Supporting Information

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SI Materials and Methods

Cell Lines and Plasmids. 293T (human embryonic kidney), Vero (monkey kidney), MDCK (Madin-Darby Canine Kidney) and C6/36 *Aedes albopictus* (mosquito) cells were grown in DMEM, A549 (human lung) cells in F12 medium, and Jurkat (human T lymphocyte) cells in RPMI 1640 medium. All cells were cultured in medium supplemented with 10% (vol/vol) FBS, at 37 °C with 5% CO_2, except for C6/36 cells, which were kept at 28 °C with 5% CO_2.

Naïve peritoneal macrophages were obtained from WT BALB/cBYJ mice via peritoneal cavity lavage. Briefly, peritoneal cavity cells were plated in 12-well plates at 10^6 cells per well in RPMI 1640 supplemented with 10% FBS and antibiotics, and incubated for 1 h at 37 °C to let macrophages adhere. After removing nonadherent cells by washing three times in PBS solution/2% FBS, macrophages were incubated overnight at 37 °C in fresh medium and infected the following day for 3 h at 37 °C with EBOV VLPs or no-GP VLPs as a control. Infected cells were detached by scraping in trypsin 0.25%/EDTA, washed, and loaded with the Bla substrate CCF4-AM as described later. Experiments involving mice have been performed according to the guidelines established by Scripps Florida Institutional Animal Care and Use Committee (protocol 14–007).

The retroviral expression plasmid pQCXIX (BD Biosciences) encoding hTIM1 and used for overexpression in 293T cells has previously been described (1).

To generate hTIM-mIg fusion constructs, the coding sequence of the extracellular domain of each hTIM was inserted into pcDNA3.1+ (Life Technologies) containing the cDNA sequence of the Fc region of mIgG2a. For the production of hMFGE8-mIg, the plasmid encoding hMFG-E8 was purchased (Sino Biological) and its coding sequence was similarly inserted into pcDNA3.1+ containing the cDNA of the mIgG2a Fc region.

Plasmids used for the production of VLPs and PVs are as follows: for LASV PVs, pQCXIX encoding eGFP protein, a plasmid encoding the gag and pol proteins of MLV, a plasmid driving the expression of LASV (Josiah) entry protein; for WNV VLPs, a plasmid encoding WNV (lineage 1, NY99) structural proteins, a plasmid encoding WNV nonstructural proteins and GFP; and for EBOV and VSV VLPs, a plasmid encoding EBOV VP40 matrix protein fused to Bla and a plasmid encoding EBOV GP (Zaire) lacking the mucin domain or VSV entry protein were used. All these plasmids have previously been described (1).

Proteins, Antibodies, and Molecular Probes. To produce mIg-fusion proteins, the plasmids encoding hTIM1-mIg, hTIM3-mIg, and hTIM4-mIg were transfected into 293T cells by using the calcium phosphate method and cultured in serum-free medium FreeStyle 293 (Life Technologies) supplemented with a mixture of protease inhibitors (Sigma) and 5 mM sodium butyrate. The culture supernatants were harvested 72 h posttransfection, and the proteins were purified by protein A-Sepharose affinity chromatography, eluted with 50 mM glycine (pH 2.5) buffer containing 150 mM NaCl, and dialyzed against PBS solution by using dialysis cassettes with a 10-kDa cutoff (Thermo Scientific Pierce). Purified proteins were quantified by using the Nanodrop 2000C spectrophotometer (Thermo Scientific). hMFG-E8-mIg was produced the same way in 293T cells, and quantified by Coomassie staining following SDS/PAGE, using purified mIg as standards.

Antibodies, other proteins, and molecular probes are as follows: biotin-Duramycin was from Molecular Targeting Technologies, biotin-annexin A5 was from BioLegend and Life Technologies, and mouse monoclonal anti-hTIM1 antibody (clone 3D1) was previously described (2). Goat polyclonal anti-TIM1 antibody was from R&D Systems (AF1750). Mouse anti-DENV2 premembrane protein (prM; clone D3-2H2-9-21) and mouse anti-adenovirus (clone 2/6) antibodies were from Millipore. Mouse anti-DENV2 prM antibody (clone DM-1) and HRP-conjugated streptavidin were from Pierce (Thermo Scientific). Rabbit polyclonal anti-AdV5 and anti-PS (clone 4B6) antibodies were from Abcam. Mouse anti-IAV (H1N1, H2N2; clone C179) antibody was from Takara. Mouse anti-WNV E protein (clone E121) antibody was from American Type Culture Collection (ATCC). Mouse anti-EBOV GP (clone 2G4) antibody was custom-made. PE-conjugated goat anti-mouse, Alexa 647-conjugated goat anti-mouse, HRP-conjugated goat anti-mouse IgG, and HRP-conjugated goat anti-mouse IgG subclass II antibodies were from Jackson ImmunoResearch Laboratories. Alexa 647-conjugated donkey anti-goat antibody, SYTOX Red dead cell stain (633 or 635 nm excitation), pHrodo green AM intracellular pH indicator (excitation/emission of 509/533 nm), and CCF4-AM LiveBLazer FRET-B/G loading kit were from Life Technologies. FITC-conjugated avidin D was from Vector Laboratories.

ELISAs. For assessment of hTIM binding to phospholipids, phospholipids in chloroform were dried under nitrogen gas and then resuspended and diluted in methanol. The indicated amounts of phospholipids were dried overnight in polystyrene ELISA plates, washed with PBS solution containing 0.9 mM Ca^{2+} [PBS(+); Life Technologies] supplemented with 0.05% Tween 20 [PBST(+)], blocked for 1 h at room temperature with PBS(+) containing 1% BSA, further washed with PBST(+), incubated for 1 h at room temperature with the indicated amounts of proteins diluted in PBS(+), and further washed with PBST(+) before being incubated with an HRP-conjugated goat anti-mouse antibody for the detection of mIg fusion proteins. HRP-conjugated streptavidin was used to detect biotin-Duramycin or biotin-annexin A5. Wells treated the same way but not coated with any phospholipids were used as background controls. Binding was visualized using UltraTMB (Thermo Scientific Pierce) and TMB BlueStop solution (Kirkegaard and Perry Labs), and read at 650 nm with a SpectraMax Paradigm Microplate Reader (Molecular Devices). For Duramycin binding to phospholipids, and when indicated, assays were done in PBS solution (no Ca^{2+}) and PBST instead of PBS(+) and PBST(+).

For Duramycin binding to captured viruses, polystyrene ELISA plates were coated overnight at 4 °C with 100 μL per well of 1 μg/mL of virus-specific antibodies diluted in PBS solution: anti-DENV2 antibody clone D3-2H2-9-21, anti-EBOV antibody clone 2G4, anti-WNV antibody clone E121, and polyclonal rabbit anti-AdV5 were used. Plates were washed with PBST, blocked for 1 h at room temperature with PBS solution containing 1% BSA, and further washed with PBST. Plates were then incubated for 1 h at room temperature with the indicated virus or VLPs that had been dialyzed against PBS solution, washed, and incubated with the indicated amounts of biotin-Duramycin diluted in PBS solution for 1 h at room temperature. Wells treated the same way but incubated with virus-free cell supernatant dialyzed against PBS solution were used as background controls. Duramycin binding was detected with HRP-conjugated streptavidin. To detect the captured AdV5, the plate was incubated with anti-AdV5 antibody clone 2/6 for 1 h at room temperature followed by HRP-conjugated anti-mIgG antibody.

To assess Duramycin binding to PE on PLC-digested DENV2 virions, ELISA plates were coated overnight at 4 °C with 100 μL

per well of 1 μg/mL of anti-DENV2 clone DM-1 (mIgG1). Plates were blocked for 1 h at room temperature with PBS solution containing 1% BSA, incubated for 1 h at room temperature with DENV2, followed by 1 h at 37 °C with the indicated amounts of PLC diluted in Tris-buffered saline solution supplemented with 0.25 μM $ZnCl_2$. Wells treated the same way but incubated with virus-free cell supernatant were used as background controls. Duramycin binding assay was then performed as described earlier using 100 nM of biotin–Duramycin. To verify that PLC digestion did not cause captured DENV2 to detach, a sandwich ELISA was performed by using a second anti-DENV2 antibody (clone D3-2H2-9-21, mIgG2a) that was detected by using an HRP-conjugated anti-mouse IgG2a antibody.

Cytotoxicity Assay. hTIM1-293T, Vero, and A549 cells were incubated for 1 h at 37 °C with the indicated concentrations of Duramycin or Triton X-100 (control). Cells were then equilibrated for 20 min at room temperature, and membrane integrity was assessed by using the CytoTox-ONE Homogeneous Membrane Integrity assay according to the manufacturer's instructions (Promega), based on the measurement of cytosolic LDH activity that had been released into the culture medium. Briefly, the substrate resazurin was added to the wells, and, upon LDH activity, converted into the fluorescent product resorufin (excitation 560 nm, emission 590 nm). Fluorescence was then measured in each well by using the SpectraMax Paradigm Microplate Reader.

Production of Replication-Competent Viruses and Infection Assays. DENV2 (ATCC VR-1584) was propagated in C6/36 cells; IAV (H1N1) (A/Virginia/ATCC1/2009; ATCC VR-1736) in MDCK cells; and human AdV5 (ATCC VR-1516) in 293T cells. All viruses were produced in serum-free OptiPRO medium (Life Technologies), 0.45-μm-filtered, and kept at 4 °C for 1–2 wk or −80 °C for long-term storage.

For infection assays, the indicated cells were infected for 1 h at 37 °C with viruses diluted in OptiPRO medium. Culture supernatants were removed and cells were further grown in fresh medium. The next day, infected cells were trypsinized, fixed with 1% formaldehyde in PBS solution, permeabilized in 0.1% saponin in PBS solution containing 2% (vol/vol) goat serum, and stained with mouse monoclonal antibodies specifically recognizing each virus. Antibodies used for staining include anti-DENV2 clone D3-2H2-9-21, anti-IAV H1N1/H2N2 clone C179, and anti-adenovirus clone 2/6. After incubation with Alexa 647-conjugated goat anti-mouse antibody, cells were washed and fixed in 2% (wt/vol) formaldehyde in PBS solution and analyzed by flow cytometry (C6 Accuri cytometer; BD Biosciences).

Production of VP40-Bla VLPs and Entry Assays. VP40-Bla VLPs were produced in DMEM containing 10% FBS by transfecting 293T cells using the calcium phosphate method at a ratio of 3:1 with a plasmid encoding EBOV VP40 matrix proteins fused to Bla, and a plasmid encoding EBOV or VSV entry protein. The culture supernatants were harvested 38–44 h after the transfection and centrifuged twice at low speed to remove cell debris. VLP-containing supernatants were kept at 4 °C and used within 2 wk. VLPs bearing no GP (i.e., no-GP VLPs) were produced and used as a control.

For entry/fusion assays, the indicated cells were infected with VLPs for 3 h at 37 °C, extensively trypsinized (0.25% trypsin) for 20 min at 37 °C to remove bound but noninternalized VLPs, washed in PBS solution, incubated with the Bla substrate CCF4-AM for 1 h at 37 °C, further washed, and analyzed by flow cytometry (Gallios cytometer; Beckman Coulter) according to the manufacturer's instructions (Life Technologies). The detection of Bla VLP infection relies on the enzymatic activity of Bla that is present in the VLPs as a fusion protein with VP40. When CCF4-AM has crossed the plasma membrane, it is cleaved by a cytoplasmic esterase and becomes negatively charged and subsequently unable to cross another membrane such as an endosomal membrane. Therefore, only VLPs that have made it through the membrane fusion step, but not those that are internalized but not fused, can cleave CCF4-AM in the cytoplasm. The cleavage product absorbs at 409 nm and emits at 447 nm.

Production of MLV-Based PVs and WNV VLPs, and Entry Assays. LASV PVs were produced in serum-free OptiPRO medium by transfecting 293T cells using the calcium phosphate method with a plasmid encoding MLV gag and pol, together with the retroviral vector pQCXIX encoding eGFP and a plasmid encoding LASV entry protein. A plasmid ratio of 1:1:1 was used. WNV VLPs were produced in the same way by using a WNV replicon encoding the nonstructural proteins NS1-5 and GFP, along with a plasmid encoding WNV structural proteins capsid, prM, and E (plasmid ratio 2:1). After 34–36 h, the culture supernatants were harvested and cleared by 0.45-μm filtration.

For entry assays, the indicated cells were infected with LASV PVs or WNV VLPs at 37 °C for 1 h, and further cultured with fresh medium. The next day, cells were detached by trypsinization, washed with PBS solution, fixed in 2% formaldehyde in PBS solution, and analyzed by flow cytometry for eGFP (LASV PVs) or GFP (WNV VLPs) expression.

Virus Attachment to hTIM1-293T Cells. 293T, hTIM1-293T, or hL-SIGN-293T cells were incubated with 1,000 U/mL of heparin (Sigma) for 30 min on ice and washed once in PBS solution containing 2% of goat serum. DENV2, preincubated or not with the indicated concentration of Duramycin, was added, and incubation on ice was continued for 1 h. After unbound virus was washed, cells were stained with anti-DENV2 antibody clone D3-2H2-9-21.

Inhibition of Infection by Liposomes. PE or PS liposomes were made by mixing at a 1:1 molar ratio of synthetic phospholipid DOPC with DOPE or DOPS, respectively, in chloroform (Avanti Polar Lipids). PC liposomes were made of DOPC by itself. Lipids in chloroform were dried under nitrogen gas, resuspended in PBS solution, vigorously shaken for 1 h at room temperature, sonicated (Avanti Polar Lipids sonicator) for clarity, and used fresh or stored at 4 °C for use within 1 wk.

For inhibition of virus infection assays, liposomes were diluted in OptiPRO medium for replication-competent viruses or DMEM containing 10% FBS for VP40-Bla VLPs, and preincubated with the cells for 20 min at room temperature. Indicated viruses were then added to these cells and incubated at 37 °C for 1 h for replication-competent viruses or for 3 h for VP40-Bla VLPs and analyzed as described earlier.

Duramycin Inhibition of Virus Infection. For Duramycin inhibition assays, replication-competent viruses, VLPs, or PVs were preincubated with the indicated concentration of biotin-Duramycin for 1 h at room temperature and then added to the cells at 37 °C. Virus-containing culture supernatants were removed 1 h later for replication-competent viruses, WNV VLPs, and LASV PVs, and 3 h later for VP40 Bla VLPs. Cells were then analyzed for viral protein expression in infected cells [replication-competent DENV2, IAV (H1N1), and AdV5], GFP expression (WNV VLPs and LASV PVs) or Bla activity (EBOV and VSV VLPs) as described earlier.

Time of addition experiments were performed by adding the indicated concentration of biotin-Duramycin diluted in OptiPRO to cells that had been infected for 1 h with WNV VLPs. After virus had been removed, cells were incubated in Duramycin for 1 h at 37 °C, supernatant was removed, and they were fed with fresh media. Infection levels were assessed next day by measuring GFP expression.

Annexin A5 or Duramycin Binding to Apoptotic Cells. Jurkat cells were incubated with 2 μM of actinomycin D (Cayman Chemical) or DMSO as a control for 16 h at 37 °C. Cells were washed and incubated for 25 min on ice with the indicated concentrations of biotin-Duramycin or BioLegend biotin-annexin A5 diluted in PBS(+), washed, incubated with FITC-conjugated avidin, and analyzed by flow cytometry (C6 Accuri cytometer; BD Biosciences). SYTOX Red Dead Cell Stain (633 or 635 nm excitation; Life Technologies) was added to the cells 10 min before the analysis as a probe for cell permeability. Cells positive for FITC-avidin staining but negative for SYTOX staining are considered apoptotic. Double-positive cells are considered necrotic.

Phagocytosis Assays. Apoptosis was induced in Jurkat cells with 0.5 μM of actinomycin D for 9 h at 37 °C. Cells were then loaded for 30 min at room temperature with 1 μM of pH-sensitive pHrodo green dye diluted in RPMI 1640 medium containing 10% FBS, washed once, and resuspended in fresh medium. 293T, hL-SIGN-, or hTIM1-293T cells were incubated for 4 h at 37 °C with a 1.5× excess of apoptotic Jurkat cells. Bound but not internalized Jurkat cells were removed by quickly and extensively washing with 200 mM glycine buffer (pH 3.0) containing 150 mM NaCl. Phagocytes (293T, hL-SIGN-, and hTIM1-293T cells) were then trypsinized with 0.25% trypsin for 20 min at 37 °C, washed, and resuspended in PBS solution containing 2% goat serum. The fluorescence of the phagocytes was immediately analyzed by flow cytometry. As pHrodo dye emits brighter fluorescence in acidic environments such as the phagosomes, only the engulfed Jurkat cells, not those merely bound, are scored. To assess the effect of Duramycin on hTIM1-mediated phagocytosis, similar assays were performed with pHrodo green-loaded apoptotic Jurkat cells that were incubated for 30 min on ice with the indicated concentrations of biotin-Duramycin.

Statistics. *P* values were calculated by using an unpaired two-tailed *t* test.

1. Jemielity S, et al. (2013) TIM-family proteins promote infection of multiple enveloped viruses through virion-associated phosphatidylserine. *PLoS Pathog* 9(3):e1003232.

2. Kobayashi N, et al. (2007) TIM-1 and TIM-4 glycoproteins bind phosphatidylserine and mediate uptake of apoptotic cells. *Immunity* 27(6):927–940.

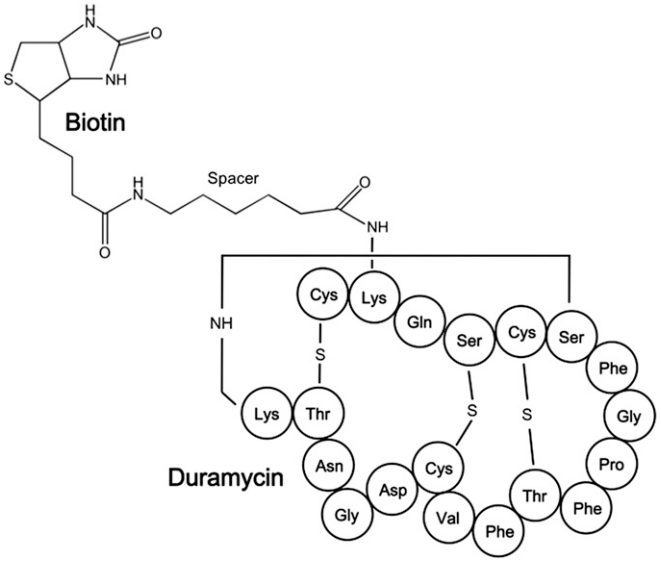

Fig. S1. Structure of biotin-Duramycin. Primary and secondary structures of the biotinylated form of Duramycin are shown. Biotin is attached to the lysine at the second position of Duramycin via a 6-carbon aminohexanoyl spacer. A lanthionine bridge between cysteine and serine, two methyl lanthionine bridges between cysteine and threonine, and a lysoalanine bond between lysine and serine are indicated.

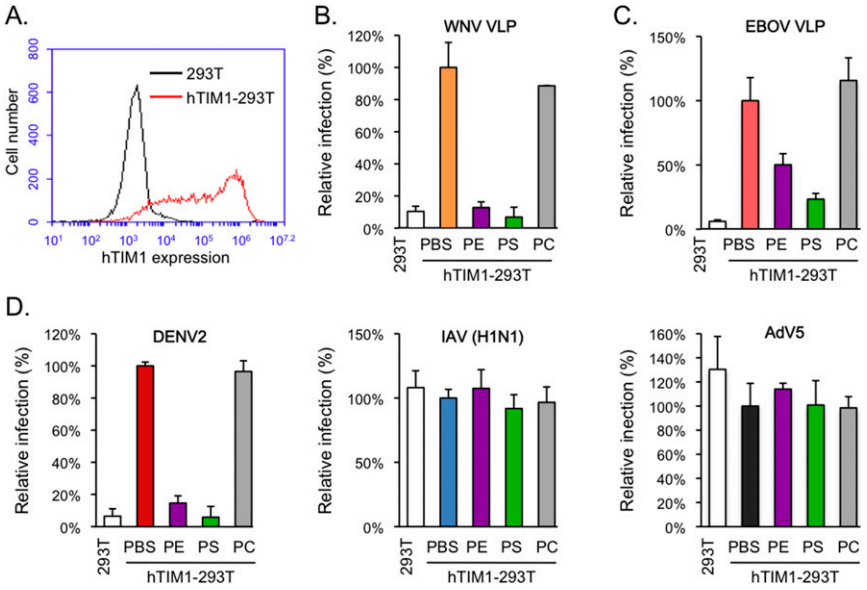

Fig. S2. PE liposomes block hTIM1-mediated viral entry. (*A*) hTIM1 expression level of transduced 293T cells (hTIM1-293T), which were used in this work, were assessed by staining the cells with an anti-hTIM1 antibody (clone 3D1). Parental 293T cells were used as a negative control. (*B–D*) hTIM1-293T cells were preincubated with 10 μM of the indicated liposomes consisting of 100% DOPC (PC), 50% DOPC and 50% DOPE (PE), or 50% DOPC and 50% DOPS (PS). Cells were then infected by adding an equivalent volume of virus or VLPs and incubated at 37 °C. Thus, the final concentration of liposomes during infection was 5 μM. Cells were infected with WNV VLPs (*B*) or EBOV VP40-Bla VLPs (*C*) or with replication-competent DENV2, IAV (H1N1), or AdV5 (*D*). Data represent infection levels normalized to that of hTIM1-293T cells preincubated with PBS solution, and the average ± SD of two or three duplicated independent experiments is shown.

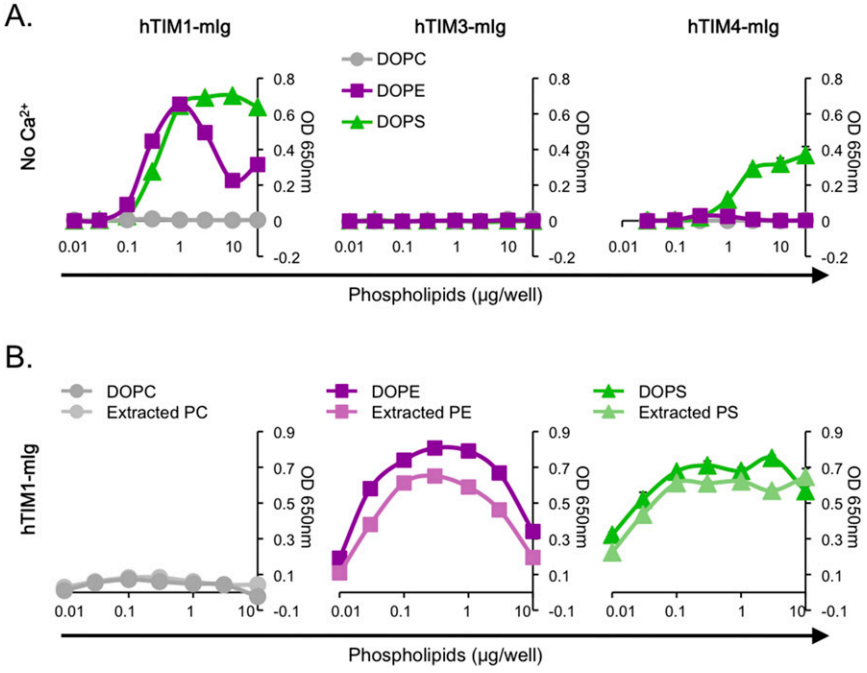

Fig. S3. PE/PS binding by hTIM proteins can be modulated by calcium but is unaffected by the source of phospholipids. (*A*) hTIM binding to PE/PS in the absence of calcium. The indicated amount of synthetic phospholipids was dried out on ELISA plates, and the binding of 1 nM hTIM1-, hTIM3-, or hTIM4-mIg was assessed in PBS solution containing no Ca^{2+}. (*B*) hTIM1 binding to PE/PS is not affected by the source of phospholipids. One microgram of synthetic or extracted PC, PE, or PS was dried out on ELISA plates, and binding of 1 nM of hTIM1-mIg was quantified in PBS(+). Data in *A* and *B*, expressed as arbitrary units of absorbance at OD_{650}, are shown as mean ± SD of duplicates, and are representative of at least three independent duplicated experiments.

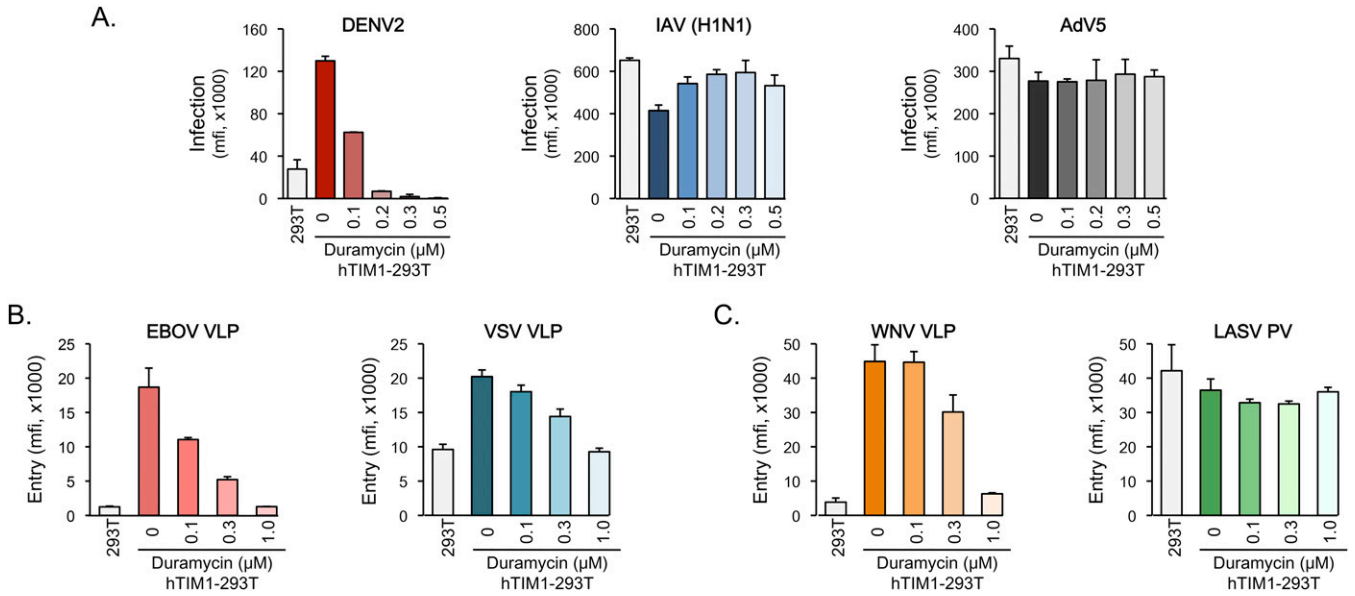

Fig. S4. PE contributes to hTIM1-mediated viral entry of EBOV, DENV2 and WNV. (*A*–*C*) DENV2, IAV (H1N1), and AdV5 (*A*); EBOV and VSV VLPs (*B*); or WNV VLPs and LASV PV (*C*) were preincubated with biotin-Duramycin and used to infect hTIM1-293T cells. Parental 293T cells were also infected to show TIM1 use of these viruses. Data are expressed as the average mean fluorescence intensity (mfi, arbitrary units × 1,000) ± SD of duplicates. Each graph is representative of one of the experiments shown in Fig. 3.

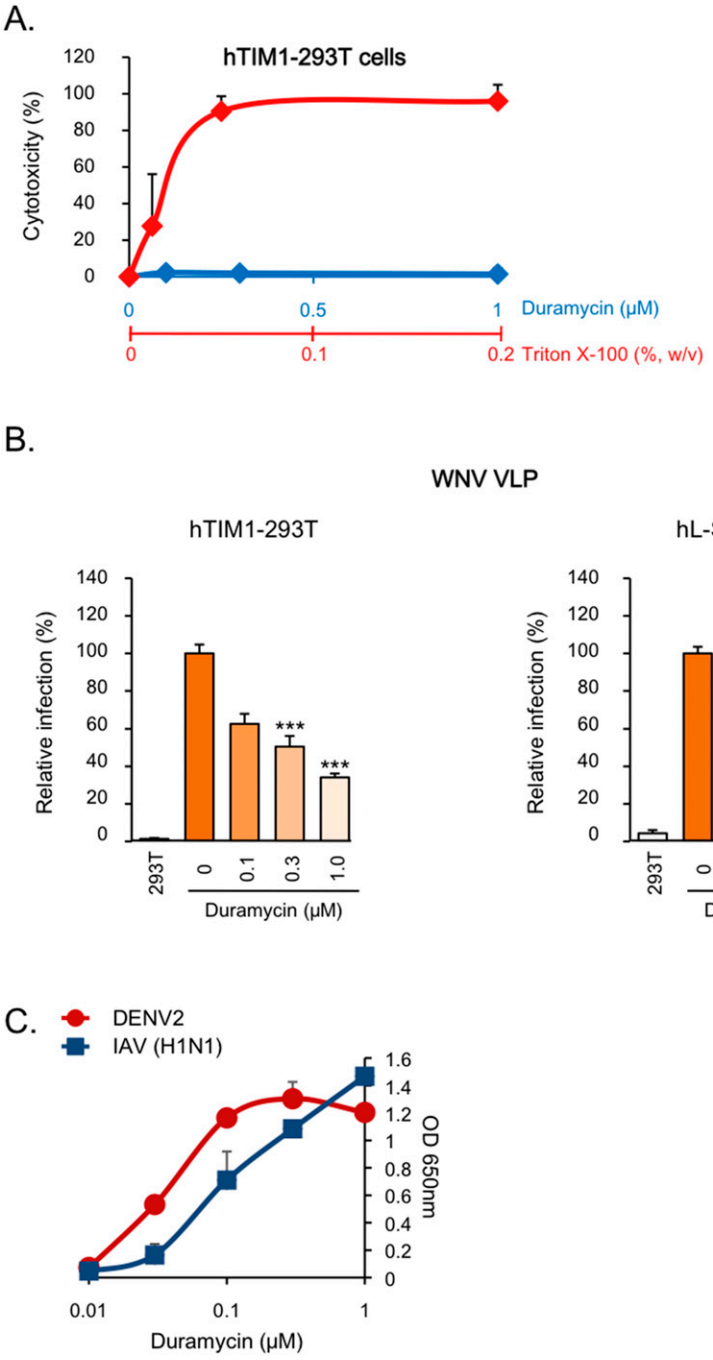

Fig. S5. Duramycin is not toxic to the cells or to the viruses. (*A*) Duramycin is not cytotoxic to cells. hTIM1-293T cells were preincubated with biotin-Duramycin (blue line) or Triton X-100 (positive control, red line) for 1 h at 37 °C. Cytotoxicity was then assessed by using the CytoTox-ONE Homogeneous Membrane Integrity assay, which measures the release of the cytosolic LDH to the culture medium. Cytotoxicity levels are normalized to that of maximum Triton X-100-mediated LDH release. The average ± SD of five independent triplicated experiments is shown. (*B*) Duramycin is not virolytic. WNV VLPs were preincubated with biotin-Duramycin and used to infect hTIM1-293T cells or hL-SIGN-293T cells. 293T cells were used to indicate the extent of hTIM1 and hL-SIGN use by WNV VLPs. Relative infection levels, normalized to that of hTIM1-293T or hL-SIGN-293T cells infected with untreated VLPs (i.e., no Duramycin), are shown. The average ± SD of four independent duplicated experiments are shown (***$P < 0.0001$). (*C*) DENV2 and IAV (H1N1) were captured by using virus-specific antibodies coated on ELISA plates, and detected using biotin-Duramycin. Duramycin binding to IAV (H1N1) shows that Duramycin also binds to viruses that do not use PS receptors and demonstrates that the inhibition of infection is not the result of any Duramycin toxicity to viruses.

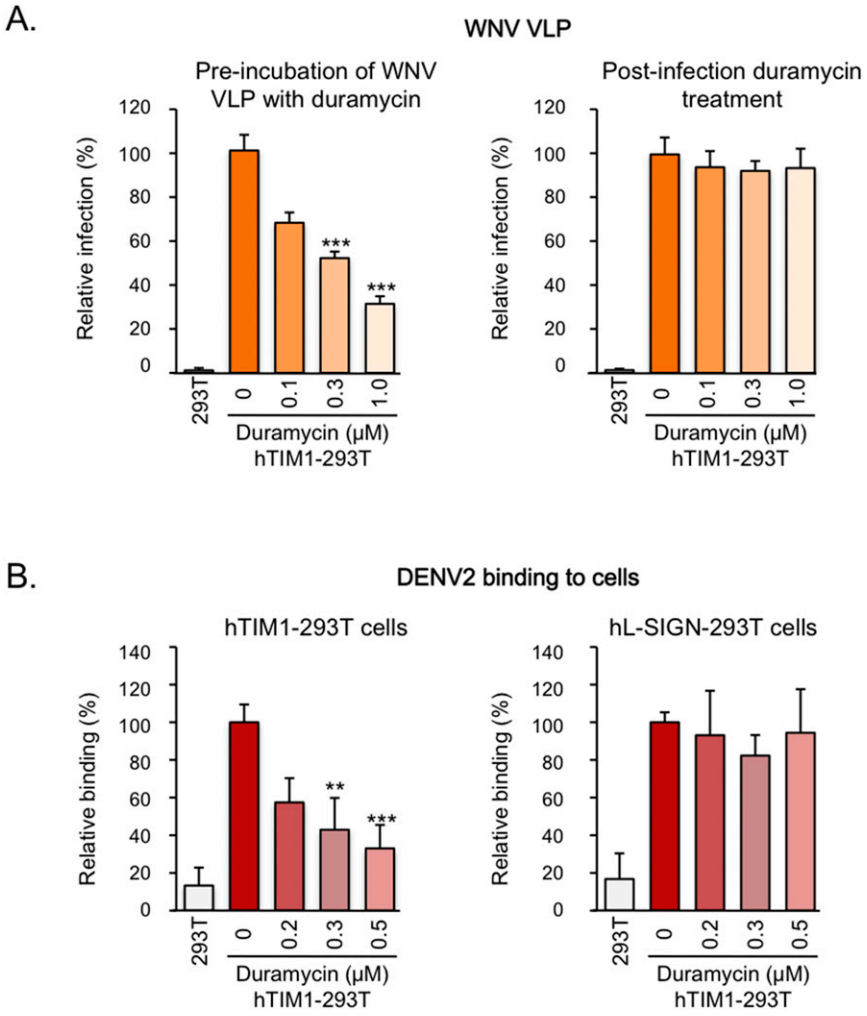

Fig. S6. Duramycin inhibits virus attachment to TIM1. (*A*) Duramycin works at early steps of virus infection. (*Left*) Preincubation of WNV VLPs with Duramycin: WNV VLPs were preincubated or not with biotin-Duramycin and used to infect hTIM1-293T cells for 1 h. (*Right*) Postinfection Duramycin treatment: hTIM1-293T cells were infected with WNV VLPs, culture supernatants were removed, media containing the indicated concentrations of biotin-Duramycin were added, and incubation was continued for 1 h. 293T cells were used to show the extent of TIM1 use by WNV VLPs. Infection levels were normalized to that of hTIM1-293T cells infected with untreated VLPs (i.e., no Duramycin). (*B*) Duramycin inhibits virus attachment to TIM1. DENV2 preincubated or not with biotin-Duramycin was added to heparin-treated hTIM1-293T or hLSIGN-293T cells for 1 h on ice. After unbound viruses were washed and cells fixed, bound viruses were detected by using the anti-DENV2 antibody clone D3-2H2-9-21. (*A* and *B*) The average ± SD of four independent duplicated experiments is shown (**$P <$ 0.001 and ***$P < 0.0001$).

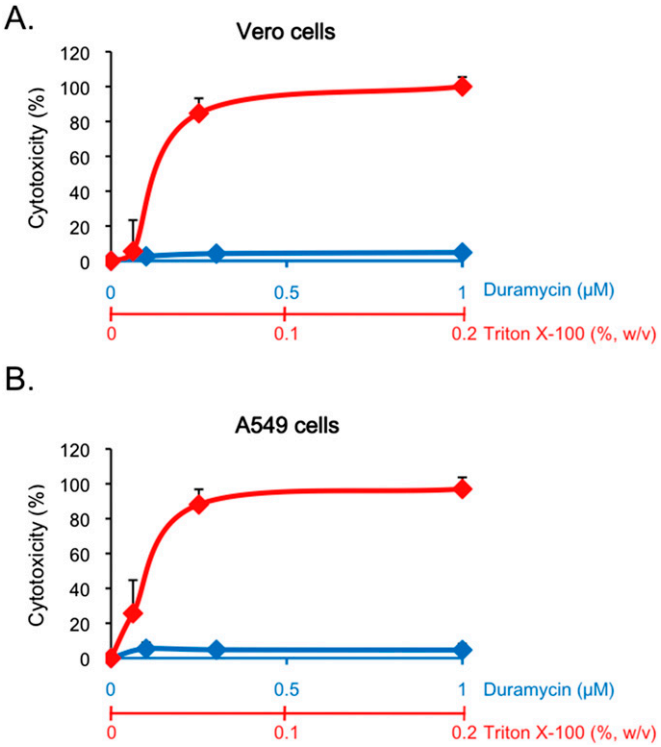

Fig. S7. Duramycin shows no cytotoxicity in Vero or A549 cells. Same experiments as those in Fig. S5*A* except that Vero (*A*) or A549 (*B*) cells were used. Cytotoxicity levels are normalized to that of maximum Triton X-100-mediated LDH release. The average ± SD of five independent triplicated experiments is shown.

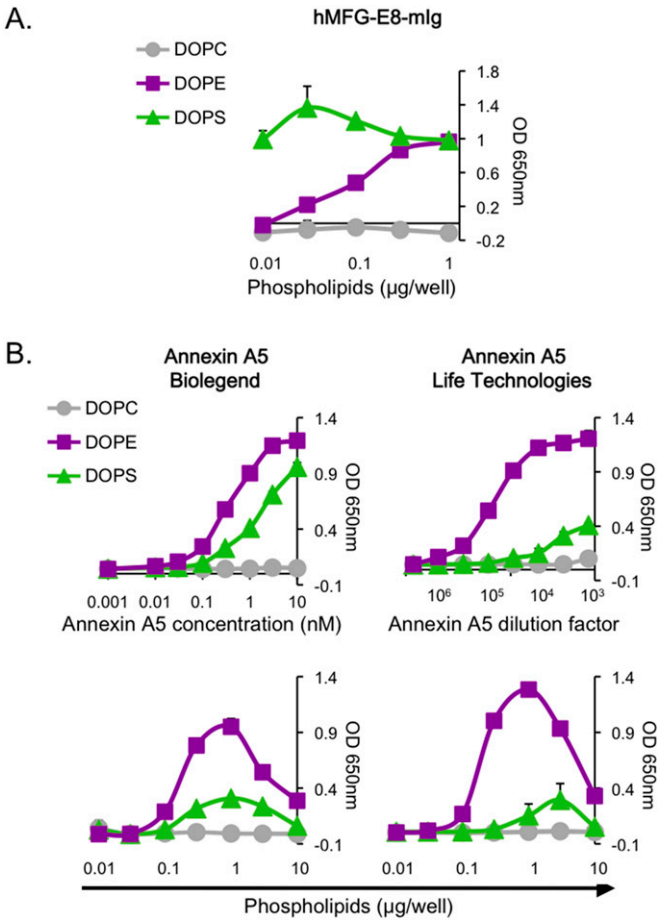

Fig. S8. PS-binding proteins MFG-E8 and annexin A5 also efficiently bind PE. (*A*) Human MFG-E8-mIg (hMFG-E8-mIg) binds PS and PE. The indicated amount of phospholipids was dried out on ELISA plates and incubated with ~1 nM of hMFG-E8-mIg in FreeStyle 293 medium. (*B*) Annexin A5 binds both PS and PE. The ability of annexin A5 (from two different suppliers) to bind phospholipids was assessed as in *A*. As the concentration of annexin A5 from Life Technologies was unknown, it was diluted similarly to annexin A5 from BioLegend. ELISAs in *A* and *B* were performed in PBS(+) (0.9 mM of Ca^{2+}). Data, expressed as arbitrary units of absorbance at OD_{650}, are shown as the mean ± SD of duplicates and are representative of three independent duplicated experiments.

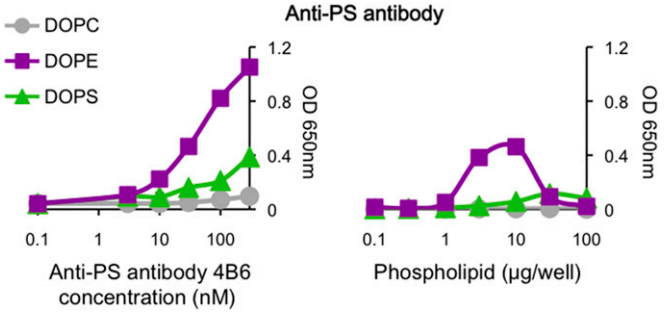

Fig. S9. Anti-PS antibody does not bind PS. Either 1 µg (*Left*) or the indicated amount (*Right*) of phospholipids was dried on ELISA plates, and the binding of increasing concentrations (*Left*) or 30 nM (*Right*) of anti-PS antibody (clone 4B6) was assessed in PBS solution containing no Ca^{2+}. Data, expressed as arbitrary units of absorbance at OD_{650}, are shown as the mean ± SD of duplicates and are representative of two independent duplicated experiments.