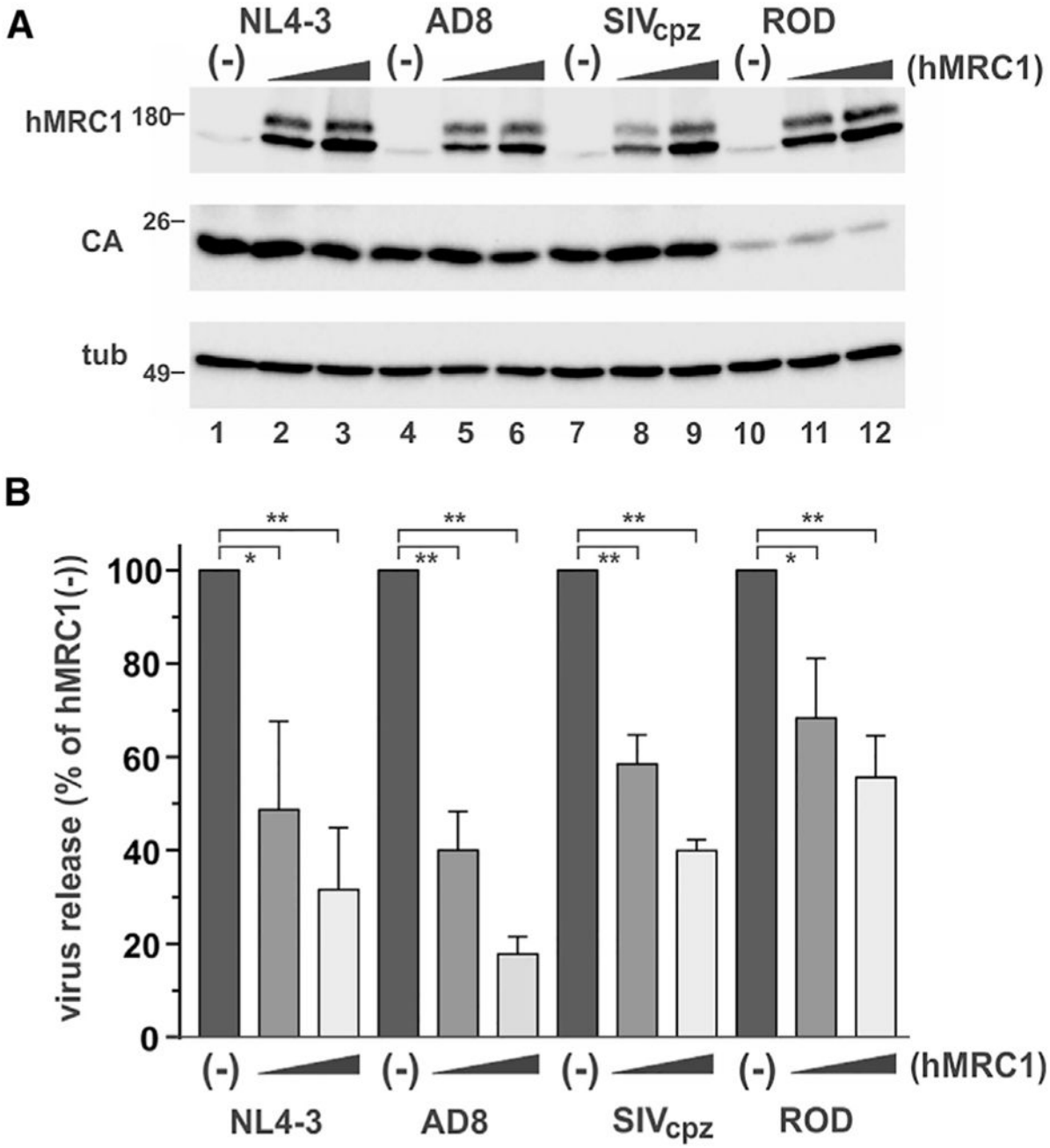

Figure 3. hMRC1 Broadly Inhibits Lentivirus Production

HEK293T cells were transfected with 3.0 μg each of pNL4-3, pAD8, pSIVcpzMB897, or pROD10 together with increasing amounts of pCMV6-hMRC1 (0, 0.5, 2.0 μg). Total amounts of transfected plasmid DNA were adjusted to 5 μg for each sample using empty vector as needed.

(A) Whole-cell extracts were prepared 24 hr post-transfection, and intracellular Gag (CA) and hMRC1 proteins were determined by immunoblotting. A tubulin blot (tub) was included as a loading control. A representative result from five independent experiments is shown.

(B) Virus production was measured by quantifying virus-associated reverse-transcriptase activity. Virus release observed in the absence of hMRC1 was defined as 100% for each virus. The results are shown as an average of five independent experiments with SDs. Statistical significance was determined using a one-tailed, paired Student's t test. The null hypothesis is rejected if the p value is less than the significance levels, i.e., *α = 0.05; **α = 0.01.

See also Figures S1–S4.

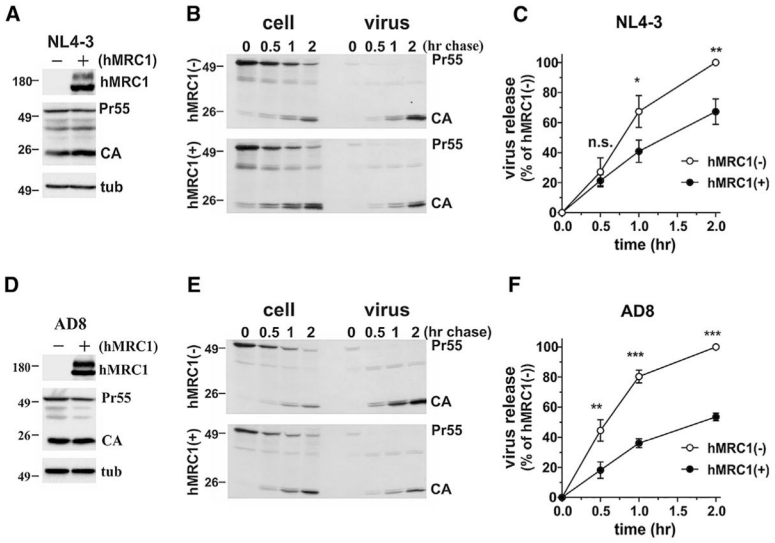

Figure 4. Inhibition of Virus Release by hMRC1 Is Phenotypically Similar to BST-2-Mediated Restriction

(A–E) HEK293T cells were transfected with 4.0 μg each of pNL4-3 (A–C) or pAD8 (D and E) together with 2.0 μg of empty vector [hMRC1(−)] or pCMV6-hMRC1 [hMRC1(+)]. Cells were harvested 24 hr later.

(A and D) A fraction (30%) of the transfected cells was processed for immunoblotting as in Figure 1B to verify comparable expression of Gag proteins. (B and E) The remaining cells were subjected to pulse-chase analysis. Cells were pulse-labeled for 30 min with [^{35}S]-labeling reagent and chased for up to 2 hr. Equal aliquots of cells and virus-containing supernatants were collected at the indicated times and immunoprecipitated separately with pooled HIV-IG. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography.

(C and F) Virus release was calculated independently for each sample as described for Figure 2C and plotted as a function of time. Maximal viral release obtained in the absence of hMRC1 (open circles) was arbitrarily defined as 100%. The results are shown as the average of four independent experiments. Error bars represent SD. Statistical significance was determined using the Student's t test with the assumption of homoscedasticity. The null hypothesis is rejected if p value is less than the significance levels, i.e., *α = 0.05; **α = 0.01; ***α = 0.001. n.s., not significant.

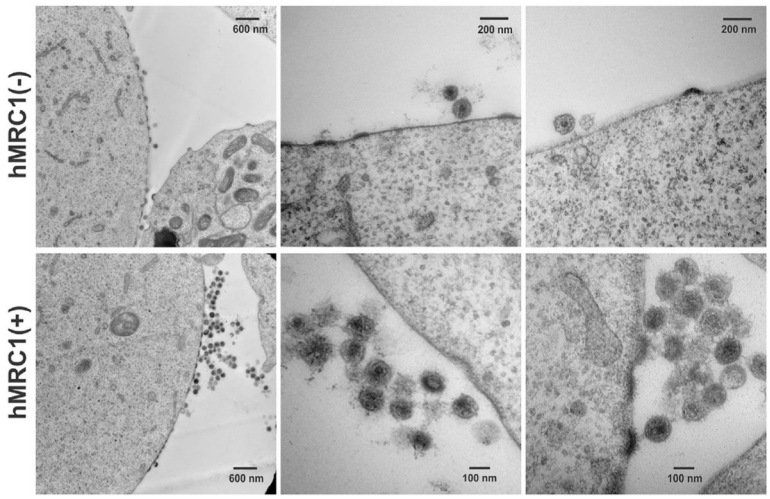

Figure 5. Expression of hMRC1 Results in the Accumulation of Clusters of Viral Particles at the Cell Surface

HEK293T cells were grown overnight on Therma-nox Plastic coverslips in a 12-well plate. Cells were then transfected with 2.0 μg each of pAD8 together with 1.0 μg of empty vector (top row) or pCMV6-hMRC1 (lower row) and cultured for 32 hr. Cells were then fixed with glutaraldehyde for 1 hr at room temperature and processed for transmission EM. Representative images are shown.

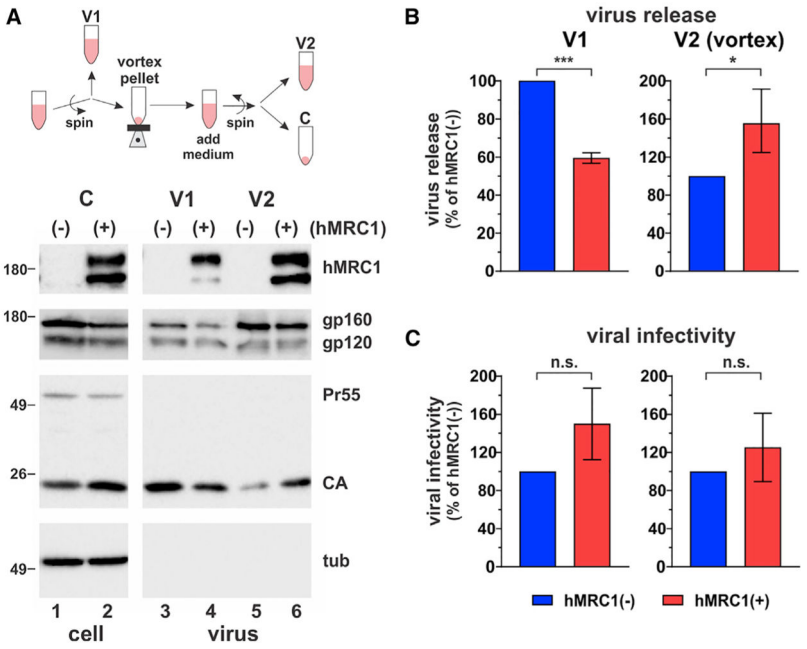

Figure 6. Particles Attached to the Cell Membrane by hMRC1 Can Be Released by Physical Force

HEK293T cells were transfected with 4.0 μg of pAD8 together with 2.0 μg of empty vector (−) or pCMV6-hMRC1 (+).

(A) A graphical presentation of the experimental procedure is shown on the top. Cells and virus-containing supernatants were collected 24 hr post-transfection. Viral fractions (V1 and V2) were collected and processed as described in the Experimental Procedures. Total cell extracts and concentrated viral supernatants were subjected to immunoblot analysis using antibodies to hMRC1, HIV-1 Gag, and tubulin.

(B) Virus release was quantified by determining virus-associated reverse-transcriptase activity. Virus release in the absence of hMRC1 was defined as 100% for each virus. The results are shown as an average of three independent experiments with SDs.

(C) A portion of the filtered unconcentrated culture supernatants was used for the infection of TZM-bl cells. Luciferase activity was measured 48 hr later and normalized for input virus. Relative infectivity observed in the absence of hMRC1 was defined as 100% for each virus. The results are shown as an average of three independent experiments with SDs. Statistical significance was determined using a one-tailed, paired Student's t test. The null hypothesis is rejected if the p value is less than the significance levels, i.e., *α= 0.05; ***α= 0.001.n.s., not significant.

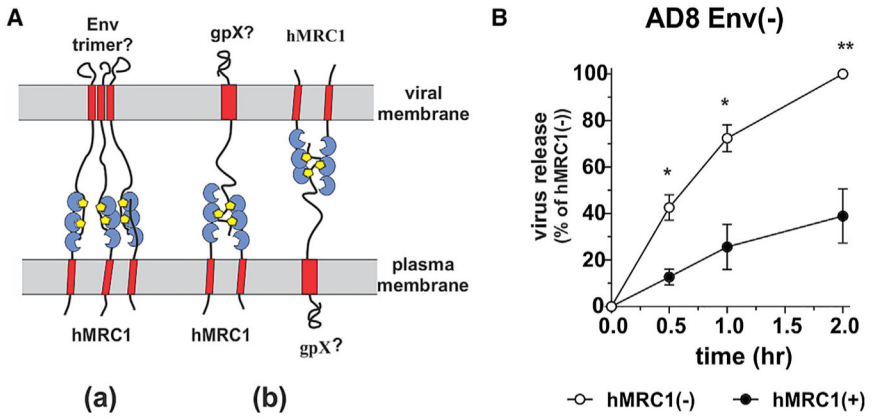

Figure 7. Deletion of the HIV-1 *env* Gene Does Not Alleviate hMRC1 Inhibition of Virus Release

(A) Hypothetical scenarios for the hMRC1-mediated inhibition of viral particle release. (a) It is conceivable that HIV-1 Env present in viral particles can interact with hMRC1 expressed at the cell surface and inhibit particle shedding. (b) It is also possible that cellular glycoprotein(s) (gpX) present in viral particles and/or plasma membrane interact with hMRC1 and inhibit particle detachment.

(B) HEK293T cells were transfected with 4.0 μg of pAD8 Env(−) together with 2.0 μg of empty vector (open circles) or pCMV6-hMRC1 (solid circles). Cells were collected 24 hr later, pulse-labeled for 30 min with [^{35}S]-labeling agent, and chased for up to 2 hr. Equal aliquots of cells and cell-free supernatants were collected at the indicated times and immunoprecipitated with pooled HIV-IG. Immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography (not shown). Virus release was calculated independently for each time point by determining the percentage of cell-free CA protein relative to the total intra-cellular and extra-cellular Gag protein and plotted as a function of time. Maximal viral release in the absence of hMRC1 was defined as 100%. The results are shown as an average of three independent experiments with SD. Statistical significance was determined using the Student's t test with the assumption of homoscedasticity. The null hypothesis is rejected if the p value is less than the significance levels, i.e., *α = 0.05; **α = 0.01.

See also Figures S5 and S6.