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The TIM and TAM Families of Phosphatidylserine Receptors Mediate Dengue Virus Entry

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SUMMARY

Dengue viruses (DVs) are responsible for the most medically relevant arboviral diseases. However, the molecular interactions mediating DV entry are poorly understood. We determined that TIM and TAM proteins, two receptor families that mediate the phosphatidylserine (PtdSer)-dependent phagocytic removal of apoptotic cells, serve as DV entry factors. Cells poorly susceptible to DV are robustly infected after ectopic expression of TIM or TAM receptors. Conversely, DV infection of susceptible cells is inhibited by anti-TIM or anti-TAM antibodies or knockdown of TIM and TAM expression. TIM receptors facilitate DV entry by directly interacting with virion-associated PtdSer. TAM-mediated infection relies on indirect DV recognition, in which the TAM ligand Gas6 acts as a bridging molecule by binding to PtdSer within the virion. This dual mode of virus recognition by TIM and TAM receptors reveals how DVs usurp the apoptotic cell clearance pathway for infectious entry.

INTRODUCTION

Dengue disease, which is caused by four dengue virus (DV) serotypes (DV1 to DV4), has emerged as a major global health problem during the last decades (Halstead, 2007; Kyle and Harris, 2008). It is estimated that 50–100 million dengue cases occur annually, with more than 2.5 billion people at risk of infection. Infection by any of the four serotypes causes disease, ranging from mild fever to life-threatening dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Halstead, 2007; Kyle and Harris, 2008). Despite the importance and increasing incidence of DV as a human pathogen, there is currently no licensed vaccine against DV, and the lack of antiviral drugs severely restricts therapeutic options.

DV entry into target cells is a promising target for preventive as well as therapeutic antiviral strategies since it is a major determinant of host-range, cellular tropism, and viral pathogenesis. During primary infection, DV enters host cells by clathrin-mediated endocytosis (Krishnan et al., 2007; van der Schaar et al., 2008), a process driven by the interaction between viral particles and cellular receptors. Virus-receptor complexes are then targeted to early endosomes, where the acidic environment triggers an irreversible trimerization of the viral E glycoprotein that results in fusion of the viral and cell membranes (Bressanelli et al., 2004; Modis et al., 2004) and the release of genomic RNA into the cytosol. To date, the molecular bases of DV-host interactions leading to virus entry are poorly understood and little is known about the identity of DV cellular receptor(s). DVs infect a wide range of cell types (Anderson, 2003; Balsitis et al., 2009; Couvelard et al., 1999; Jessie et al., 2004; Wu et al., 2000) and may therefore exploit multiple different receptors or use widely expressed entry molecules. Earlier studies indicated that DV virions make initial contact with the host by binding to membrane-associated heparan-sulfate proteoglycans (Chen et al., 1997). These molecules recognize positively charged residues on the surface of E protein and are thought to concentrate the virus at the target cell surface prior to its interaction with entry factors. Numerous cellular proteins such as heat shock protein 70 (HSP70), HSP90, GRP78/Bip, the lipopolysaccharide receptor CD14, or the 37/67 kDa high-affinity laminin have been proposed as putative DV entry receptors (Ansarah-Sobrinho et al., 2007; Cabrera-Hernandez et al., 2007; Jindadamrongwech et al., 2004; Thepparit and Smith, 2004). However, their function in viral entry remains obscure. To date, the known cellular receptors that promote DV infection are DC-SIGN in dendritic cells (Fernandez-Garcia et al., 2009; Lozach et al., 2005; Tassaneetrithep et al., 2003), L-SIGN in liver endothelial cells (Tassaneetrithep et al., 2003), and the mannose receptor in macrophages (Miller et al., 2008a), all of which are C-type lectins (Robinson et al., 2006) that bind glycans on the viral envelope glycoprotein E (Dejnirattisai et al., 2011; Lozach et al., 2005; Miller et al., 2008a). However, DVs also infect cells that lack these lectins, indicating that other unidentified receptor(s) should exist.

In this study, we have conducted a gain-of-function cDNA screen to identify plasma membrane proteins that enhance DV infection. We discovered that TIM and TAM proteins, two distinct

families of transmembrane receptors that participate in the phosphatidylserine (PtdSer)-dependent phagocytic engulfment and removal of apoptotic cells, are DV entry factors. We found that TIM proteins bind directly to PtdSer on the surface of DV particles, while TAM proteins bind indirectly to viral PtdSer via their natural ligands Gas6 and ProS, which act as bridging molecules. These results indicate that DVs have evolved to exploit TIM and TAM receptors and the apoptotic cell clearance pathway for entry into target cells and suggest that inhibitors of these receptors may represent a promising class of antiviral compounds.

RESULTS

A cDNA Screen Identifies Cell Surface Receptors that Enhance DV2 Infection

To identify DV entry factors, we carried out a gain-of-function cDNA screen for human genes that render the poorly susceptible cell line 293T (Lozach et al., 2005) infectable by the DV2 strain JAM (DV2-JAM). This screen identified L-SIGN, confirming the validity of our approach, but also T cell immunoglobulin domain and mucin domain (TIM)-3, TYRO3, and AXL as potential DV receptors (data not shown). These belong to two distinct families of transmembrane receptors that bind directly (TIMs) or indirectly (TAMs) to phosphatidylserine (PtdSer), an ''eat me'' signal that promotes the engulfment of apoptotic cells (Freeman et al., 2010; Lemke and Rothlin, 2008; Ravichandran, 2011). We further characterized the role of these receptors and of PtdSer during DV infection.

TIM Receptors Enhance DV Infection

TIM-3, along with TIM-1 and TIM-4, is a cell surface glycoprotein that binds to PtdSer on the surface of apoptotic cells (Freeman et al., 2010) and are thought to be key regulators of immune tolerance (Freeman et al., 2010; Umetsu et al., 2008). To examine whether expression of TIM receptors enhances DV infection, 293T cells stably expressing TIM-1 and TIM-4 (which were not initially present in our cDNA library) or TIM-3 (Figure 1A) were generated and challenged with mosquito-derived DV2-JAM at different multiplicities of infection (moi) (Figure 1B). Parental cells were minimally infected by the virus even at high moi. TIM-3 expression resulted in a modest increase in the percentage of infected cells. Strikingly, TIM-1 or TIM-4 expression potentiated infection up to 500-fold. Of note, infection was assessed by measuring newly synthesized NS1 proteins, indicating that TIMs mediate productive DV infection. We obtained similar results using DV2 particles grown in Vero cells (Figure S1A). Titration of cell-free supernatants collected from cells challenged with DV2-JAM showed that TIM-1- and TIM-4-infected cells released large amounts of infectious particles (Figure 1C). Interestingly TIM-mediated enhancement of DV infection was also observed when HeLa cells were used as recipients (Figure S1B) and did not occur in cells expressing BAI1 (Figure S1C), a recently identified PtdSer receptor (Park et al., 2007). TIM-1 or TIM-4 also enhances infection by the three other DV serotypes (Figure 1D). The laboratory-adapted DV2 New Guinea C (NGC) and 16681 strains infected parental 293T cells, suggesting that some DV isolates may use other receptor(s) (Figure S1D). However, DV2 NGC or 16681 infection was also strongly enhanced by TIM-1 or TIM-4 (Figure S1D). To determine whether TIM receptors mediated infection by other viral species, we challenged TIM-1- and TIM-4-expressing cells with West Nile virus (WNV), yellow fever virus vaccine strain (YFV-17D), and herpes simplex virus 1 (HSV-1). Viral infection was quantified by flow cytometry using specific Ab (Figure 1E). TIM-1 and TIM-4 massively enhanced WNV infection, slightly upregulated sensitivity to YFV-17D, but had no effect on HSV-1. Together, these data indicate the PtdSer receptors TIM-1 and TIM-4, and to a lesser extent TIM-3, are cellular factors promoting flavivirus infection.

TIM Receptors Directly Interact with DV Particles and Enhance Virus Internalization

To determine whether DV virions directly bind TIM receptors, we conducted a pull-down assay with soluble TIM-Fc. We incubated DV2 particles with TIM-1-Fc or TIM-4-Fc, or as a positive control with DC-SIGN-Fc and protein G-sepharose beads. Precipitated virus was analyzed by western blotting using the anti-DV E protein mAb 4G2. DV bound to TIM-1, TIM-4, and DC-SIGN constructs and not to NKG2D-Fc or IgG1-Fc negative control constructs (Figure 2A). DV-TIM-1 interaction was also confirmed by ELISA using TIM-1-Fc coating on well plates (Figure 2B). The binding of DV to TIM-1-Fc was inhibited by anti-TIM-1 polyclonal Ab but not by control Ig (Figure S2). DV/TIM-1-Fc interaction was inhibited by EDTA (Figure S2), suggesting that it is Ca^{2+} dependent. Moreover, DV efficiently attached to 293T-TIM-1 and 293T-TIM-4 but not to control cells (Figure 2C). Together, these results show that TIM-1 and TIM-4 bind DV and mediate virus attachment to target cells.

We then investigated whether TIM expression enhances DV internalization. We challenged parental, TIM-1, or TIM-4-expressing 293T cells with DV2-JAM for 2 hr at 37°C. Cells were treated with proteinase K to eliminate bound virus, as previously described (Fernandez-Garcia et al., 2011). Total RNA was extracted and viral RNA levels were quantified by qPCR. TIM receptors strongly increased DV RNA uptake into 293T cells (18-fold and 10-fold enhancement with TIM-1 and TIM-4, respectively) (Figure 2D). In a second approach, we expressed TIM-1, TIM-4, and DC-SIGN into CHO-745 cells, which are a mutant CHO cell line that does not express cell surface heparan sulfate proteoglycans (Esko et al., 1985). Cells were incubated with purified DV2-JAM particles for 1 hr at 4°C and shifted to 37°C for 45 min to allow endocytosis, as previously described (Fernandez-Garcia et al., 2011). Virus uptake was monitored by fluorescence microscopy using anti-DV E mAb 4G2 in cells that were permeabilized, or not, with saponin (Figure 2E). We observed a massive intracellular accumulation of DV E protein in permeabilized cells expressing TIM-1, TIM-4, or DC-SIGN, but not in parental CHO (Figure 2E). Few virus particles were detected in cells that were not permeabilized. To study the role of TIM-1 cytoplasmic tail in enhancement of infection, we transfected 293T cells with TIM-1 WT or a TIM-1 mutant lacking its entire cytoplasmic tail (TIM-1 Δcyt). Cell surface expression levels of TIM-1Δcyt were found to be similar to WT TIM-1 (Figure 2F), however our infection studies show that TIM-1Δcyt is still able to enhance DV2-JAM infection. Thus, TIM-1 enhances DV entry via a mechanism that is independent of its cytoplasmic tail.

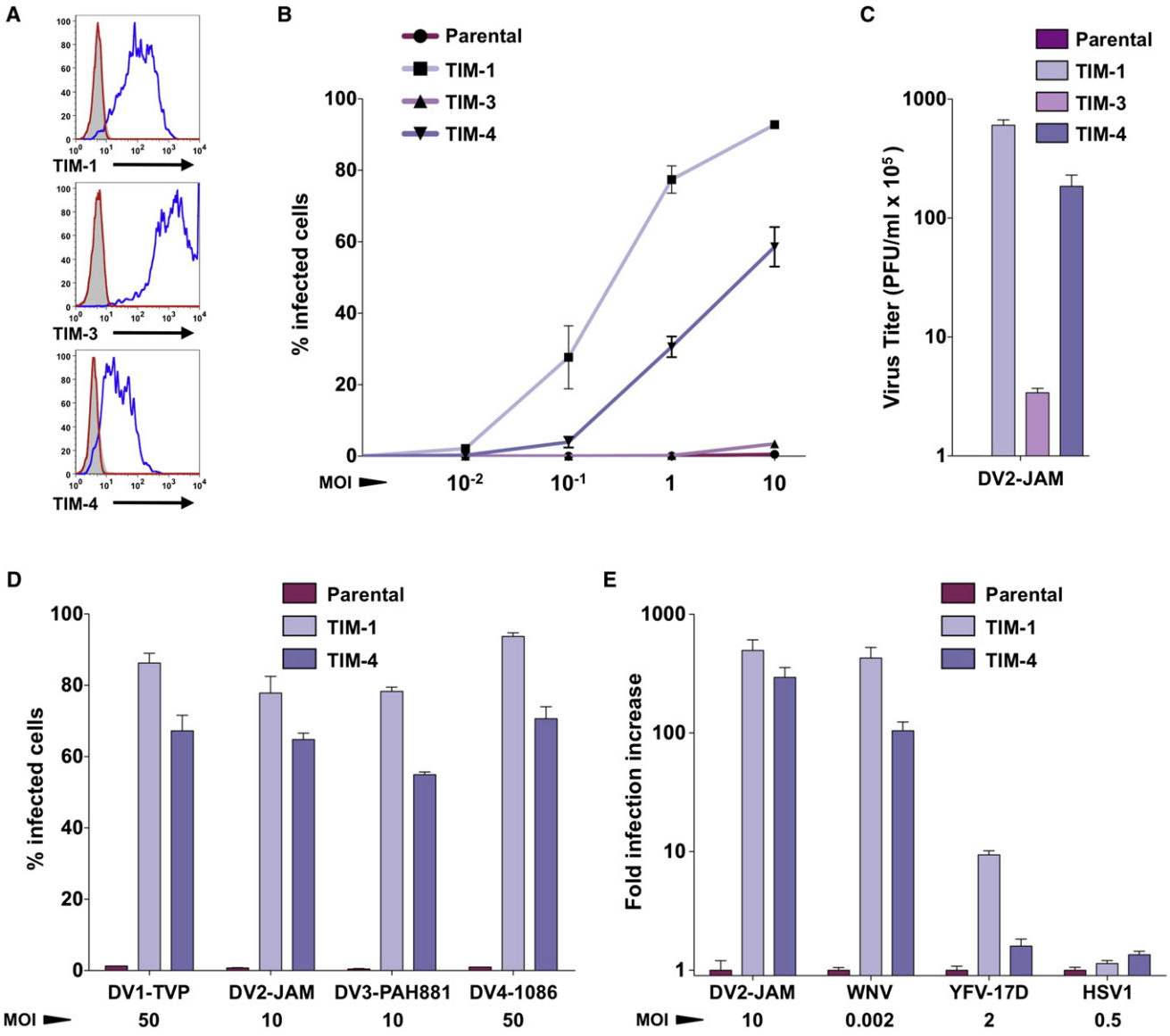

Figure 1. TIM Receptors Enhance DV Infection

(A) Surface levels of TIM molecules on 293T parental (red line) and transduced cells (blue line) were evaluated by flow cytometry using polyclonal anti-TIM antibodies. Gray shading represents cell staining with a control IgG.

(B–E) Parental and 293T cells expressing TIM receptors (B) were challenged with DV2-JAM. Infection levels were assessed by flow cytometry using the anti-NS1 mAb. Parental, TIM-expressing 293T (C) were infected with DV2-JAM. Supernatants were collected 48 hr later. Virus titers were determined by plaque assay and expressed as plaque forming unit per ml. TIM receptors are used by the four DV serotypes (D). TIM receptors are shared by other flaviviruses (E). Data are represented as mean ± SEM of at least three independent experiments. See also Figure S1.

TIM Receptors Recognize PdtSer Associated with DV Particles

TIMs specifically recognize PtdSer exposed on the surface of apoptotic cells (Freeman et al., 2010; Kobayashi et al., 2007; Santiago et al., 2007a, 2007b). We therefore investigated whether PtdSer is associated with DV virions. For this aim, we purified DV2-JAM particles using sucrose gradient as previously described (Navarro-Sanchez et al., 2003). Purified viruses retain their ability to use TIM receptors (Figure S3). Sucrose gradient purified DV2-JAM were coated on ELISA wells and incubated with the anti-PtdSer mAb 1H6. We found that the 1H6 mAb, but not its isotype control, bound to DV-coated ELISA plates (Figure 3A). To examine if TIM-mediated DV infection is dependent on viral PtdSer, we preincubated purified DV2 with annexin V (ANX5), a PtdSer-binding protein. ANX5 inhibited infection of 293T-TIM-1 and 293T-TIM-4 but not of 293T-DC-SIGN cells (Figure 3B). Structural studies of the TIM immunoglobulin (Ig) domain previously demonstrated that PtdSer binds in a cavity built up by the CC′ and FG loops, termed the metal ion-dependent ligand-binding site (MILIBS) (Kobayashi et al., 2007; Santiago et al., 2007a, 2007b). We designed mutants of highly conserved amino acids (TIM-1 N114A or D115A, TIM-4 N121A)

that line the binding cavity and are essential to PtdSer recognition (Kobayashi et al., 2007; Santiago et al., 2007a, 2007b). We found that the mutated TIM receptors were unable to mediate DV2 infection even though they were correctly expressed at the cell surface (Figure 3C). Together, these data suggest that PtdSer is associated with the surface of DV virions and is required for TIM-mediated DV infection.

TAM Ligands Bind to Viral PtdSer and Bridge DV Particles to TAM Receptors

TYRO3 and AXL belong to the TAM family, a group of three receptor protein tyrosine kinases that mediate the clearance of apoptotic cells (Lemke and Rothlin, 2008). The TAM ligands Gas6 and ProS are essential to this process (Fernández-Fernández et al., 2008; Lemke and Rothlin, 2008). Via their N-terminal Gla domain, they recognize the PtdSer expressed on apoptotic cells and bridge these cells to a TAM receptor on the surface of phagocytes (Lemke and Rothlin, 2008).

We found that 293T cells expressing high levels of TYRO3 or AXL (Figure 4A) were efficiently infected by DV2-JAM as assessed by immunofluorescence (Figure 4B) and flow cytometry using anti-NS1 Ab (Figure 4C) and released many infectious particles (Figure S4A). Ectopic expression of AXL and TYRO3 in 293T cells strongly potentiated infection by the four different DV serotypes and by WNV, but not by HSV-1 (Figures S4B and S4C). In addition, TYRO3 and AXL act at a very early stage during DV infection by enhancing virus uptake (Figure S4D). We expressed WT, kinase-dead forms of AXL (K567A mutation of the ATP-binding site) and AXL ΔCyt (complete cytoplasmic domain deletion) in 293T cells and compared their ability to mediate endocytosis and infection. Interestingly, our data indicated that tailless and kinase-dead AXL variants are unable to enhance DV infection (Figure S4E). However, these mutated receptors mediate DV internalization as efficiently as AXL WT (Figure S4F). This suggests that the AXL cytoplasmic tail and tyrosine kinase activity are essential for enhancement of DV infectivity but dispensable for virus endocytosis.

We found that DV did not interact directly with TYRO3 or AXL, since we were unable to immunoprecipitate virus particles with TAM-Fc constructs (Figure S4G). However, incubation of DV particles with purified Gas6 (Figure 4D) or with fetal bovine serum (FBS) (Figure S4H), in which ProS is present at ~300 nM (Fernández-Fernández et al., 2008), potentiated virus binding to TAM-expressing cells. Thus, DV attachment to TYRO3 and AXL likely occurs only in the presence of TAM ligands. Moreover, AXL-mediated enhancement of DV infection was no longer detectable in serum-free medium, but was restored after preincubating DV particles with Gas6 (Figure 4E) or FBS (Figure S4I). Similar results were observed with TYRO3 (Figure S4J). Consistent with these findings, an AXL receptor mutated in the Gas6 binding site (AXL E66R/T84R) (Sasaki et al., 2006) did not enhance infection of DV complexed to Gas6, even though it was expressed at the cell surface (Figure 4E). To further investigate the importance of viral PtdSer in TAM-mediated enhancement of DV infection, we used Gas6ΔGla, a Gas6 variant impaired for PtdSer binding (Figure S4K). Gas6ΔGla did not interact with DV particles coated onto plate wells (Figure 4F) and failed to bridge DV virions to TYRO3-Fc and AXL-Fc (Figure 4G). Pretreatment of TAM-expressing 293T cells with Gas6ΔGla abrogated DV infection (Figure 4H). As for TIM receptors, ANX5 inhibited DV infection of 293T cells expressing TYRO3 or AXL (Figure S4L), strongly suggesting that the TAM effect is PtdSer-dependent. Together, these data support a tripartite model, whereby TAM ligands bind to PtdSer associated with DV particles and bridge virions to TAM receptors.

TIM-1 and AXL Expression Correlate with DV Susceptibility

We next investigated the relationship between TIM and TAM expression on the cell surface and the efficiency of DV infection in various cell lines. We detected high levels of TIM-1 and/or AXL expression on the surface of A549, Vero, Cos-7, and Huh7 5.1 cells (Figure 5A), all of which are readily infected by DV2-JAM (Figure 5B). 293T, U937, or RAJI cells, which are poorly susceptible to DV2-JAM (Figure 5B), lack TIM-1 and AXL (Figure 5A). To investigate whether enhanced infectivity associated with TIM and TAM expression correlate with increased virus binding, we performed binding studies using the A549 cell line that endogenously expresses TIM-1 and AXL (Figure 5A). A549 were also chosen because they express low levels of heparan sulfate proteoglycan, which has been described as an attachment receptor for DV (Chen et al., 1997) and thus may interfere with the binding studies. We found that DV2 interacts efficiently with A549 cells. Interestingly, we observed that anti-TIM1 Ab significantly inhibits DV2 binding, while anti-AXL polyclonal has only a modest effect (Figure 5C). These data suggest that TIM-1 is an important receptor that mediates DV attachment to A549 cells.

Endogenous TIM-1 and AXL Mediate DV Infection

We then analyzed the effects of neutralizing anti-TIM1 and/or anti-AXL Abs on DV infection of different cell lines. The Huh7 5.1 cell line expresses only TIM-1 (Figure 5A). An anti-TIM-1 Ab inhibited DV2 infection but not HSV-1 infection (Figure 6A). The A549 cell line expresses both TIM-1 and AXL (Figure 5A). DV2 infection was partly reduced with an anti-TIM-1 or anti-AXL Ab administrated alone, while the two Abs in combination fully inhibited DV2 (Figures 6B and 6C) and DV3 (Figure S5A) but not HSV-1 infection. Similar results were obtained on Vero cells, which express TIM-1 and AXL (Figure S5B). We then silenced TIM-1 or AXL expression by RNA interference in A549 cells (Figure 6D). DV infection was reduced in AXL-silenced cells and almost totally inhibited in TIM-1 silenced cells (Figure 6D), indicating an important role of TIM-1 in DV infection of A549. Notably, as for TIM- and TAM-293T-transfected cells, ANX5 blocked DV infection of A549 cells (Figure 6E). Altogether, these results show that TIM and TAM receptors may naturally cooperate to promote DV infection and that viral PtdSer mediates infection in cells endogenously expressing the receptors.

Epithelial cells and astrocytes are DV targets in vivo (Balsitis et al., 2009; Ramos et al., 1998; Limon-Flores et al., 2005). Primary kidney epithelial cells and astrocytes express AXL (Figures 6F and 6G) but not TYRO3, TIM-1 or TIM-4 (not shown). DV infection was significantly reduced by an anti-AXL Ab in both cell types (Figures 6F and 6G). Silencing AXL in astrocytes also significantly decreased DV3 infection (Figure S5C). Therefore, as illustrated for AXL, the PtdSer receptors identified in our

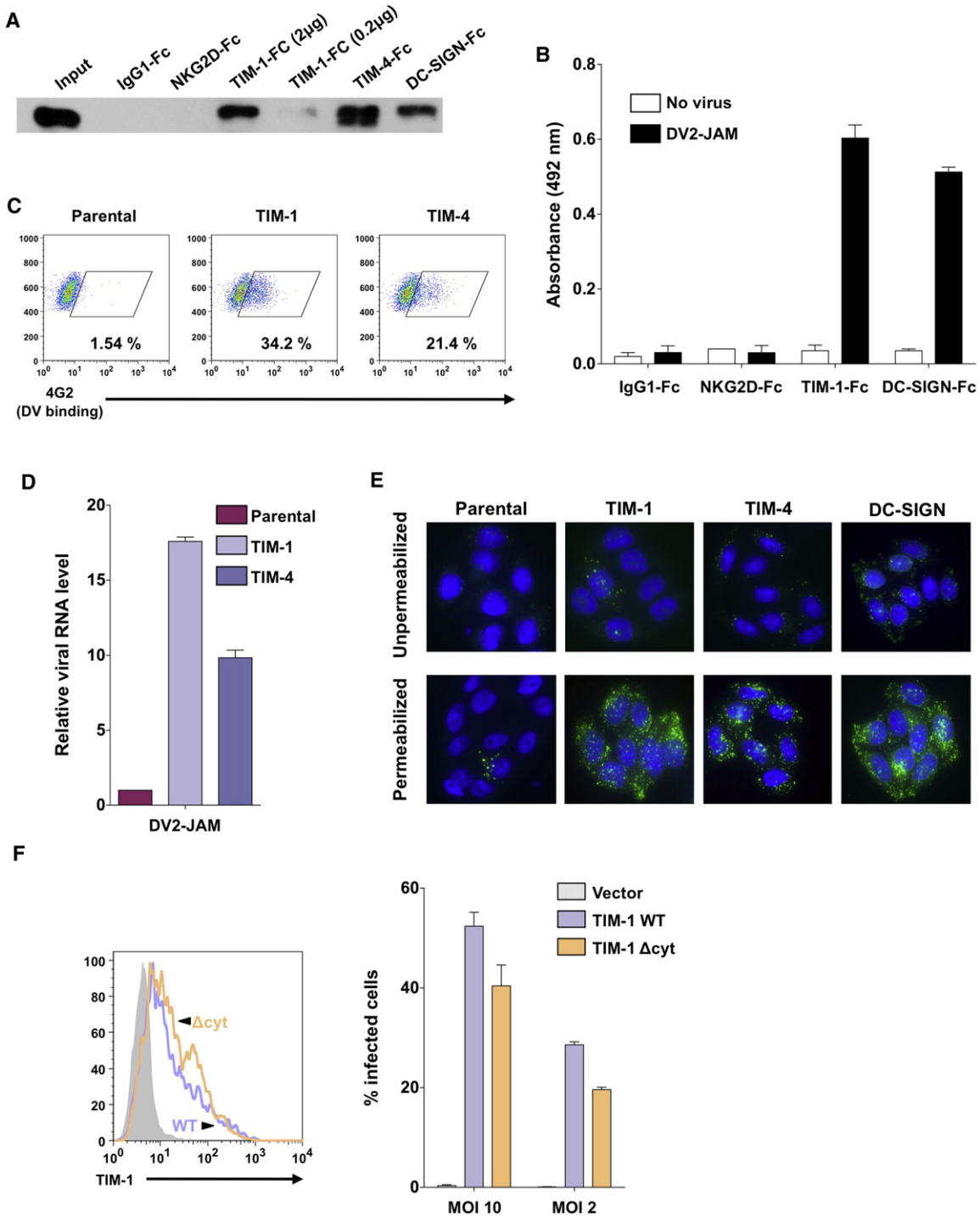

Figure 2. TIM-1 and TIM-4 Molecules Bind to DV and Enhance Virus Endocytosis

(A) Western blot analysis of DV2-JAM preincubated with control Fc, NKG2D-Fc, TIM-1-Fc, or TIM-4-Fc bound to protein A-agarose beads. Pulled-down virus was detected using the 4G2 anti-DV E protein mAb.

(B) Control Fc, NKG2D-Fc, or TIM-1-Fc were coated on 96-well plates and incubated with DV2-JAM for 1 hr at 4°C. Bound virus was detected using the biotinylated 4G2 mAb and HRP-conjugated streptavidin See also Figure S2.

(C) Cells transfected with an empty vector or plasmids encoding TIM-1 or TIM-4 were incubated 1 hr at 4°C with DV2-JAM. Bound virus was detected by flow cytometry using the 4G2 mAb.

(D) Cells were incubated with DV2-JAM for 2 hr at 37°C and treated with proteinase K. Total RNA was extracted from infected cells, and DV2 viral RNA level was determined by real-time quantitative PCR with human GAPDH as endogenous control. Results are expressed as the fold difference using expression in 293T infected cells as calibrator value.

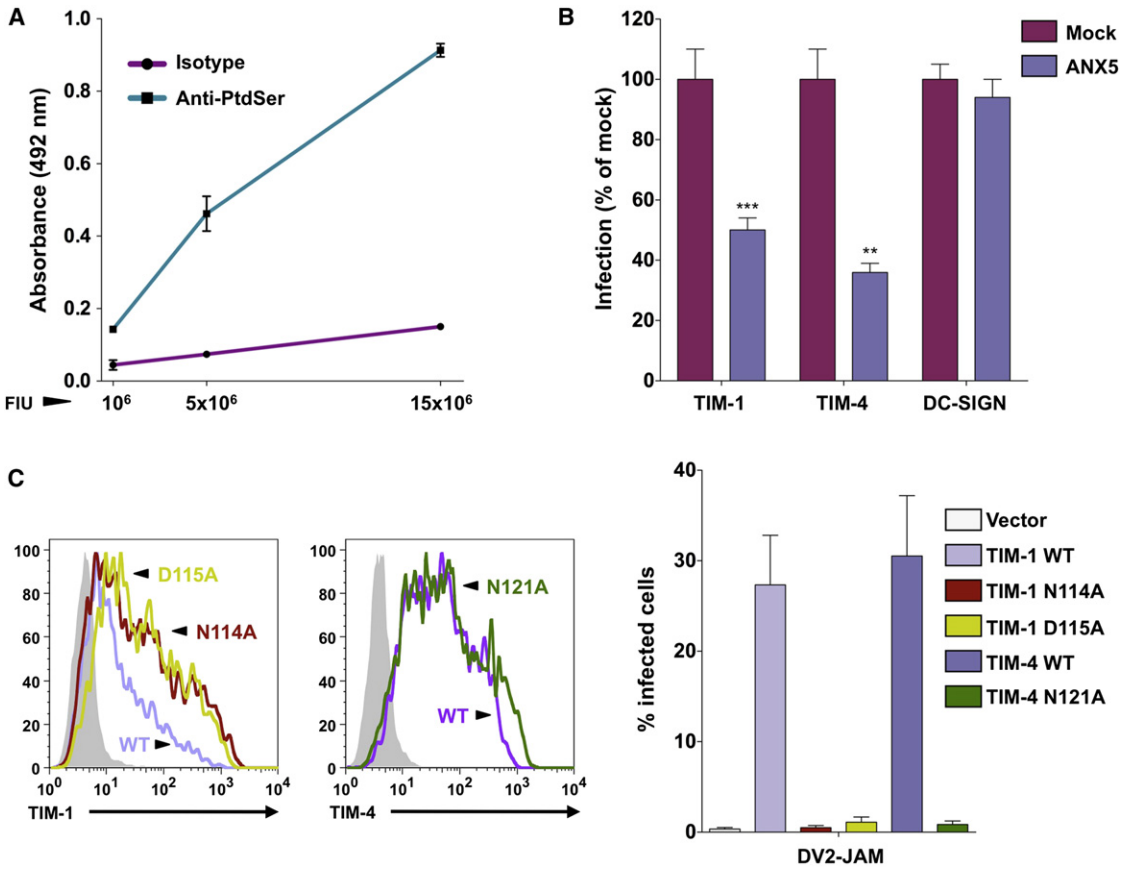

Figure 3. TIM-1 and TIM-4 Molecules Recognize PtdSer Associated with DV Particles

(A) Sucrose gradient purified DV2 particles were coated on well plates and incubated with the anti-PtdSer 1H6 mAb. See also Figure S3.

(B) DV2-JAM preincubated with Annexin V (ANX5; 25 μg/ml) was used to infect the indicated cells. Infection was normalized to infection without ANX5.

(C) TIM molecules mutated in the PtdSer binding domain do not mediate DV infection. Left panel: Cell surface expression of WT and TIM-1 or TIM-4 mutants. Right panel: Transfected cells were infected with DV2-JAM. Data are represented as mean ± SEM of at least three independent experiments.

screening are involved in the infection of human primary cells, an observation that should be relevant for DV pathogenesis.

DISCUSSION

The present study adds significant insights into the molecular interactions that occur between DV and the host cell during viral entry. We show that TIM and TAM proteins, two receptor families involved in apoptotic cell recognition and clearance, mediate DV infection. TIM- and TAM-mediated enhancement of virus infection is dependent on PtdSer associated with DV particles. This supports a model by which DV use a strategy of ''apoptotic mimicry'' to infect target cells (Laliberte and Moss, 2009; Mercer and Helenius, 2008).

We provide strong evidence indicating that TIM and TAM receptors are cellular factors mediating DV binding and infection. First, ectopic expression of TIM and TAM rendered human cells susceptible to DV infection. Second, Ab against TIM and TAM receptors inhibited DV infection in cells naturally expressing these proteins. Third, in analyses of DV infectivity, evidence of loss-of-function was obtained using RNA interference. Finally, binding studies revealed that TIM and TAM molecules interact with DV particles and enhance virus endocytosis. However, our data suggest that TIM and TAM proteins do not directly promote DV internalization. We observed that the TIM-1 cytoplasmic tail is dispensable for DV entry, indicating that TIM-mediated enhancement of infection relies mainly on the receptor ectodomain. We propose that TIM-1 may act primarily as a tethering/attachment receptor that binds and enhances DV infection without any obligatory role in virus endocytosis. This is consistent with the proposed role of TIM receptors during the engulfment of apoptotic cells (Park et al., 2009; Toda

(E) Parental and CHO-745 cells expressing TIM-1, TIM-4, or DC-SIGN were incubated with DV2-JAM for 1 hr at 4°C and shifted at 37°C for 45 min. Cells were labeled with DAPI (blue) and the anti-DV E protein 4G2 (green) to detect virus uptake in unpermeabilized and permeabilized cells.

(F) 293 T cells were transfected with plasmid encoding for TIM-1 WT, TIM-1 lacking its entire cytoplasmic tail (TIM-1 Δcyt), or an empty vector (pcDNA3). Cells were stained for TIM-1 expression (left panel) and challenged with DV2-JAM (right panel). Data are represented as mean ± SEM of at least three independent experiments.

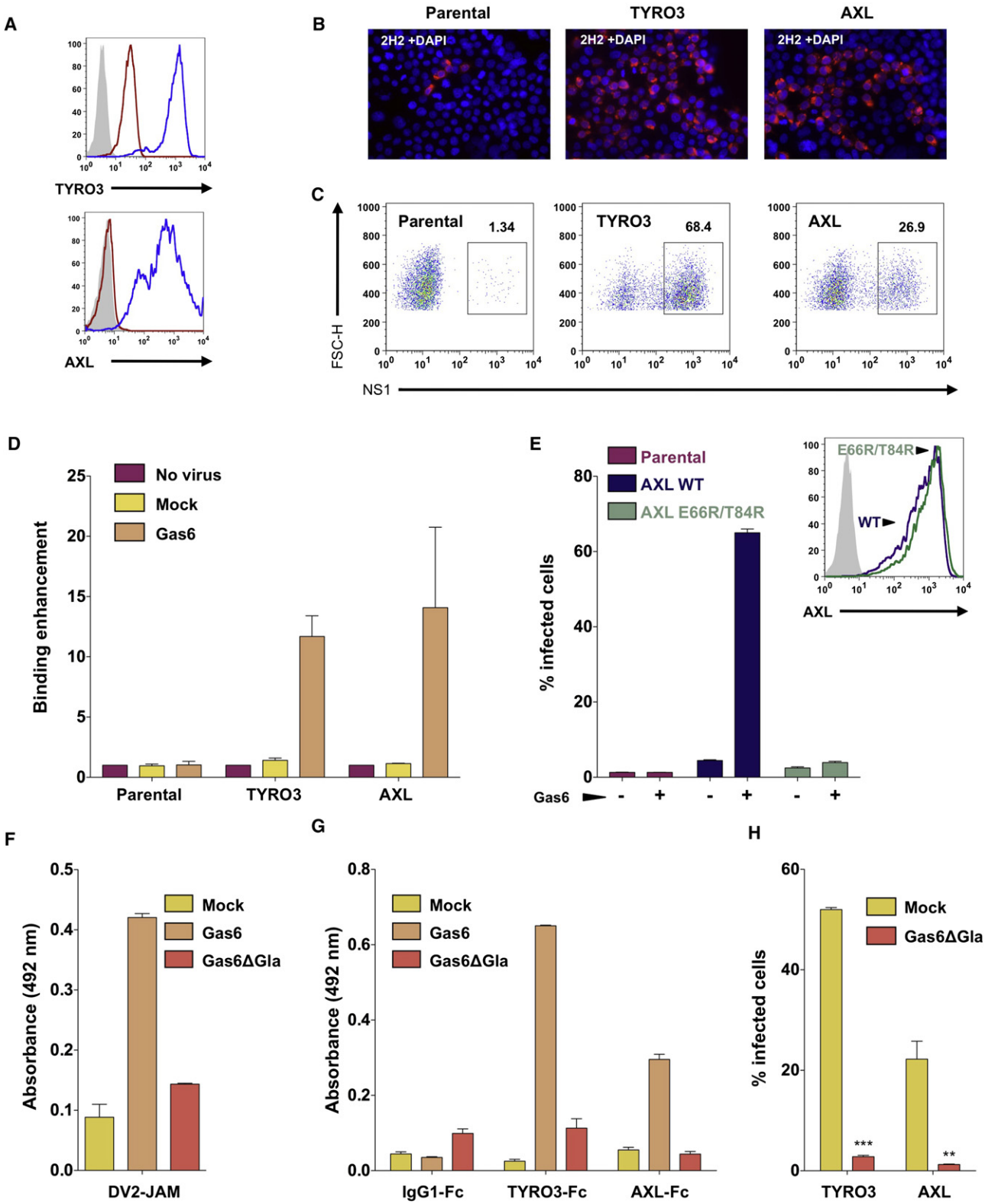

Figure 4. TYRO3 and AXL Enhance DV Infection

(A) Surface levels of TAM molecules on 293T parental (red) and cells stably expressing human TYRO3 and AXL (blue). Gray: isotype control Ab.

(B and C) Cells were challenged by DV2-JAM and infection was assessed 48 hr later. Immunofluorescence (B) with an anti DV prM glycoprotein (2H2) (red) and DAPI (blue). Flow cytometry analysis (C) with an anti-NS1 mAb. See also Figure S4.

et al., 2012). Our results also suggest that TIM-mediated enhancement of DV infection relies on still uncharacterized cellular receptors. It is possible that TIM receptors pass the virus in *cis* to another molecule that is ultimately responsible for virus endocytosis, as we proposed for DC-SIGN-mediated DV entry (Lozach et al., 2005). Alternatively, TIMs could also directly associate with unknown molecules to form an entry complex that coordinates virus endocytosis. Our data suggest that TIM and TAM receptors may play distinct roles during DV infection. We found that tailless and kinase-dead AXL variants are still able to enhance DV endocytosis. However, and as shown for Ebola and Lassa virus entry (Shimojima et al., 2007, 2012), both mutated AXL molecules are unable to enhance DV infection, indicating that tyrosine kinase activity is important for AXL potentiation of viral infection. These data suggest that AXL function is not restricted to enhance virus endocytosis but also to initiate signaling that facilitates infection of the target cells. Of note, Gas6 binding to TAM receptors has been shown to inhibit innate inflammatory immune responses (Lemke and Rothlin, 2008), including signaling downstream of TLRs (Rothlin et al., 2007). It is thus tempting to speculate that TAM ligation by DV during entry may initiate signal transduction events that may modulate host immune responses to potentiate a postendocytic step of the DV life cycle.

In cells expressing both TIM-1 and AXL (such as A549 and Vero), DV infection was partly reduced by anti-TIM-1 or anti-AXL Ab administrated alone and completely inhibited by the two Ab in combination. Thus, TIM-1 and AXL may cooperate to mediate optimal DV infection. TAM receptors are known to physically associate with non-TAM receptors by heterotypic dimerization (Linger et al., 2008; Rothlin et al., 2007). One can speculate that they may recruit TIM and/or additional receptors to form a viral entry complex required for DV uptake and infection (Figure 7A). DVs use the clathrin-mediated endocytosis as a major entry route for internalization (Krishnan et al., 2007; van der Schaar et al., 2008). It will be worth further assessment of the role of the clathrin-dependent pathway and other endocytic routes during TIM- and TAM-mediated enhancement of DV infection.

The role played by TIM and TAM proteins during DV pathogenesis in vivo is currently unknown. In vivo, TIM and TAM receptors are expressed in many cell types relevant to DV infection. AXL is widely expressed (Lemke and Rothlin, 2008) with prominent expression in macrophages, dendritic cells, and vascular endothelia, and we show here that DV infection of cultured epithelial cells and astrocytes is AXL-dependent. TIM-1 is abundant on Th-2 T cells, mucosal epithelial cells, and mast cells; the last have been recently identified as important DV target cells (Freeman et al., 2010; Kondratowicz et al., 2011; St John et al., 2011). TIM-4 is present exclusively on antigen-presenting cells (Kobayashi et al., 2007; Wong et al., 2010), which are sensitive to DV infection and thought to play an important role in DV pathogenesis. Our data indicate that TIM and TAM usage is not sufficient to explain DV tropism. Indeed, we have shown that DVs can infect cells lacking these proteins, suggesting that other cellular receptors that dictate virus tropism remain to be characterized. However, our results suggest that TIM and TAM receptors contribute to enhance infection of relevant target cells, which may have important consequences for virus spread.

We reveal an unexpected role for PtdSer during DV infection. Our data suggest that PtdSer is displayed on the surface of DV particles and is important for TIM- and TAM-mediated infection. In agreement with the binding of apoptotic cells to PtdSer receptors (Ravichandran, 2011; Ravichandran and Lorenz, 2007), we describe a dual mode of recognition of DV by TIM and TAM receptors. TIM molecules directly interact with DV. Our mutagenesis study of TIM strongly suggests that PtdSer associated with DV particles binds to the MILIBS of the molecule. Indirect recognition of DV is mediated by a TAM ligand such as Gas6, which recognizes through its Gla domain viral PtdSer and bridges DV virions to AXL and TYRO3. Together, these observations suggest that, by mimicking apoptotic bodies, DVs subvert the apoptotic clearance function of TIM and TAM receptors to enhance infection.

TIM and TAM proteins mediate the entry of other viruses (Feigelstock et al., 1998; Kondratowicz et al., 2011; Morizono et al., 2011; Shimojima et al., 2012, 2006). AXL and TIM-1 are receptors for Ebola virus, which, like DV, infects a broad range of cell types and causes hemorrhagic fever (Kondratowicz et al., 2011; Shimojima et al., 2006). TIM-1-mediated Ebola infection depends on a direct interaction between the viral glycoprotein GP through residues outside the MILIBS (Kondratowicz et al., 2011), indicating that DV and Ebola virus may use distinct TIM-1 regions. A recent study also identified Gas6 and ProS as "bridging factors" that link PtdSer expressed on the viral envelope of lentiviral pseudotypes and vaccinia virus to AXL expressed on the target cell (Morizono et al., 2011). Collectively, these data suggest that TIM and TAM facilitation of viral infection may represent a general mechanism exploited by viruses that incorporate PtdSer in their membrane for optimal infection.

An interesting direction for future work is to identify how DVs acquire this lipid and how PtdSer becomes accessible to TIM and TAM receptors. The DV membrane is derived from the ER of the infected cell upon budding. The ER membrane is known to contain PtdSer in the lumenal leaflet (Leventis and Grinstein, 2010), which suggests an obvious mechanism through which

(D) Gas6 enhances DV binding to TYRO3- and AXL-expressing cells. Cells were incubated for 90 min at 4°C with DV2-JAM in serum-free medium containing Gas6 (1 μg/ml) or PBS (mock). Mean fluorescent intensity was measured by flow cytometry and normalized to that in noninfected cells.

(E) 293T cells expressing WT AXL or a molecule mutated in the Gas6-binding site (E66R/T84R) were incubated with DV2-JAM in serum-free medium containing Gas6 (1 μg/ml) or PBS (mock). After 3 hr, medium was replaced by medium supplemented with 10% FBS. The inset displays wild-type and mutant AXL surface expression.

(F) Plastic-coated DV2-JAM (10^7 FIU) was incubated with Gas6 or Gas6ΔGla (2 μg/ml) for 1 hr. Bound Gas6 was detected by ELISA using a goat polyclonal anti-Gas6 (10 μg/ml) Ab.

(G) Plastic-coated DV2-JAM particles (10^7 FIU) were incubated with the indicated Fc-chimeras (2 μg/ml) in the presence or absence of Gas6 or Gas6ΔGla (2 μg/ml). Bound Fc-chimeras were detected using an HRP-conjugated anti-human IgG.

(H) Cells were incubated with Gas6ΔGla (1 μg/ml) for 30 min before and during a 3 hr-incubation with DV2-JAM. Data are represented as mean ± SEM of at least three independent experiments. $^{**}p < 0.001$, $^{***}p < 0.0001$. See also Figure S5.

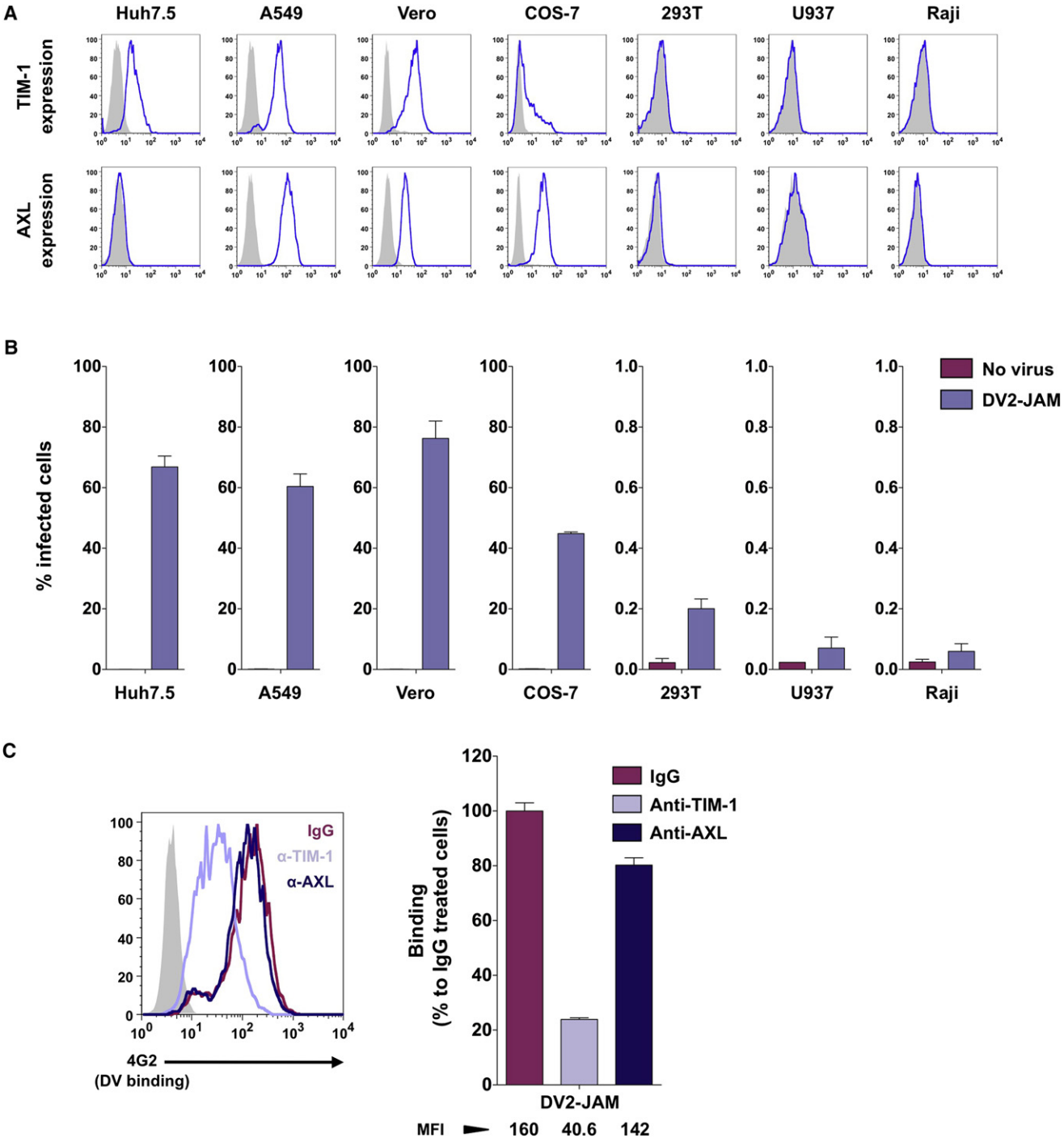

Figure 5. TIM-1 and AXL Expression and DV Infection in Cell Lines

(A) Surface level of TIM-1 and AXL on cell lines was monitored by flow cytometry. Gray shading represents cell staining with a control Ab.

(B) The indicated cells were challenged with DV2-JAM.

(C) A549 cells were incubated with DV2-JAM for 1 hr at 4°C in the presence of goat polyclonal anti-TIM-1, anti-AXL, or normal goat IgG as control. Mean fluorescent intensity was measured by flow cytometry and normalized to binding in presence of control IgG. Data are shown as one representative flow cytometry analysis (left panel) and are represented as mean ± SEM of at least three independent experiments (right panel).

PtdSer may be incorporated into DV virions (Figure 7B). However, the viral membrane does not appear to be readily exposed to receptors at the virion surface. This is shown by structural studies, which have revealed that a closed icosahedral shell of the envelope protein completely encloses the viral membrane in mature DV particles (Kuhn et al., 2002). It is

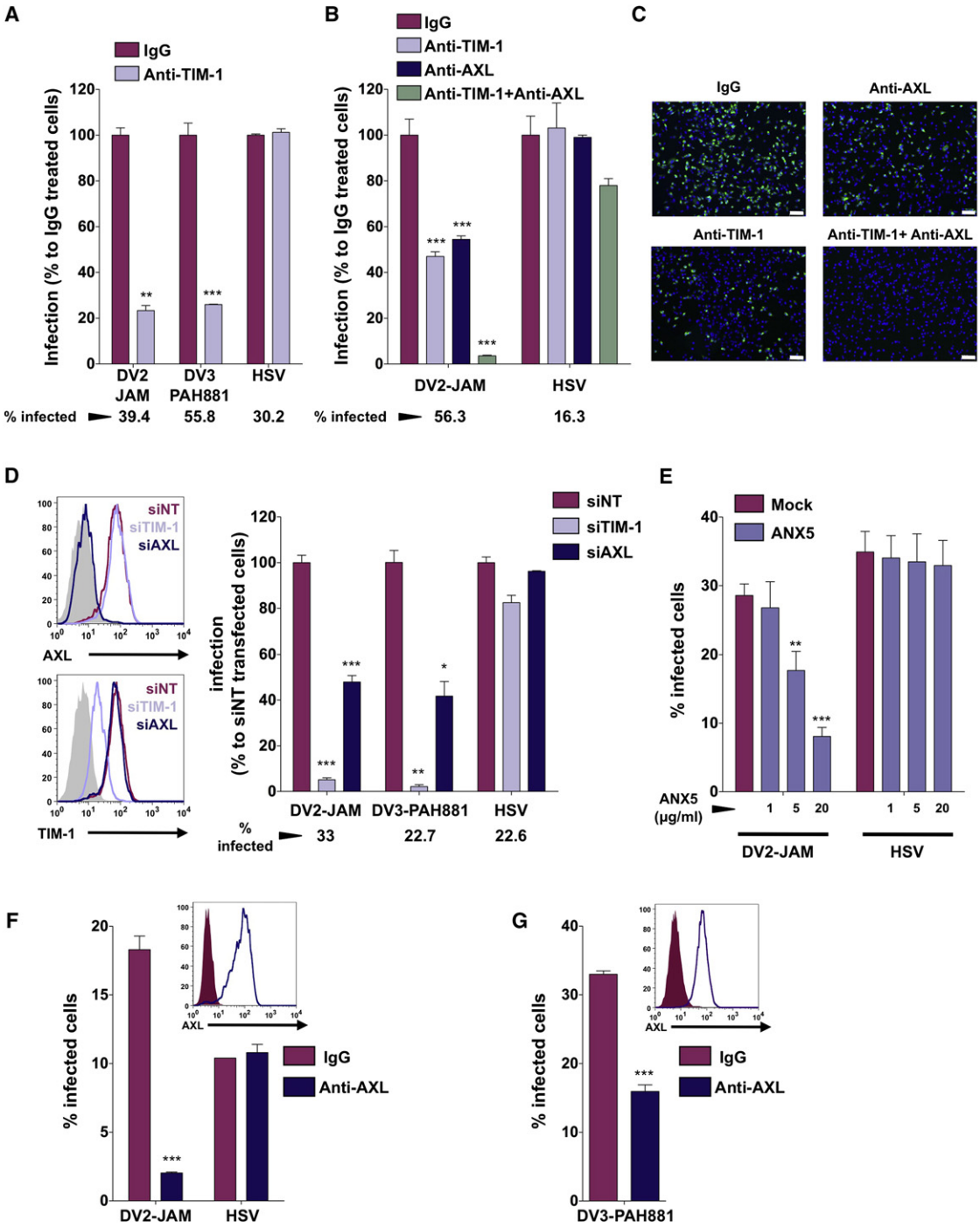

Figure 6. Endogenous TIM-1 and AXL Molecules Mediate DV Infection

(A and B) Huh7.5.1 (A) and A549 (B) cells were infected with the indicated DV strains or HSV-1 in the presence of polyclonal goat anti-TIM-1, anti-AXL, or normal goat IgG as control. The levels of infected cells were normalized to infection in presence of control IgG.

(C) Representative immunofluorescence analysis of A549 infected with DV2-JAM in the presence of the indicated Ab. Green anti-prM 2H2, Blue DAPI. Scale bar: 100 μm.

(D) A549 cells were transfected by the indicated siRNA, and TIM-1 and AXL expression was assessed by flow cytometry after 2 days, at the time of infection. Cells were infected with DV2-JAM or HSV-1. Infection was normalized to infection in nontargeting (siNT) siRNA-transfected cells.

(E) A549 cells were infected with DV2-JAM or HSV-1 preincubated with different concentrations of ANX5.

(F) and (G) Human primary kidney epithelial cells (F) or astrocytes (G) were infected with DV in the presence of control IgG or an anti-AXL Ab. Insets display AXL cellular expression at the time of infection. Data are represented as mean ± SEM of at least three independent experiments. $^{**}p < 0.001$, $^{***}p < 0.0001$. See also Figure S6.

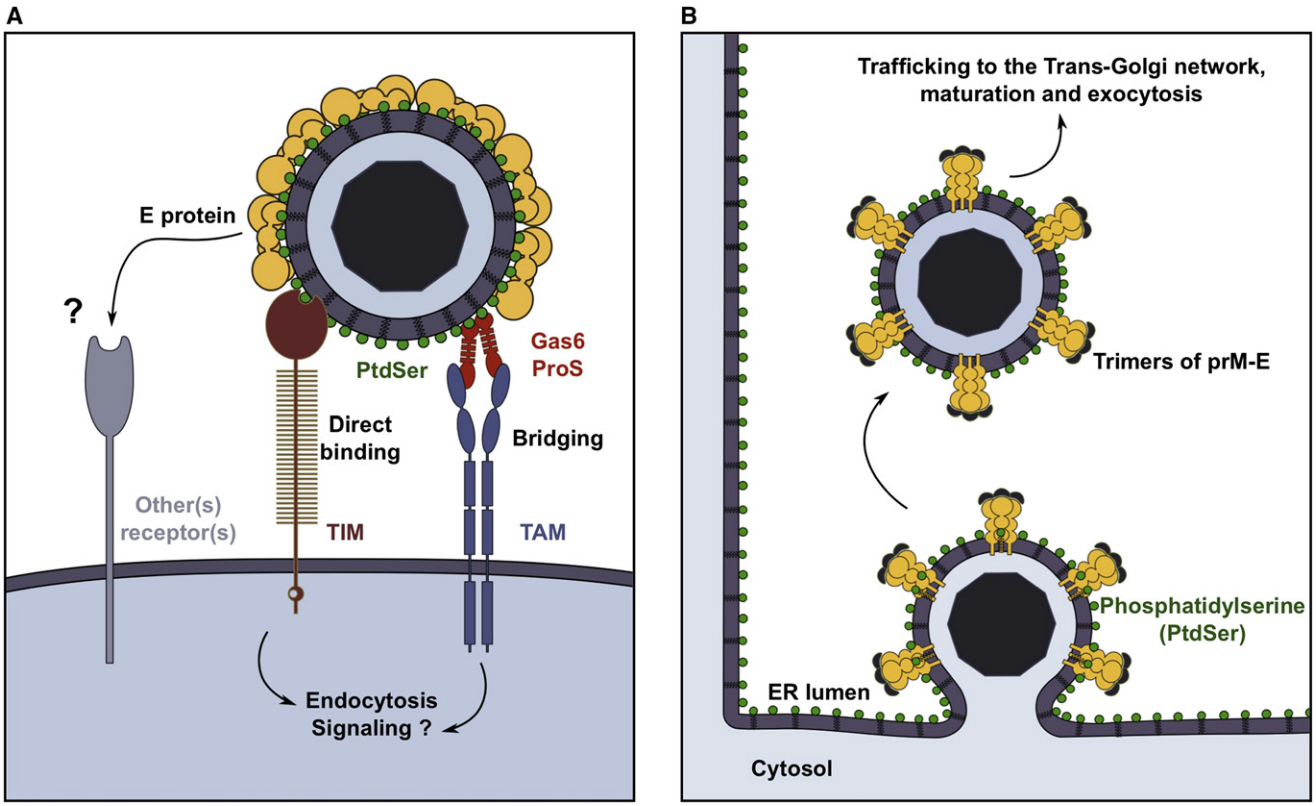

Figure 7. Model of PtdSer Acquisition by DV Particles and the TIM and TAM Receptor-Mediated Enhancement of DV Infection

(A) Proposed model of direct and indirect (bridging) recognition of DV by the TIM and TAM receptors.

(B) Schematic representation of DV particle acquiring phosphatidylserine (PdtSer) upon budding into the ER lumen.

plausible that TIM and TAM molecules or other yet-to-be identified receptors may display weak interactions with the E protein that trigger opening of the icosahedral shell, leading to exposure of viral membrane, as recently suggested by studies with Ab complexes (Cockburn et al., 2012). Also, recent studies have indicated that the majority of the DV particles released from infected cells are partially mature virions, which display a mixture of immature and mature surfaces (Junjhon et al., 2010; Pierson and Diamond, 2012; Plevka et al., 2011). These immature-like regions could expose membrane patches, such that PtdSer would be accessible for TIM and TAM interactions. Partially mature virions result in an incomplete cleavage of the envelope glycoprotein prM. Additional studies are thus required to evaluate whether modulation of the efficiency of the prM cleavage impacts TIM and TAM receptor usage.

EXPERIMENTAL PROCEDURES

cDNA Library Screening

We used sequence databases (SWISS-PROT, Uniprot, Human Protein Reference Database) and selected 1728 full-length cDNAs encoding plasma membrane receptors from an arrayed cDNA library cloned into the vector pCMVSPORT6 (Porcel et al., 2004). In a first round of screening, 293T cells were transfected for 4 hr with 216 pools of 8 cDNAs (1 μg each) in a 24-well plate format following Lipofectamine LTX protocol. An equal amount of a DC-SIGN cDNA dilution (diluted 1/8 in empty plasmid) was used as a positive control. Empty vector was used as a negative control. Transfected cells were challenged with the DV2-JAM primary strain (moi = 2) and infection was quantified 48 hr later by flow cytometry using the 2H2 anti-prM mAb. Pools of cDNA that rendered 293T cells positive for prM protein intracellular staining entered the second round of screening, in which single cDNAs from each positive pool were tested as described above.

Viruses and Cells

The DV-1-TVP5175 strain, DV2-JAM strain (Jamaica), DV2-New Guinea C strain, DV2-16881 strain, DV3-PAH881/88 strain (Thailand), DV4-1086 strain and WNV (Israeli IS-98-STI strain) were propagated in AP61 cell monolayers after having undergone limited cell passages. Virus titers were assessed by flow cytometry analysis (FACS) on C6/36 cells and were expressed as FACS infectious units (FIU). YFV was grown and titrated on Vero cells. HSV-1(F) was propagated and titrated on Vero cells as described elsewhere (Taddeo et al., 2004). HEK293T, CHO745 cells, Cos-7, A549, VERO, and Huh7 5.1 cells (a gift of C.Rice, New York, USA) were maintained in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin. Human primary astrocytes and epithelial cells were purchased from LONZA and cultured according to the manufactured conditions.

Virus Pull-Down

DV particles (10^7 FIU) were incubated overnight at 4°C with 2 μg of Fc-chimera proteins in TBS, 10 mM CaCl2. BSA-saturated Protein G Sepharose beads (GE Healthcare) were added and incubated for 4 hr at 4°C. Beads were washed with TBS, 10 mM $CaCl_2$, 0.05% Tween, and bound material was resolved in 1X Laemmli buffer in nonreducing conditions. Nitrocellulose-bound E envelope glycoprotein was detected with the 4G2 mAb and HRP-conjugated rabbit anti-mouse IgG antibody (Sigma-Aldrich).

Cell-Binding Assay

Cells (4×10^5) were incubated with DV for 90 min at 4°C in binding buffer (DMEM, NaN3 0.05%) containing either 2% BSA or 5% FBS. Cells were

treated with 100 U heparin for 30 min at room temperature, before incubation with the virus. The cells were washed twice with cold binding buffer and fixed in PBS-PFA 2% at 4°C. Cell surface-absorbed DV particles were stained with the 4G2 Ab, then analyzed by flow cytometry. For bridging assays, cells were simultaneously incubated with virus and Gas6 (10 μg/ml).

ELISA Binding

For detecting direct interactions between TIM-Fc and DV, Fc-fused proteins were first coated (400 ng/well) in TBS supplemented with 10 mM $CaCl_2$ on 96-well plates overnight at 4°C. Wells were washed with TBS 10 mM $CaCl_2$ and saturated for 2 hr at 37°C with TBS 10 mM $CaCl_2$, 2% BSA. DV particles (5.10^6 FIU/well) were added and incubated for 2 hr at 4°C. Bound particles were detected with the biotinylated 4G2 antibody (1 μg/ml) and Horseradish peroxydase (HRP)-conjugated Streptavidine (R&D systems). For Gas6 bridging experiments, DV particles were incubated with Gas6 proteins (2 μg/ml) and Fc-chimera proteins (2 μg/ml) for 1 hr at 37°C in TBS 10 mM $CaCl_2$, 0.05% Tween. Bound Fc-chimeras were detected with HRP-conjugated rabbit anti-human IgG antibody. For Gas6 binding experiments, DV particles (10^7 FIU) or PtdSer (3-*sn*-Phosphatidyl-L-serine from bovine brain) were coated overnight at 4°C. Wells were incubated with Gas6 proteins (2 μg/ml) and extensively washed. Bound Gas6 proteins were labeled with a goat anti-Gas6 polyclonal antibody and detected with a HRP-conjugated donkey anti-goat IgG antibody. PtdSer was detected on coated DV particles (10^7 FIU) using anti-PtdSer 1H6 mAb (10 μg/ml) and a HRP-conjugated rabbit anti-mouse IgG antibody in PBS BSA 2%.

Immunofluorescence Assay

Cells were cultured on Lab-Tek II-CC^2 Chamber Slide (Nunc, Roskilde, Denmark) and infected with DV2-JAM. Cells were fixed with PBS-PFA 4%, permeabilized with 0.05% saponin in PBS, and incubated with the anti-prM Ab 2H2. Slides were mounted with ProLong Gold antifade reagent containing 4,6-diamidino-2-phenylindole (DAPI) for nuclei staining. For internalization assay, prechilled CHO745 cells were incubated with viral particles at 4°C for 1 hr. Cells were washed with cold PBS to remove unbound particles and shifted at 37°C for 30 min. Cells were fixed with PBS-PFA 4% before immunostaining with 4G2 anti-E antibody under permeabilized and unpermeabilized conditions.

RNA Interference

Cells were transiently transfected using the Lipofectamine RNAiMax protocol (Life Technologies) with 10 nM final siRNAs as described previously. After 48 hr, cells were infected at the indicated moi, and infected cell percentages were quantified 24 hr postinfection by flow cytometry. Pools of siRNAs (ON-TARGETplus SMARTpool) used in this study were from Dharmacon: TIM-1 (L-019856-00), AXL (L-003104-00). Nontargeting negative control (NT) was used as control.

Flow Cytometry Analysis

Flow cytometry analysis was performed as previously described (Lozach et al., 2005). DV infection was detected using the anti-prM 2H2 mAb or an NS1 mAb. HSV-1 infection was detected using the anti-ICP4 mAb (clone 10F1, Santa Cruz Biotechnology).

Inhibition of Infection Assay

Cells were incubated for 30 min prior to infection with media containing the indicated quantities of goat anti-TIM and/or anti-TAM polyclonal antibodies. Identical concentrations of purified normal goat IgG were used as control. Cells were then infected for 3 hr incubation in the presence of inhibitors, washed, and incubated with culture medium. Infection was quantified by flow cytometry as indicated above.

RNA Purification, Reverse Transcription, and Real-Time qPCR

Total RNA was extracted from infected cells, using an RNeasy Mini Kit (QIAGEN, Courtaboeuf, France) with on-column DNase digestion, and stored at −20°C. cDNA was synthesized from 500 ng total isolated RNA by random priming-reverse transcription with the SuperScript VILO cDNA Synthesis Kit (Invitrogen by Life Technologies). Real-time quantitative PCR (qPCR) was performed using the Fast SYBR Green Master Mix Kit (Invitrogen by Life Technologies) on an Applied Biosystems 7500 Fast real-time PCR system (Life Technologies). The primers for viral RNA quantification were designed with PrimerExpress software V2.0 (Life Technologies) within the conserved region of the capsid gene of DV2-JAM (Forward primer 5′-TTCTCACTTGGA ATGCTGCAA-3′; Reverse primer 5′-GCCACAAGGGCCATGAAC-3′) and QuantiTect primers for GAPDH were purchased from QIAGEN. Relative expression quantification was performed based on the comparative C_T Method, using GAPDH as endogenous reference control.

Statistical Analyses

Graphical representation and statistical analyses were performed using Prism5 software (GraphPad Software). Unless otherwise stated, results are shown as means ± standard deviation (SD) from three independent experiments. Differences were tested for statistical significance using the paired two-tailed t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2012.08.009.

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