



Phosphatidylserine-exposing extracellular vesicles in body fluids are an innate defence against apoptotic mimicry viral pathogens

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Supplementary Table 1: Shedding into body fluids and modes of human to human transmission for viruses using or not using viral apoptotic mimicry. VAM – using viral apoptotic mimicry as a primary entry mechanism.

Virus	VAM	Sexual transmission		Oral transmission		Blood-borne	
		Semen	Frequency	Saliva	Frequency	Blood	Frequency
Zika	Yes ¹	Yes ²⁻⁴	Rare ^{2,4}	Yes ⁴	None reported	Yes ⁵	Dominant via mosquito vectors ⁶
Dengue	Yes ^{1,10}	Yes ¹¹	Anecdotally ¹¹	Yes ^{12,13}	None reported	Yes ¹⁴	Dominant via mosquito vectors ^{6,15}
West Nile	Yes ^{1,10}	None reported	None reported	Yes ¹³	None reported	Low ¹⁶	Dead end hosts, transfusion possible ^{16,17}
Chikungunya	Yes ^{1,10}	Yes ³	None reported ³	Yes ^{13,19}	None reported	Yes ²⁰	Dominant via mosquito vectors ^{15,21}
Ebola	Yes ^{1,10}	Yes ³	Rare ²³	Yes ^{13,23,24}	Possible route ²³	Yes ^{23,24}	Possible route ²³
SARS-CoV-2	No ^{1,25}	Anecdotally ²⁶	None reported	Yes ²⁷	Common ^{28,29}	Low ³⁰	No evidence ³¹
HIV-1	No ¹	Yes ³	Dominant route ³⁴	Yes ³⁵	Unlikely ³⁶	Yes ³⁶	Possible route ^{34,36}
CMV	No ³⁹	Yes ³	Possible route ⁴⁰	Yes ^{13,41}	Common route ⁴¹	Yes ⁴¹	Possible route ^{41,42}
HSV-1	No ^{10,43}	Yes ³	Possible route ⁴⁴	Yes ^{45 13}	Dominant route ⁴⁵	Low ⁴²	No evidence ⁴²
HSV-2	No ⁴³	Yes ³	Dominant route ⁴⁴	Yes ¹³	Possible route ⁴⁴	Low ⁴²	No evidence ⁴²
Hepatitis C	No ⁴⁶	Yes ³	Possible route ⁴⁷⁻⁴⁹	Yes ^{13,50}	None reported ⁵⁰	Yes ⁵¹	Possible route ⁵¹

Virus	VAM	Breast feeding		Urine	
		Milk	Frequency	Urine	Frequency
Zika	Yes ¹	Yes ⁷	Rare ^{4,8,9}	Yes ⁵	None reported
Dengue	Yes ^{1,10}	Yes ⁷	None reported ⁷	Yes ¹¹	None reported
West Nile	Yes ^{1,10}	Yes ⁷	None reported ⁷	Yes ¹⁸	None reported
Chikungunya	Yes ^{1,10}	Anecdotally ²²	None reported	Yes ¹⁹	None reported
Ebola	Yes ^{1,10}	Yes ^{23,24}	Rare ²³	Yes ^{23,24}	Possible route ²³
SARS-CoV-2	No ^{1,25}	Yes ³²	None reported ³²	Yes ^{26,33}	None reported
HIV-1	No ¹	Yes ³⁷	Possible route ^{37,38}	Yes ³⁶	Unlikely ³⁶
CMV	No ³⁹	Yes ⁴¹	Possible route ^{38,41}	Yes ⁴¹	Possible route ⁴¹
HSV-1	No ^{10,43}	n/a	None reported	None reported	None reported
HSV-2	No ⁴³	n/a	None reported	None reported	None reported
Hepatitis C	No ⁴⁶	Yes ⁵²	Rare ⁵²	Yes ⁵³	None reported

Supplementary Table 2: Purified EVs from body fluids and cell culture supernatant, related to Figure 3. The number of preparations refers to unique isolation procedures performed per source; for sources with multiple preparations the range of donors, volume, particle concentration, concentration factor and particle recovery are given. Recovery of particles is calculated by NTA-tracked particles in the source fluid (after centrifugation) and the final EV preparation; due to presence of non-EV-particles especially in the source fluid which may be tracked by NTA, this does not necessarily reflect the recovery of EVs specifically. N/A = not applicable. * semen used for preparation of seminal plasma was obtained as excess material from a fertility clinic as unlabeled aliquots; the number of donors shown assumes that all aliquots are from separate donors, but repeated donations and multiple aliquots per donation are possible and cannot be verified.

Source	Isolations	Donors	Source volume [ml]	Particles/ml source	Particles/ml EV preparation	Conc. by volume	Recovery particles [%]
Semen	3	35 - 100*	33 - 90	$3.7 - 4.0 \times 10^{12}$	$4.3 - 9.9 \times 10^{12}$	8 - 11x	10.3 - 25.2
Saliva	5	9 - 10	100 - 300	$3.3 - 8.5 \times 10^{10}$	$1.7 - 9.9 \times 10^{11}$	100 - 429x	1.6 - 18.2
Urine	1	6	3,125	8.2×10^9	4.4×10^{12}	2,232x	23.9
Breast milk	1	1	49	2.3×10^{12}	2.5×10^{13}	33x	33.2
Blood	3	N/A	20 - 40	2.2×10^{12}	1.3×10^{12}	20x	3.0

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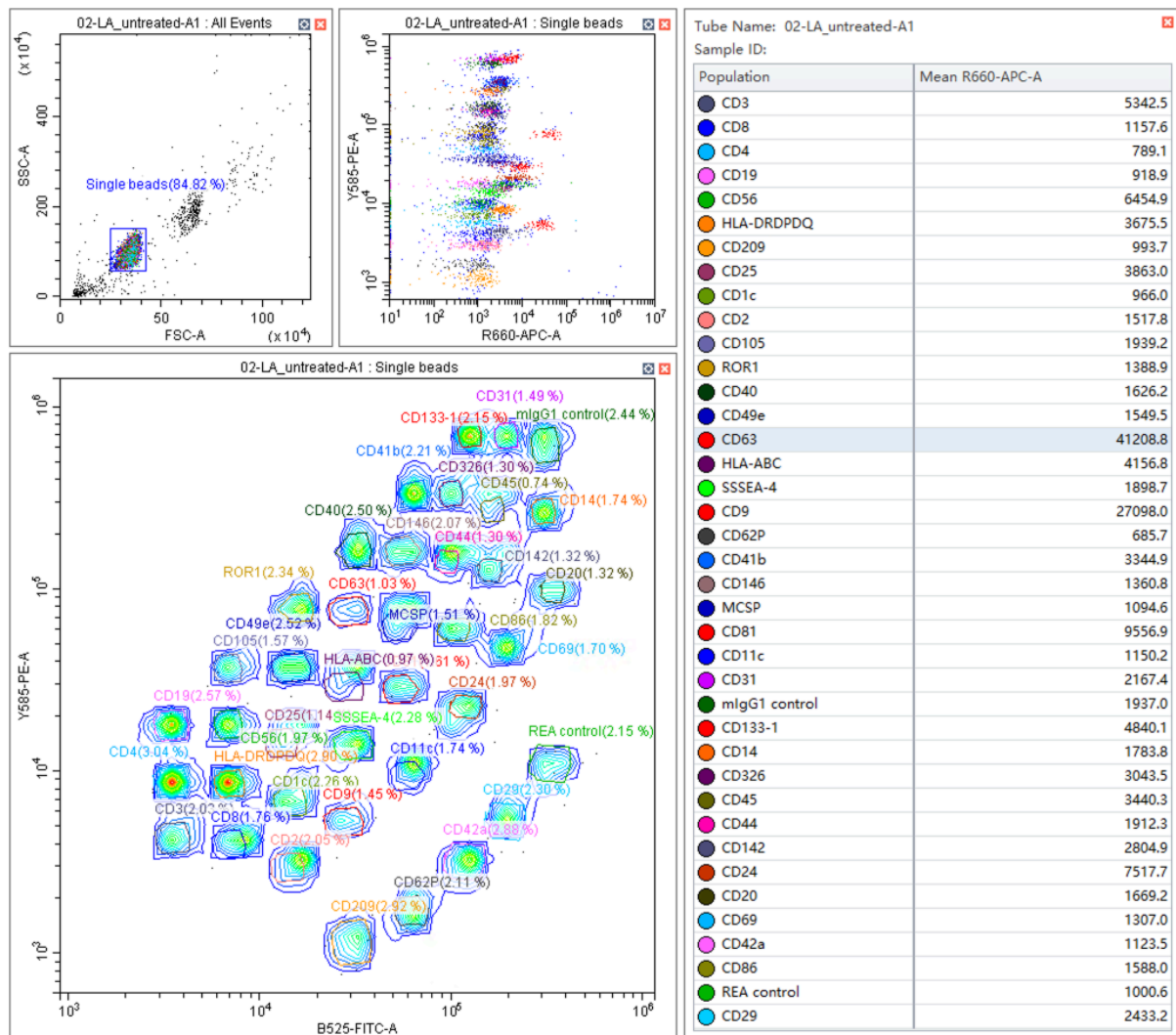
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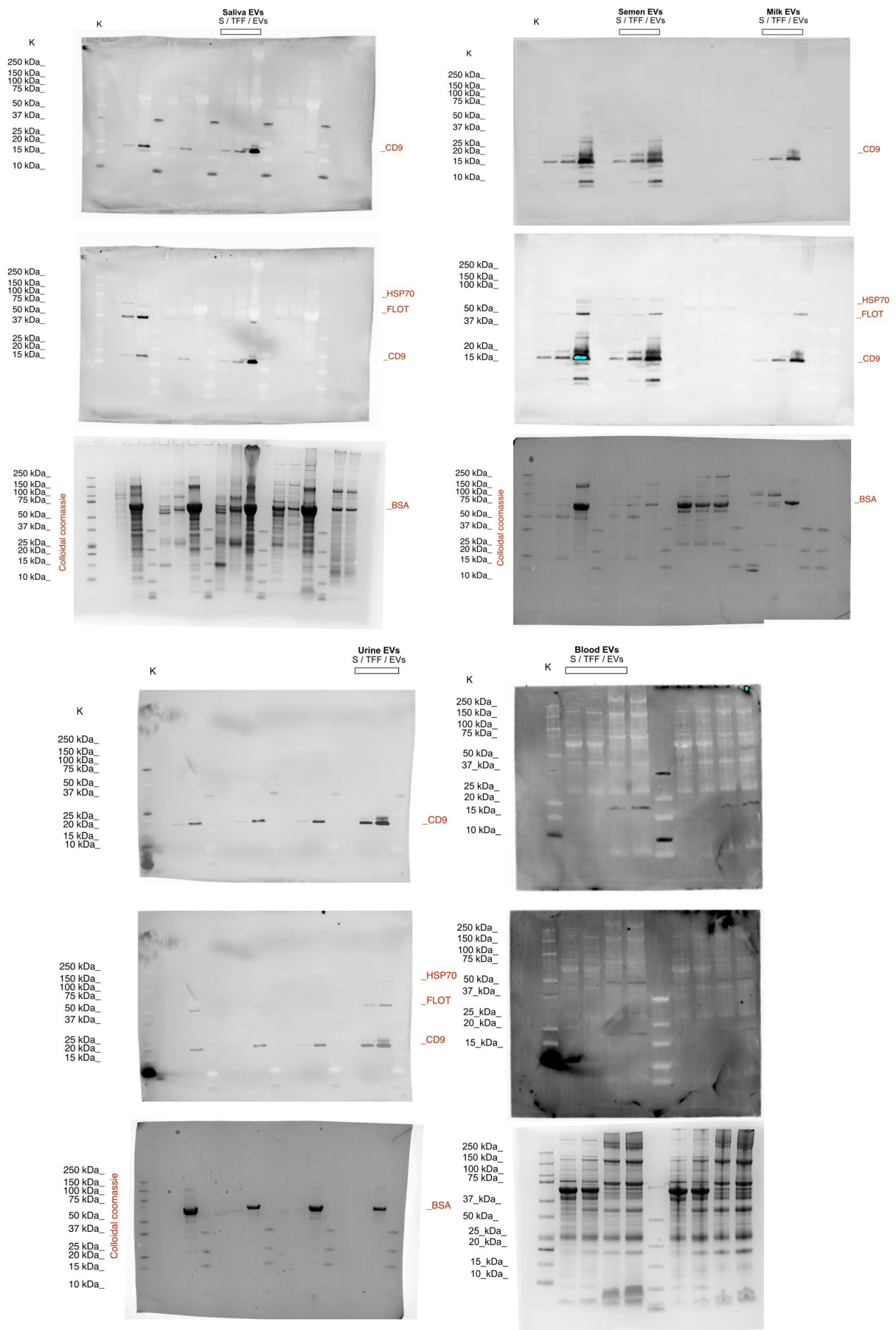
Gating strategy

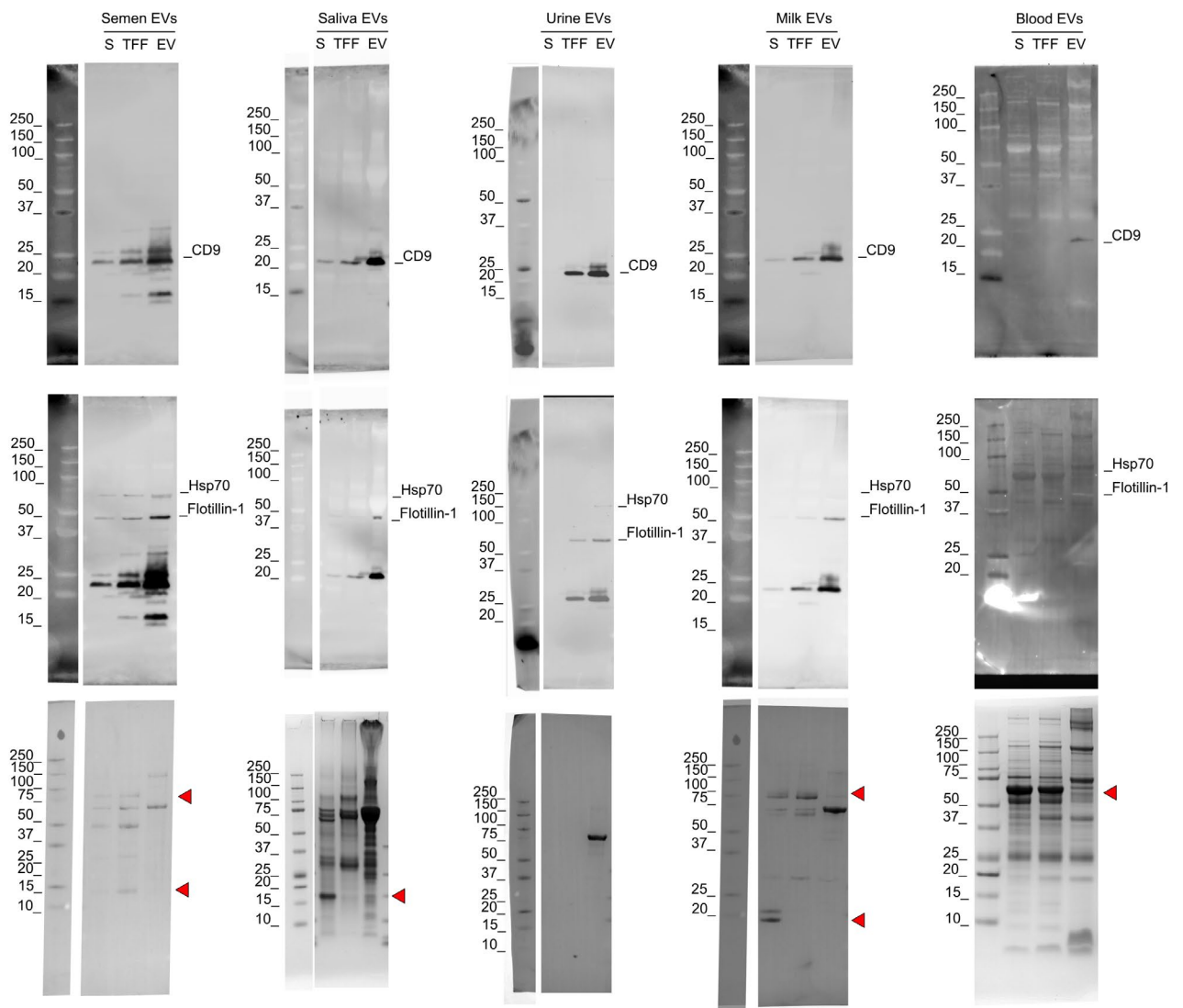


Gating strategy for bead-assisted flow cytometry as shown in Figure 3H, 4G, Extended Data Figure 4E. Representative raw data of EV-exposed PS using fluorescently tagged lactadherin. Single beads are first gated by SSC/FSC-A (top left) and individual beads containing antibodies directed against typical EV surface markers then separated by their endogenous PE/FITC-signal, enabling discrimination of 39 bead populations (37 markers + 2 isotype controls, large graph). The mean R660-APC/Alexa Fluoro 647 fluorescence intensity of each bead population is then analyzed to reveal the proportion of PS-containing vesicles which simultaneously carry the protein marker of respective beads. Performed according to Wiklander et al. Systematic Methodological Evaluation of a Multiplex Bead-Based Flow Cytometry Assay for Detection of Extracellular Vesicle Surface Signatures. *Front. Immunol.* 9, 1326 (2018).

Uncut western blots

A



B

Uncut western blot membranes and total protein stain corresponding to cropped images shown in Figure 3B. Protein concentrations are 1 $\mu\text{g}/\text{lane}$ (urine), 10 $\mu\text{g}/\text{lane}$ (seminal plasma, breast milk, saliva, serum). For urine, the source fluid contained undetectable ($< 25 \mu\text{g}/\text{ml}$) protein levels and was thus used undiluted; TFF and BE-SEC fractions loaded are 1 $\mu\text{g}/\text{lane}$ protein. For all preparations, membranes were stained for EV marker proteins CD9, Hsp70 and flotillin-1. Separate gels were stained for total protein by colloidal Coomassie (bottom row). Note that after determining protein concentrations, all EV preparations were supplemented with BSA (0.1%, v/v) as a stabilizing agent (seen as a band at $\sim 66 \text{ kDa}$ in total protein stain). For serum, a sample without added BSA was separated on SDS-PAGE to visualize loss of HSA by purification. Proteins abundant in source fluids with notable reduction after TFF/BE-SEC are indicated by red triangles. Shown are A) uncut membranes and with B) and relevant lanes and markers.

Resource Table

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Flavivirus group antigen [D1-4G2-4-15 (4G2)]	Absolute Antibodies	Cat. # Ab00230-2.0 RRID:AB_2715504
CD9 (D8O1A) Rabbit mAb 13174	Cell Signaling Technology	Cat. # 13174 RRID: AB_2798139
Flotillin-1 (D2V7J) XP® Rabbit mAb 18634	Cell Signaling Technology	Cat. # 18634 RRID: AB_2773040
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP	Thermo Fisher Scientific	Cat. # 31430 RRID: AB_228307
Goat anti-mouse (H+L) Alexa Fluor 488	Thermo Fisher Scientific	Cat. # A11001 RRID: AB_2534069
Goat anti-Rabbit Affinity HRP	Thermo Fisher Scientific	Cat. # 31460 RRID: AB_228341
Goat anti-Mouse IgG (H+L) Superclonal Recombinant Secondary AB Alexa Fluor 647	Thermo Fisher Scientific	Cat. # A27029 RRID: AB_2536092
Goat anti-rabbit IgG StarBright 520	Bio-Rad	Cat. # 12005870 RRID: AB_2884949
Goat anti-rabbit IgG StarBright 700	Bio-Rad	Cat. # 12004162 RRID: AB_2721073
HSP70 (D69) Antibody 4876	Cell Signaling Technology	Cat. # 4876 RRID: AB_2119693
SARS-CoV/SARS-CoV-2 N 40143-MM05 Mouse mAb	SinoBiological	Cat. # 40143-MM05 RRID: AB_2827977
Axl Monoclonal Antibody (DS7HAXL), PE	Thermo Fisher Scientific	Cat. 12-1087-42 RRID: AB_2723961
CD9 (HI9a) FITC mouse mAb 312104	Biolegend	Cat. # 312104 RRID: AB_2075894
CD81 (TAPA-1) FITC mouse mAB 349504	Biolegend	Cat. # 349504 RRID: AB_2075894
CD63 (H5C6) FITC mouse mAB 353005	Biolegend	Cat. # 353005 RRID: AB_10898319
IgG1, κ (MOPC-21) mouse mAB 400109	Biolegend	Cat. # 400109 RRID: AB_2861401
IgG2a, κ (MOPC-173) mouse mAB 400207	Biolegend	Cat. # 400207 RRID: AB_2884007
Chemicals, peptides, and recombinant proteins		
18:1 (Δ9-Cis) PC (DOPC)	Avanti Polar Lipids	Cat. # 850375C
18:1 Cy5 PC	Avanti Polar Lipids	Cat. # 850483C
18:1 PS (DOPS)	Avanti Polar Lipids	Cat. # 840035C
Bovine Lactadherin Alexa 647	Haematologic Technologies, Inc.	Cat. # BLAC-ALEXA647
BSA	Gibco	Cat. # 15260-037
Choline	Sigma Aldrich	Cat. # C7527
Chloroform	Sigma Aldrich	Cat. # 34854

Clarity Max Western ECL Substrate	Bio-Rad	Cat. # 1705062
DSB-3	Bunz Lab	Choi et al., 2018 ⁵⁴
DPBS	Gibco	Cat. # 14190-144
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat. # 41965039
Fetal bovine serum (FBS)	Gibco	Cat. # 10270106
GelCode Blue	Thermo Fisher Scientific	Cat. # 24590
HEPES	Gibco	Cat. # 15630-080
Hoechst 33342	Thermo Fisher Scientific	Cat. # H1399
Immobilon-FL PVDF	Merck	Cat. # IPFL00010
Isopropanol	Sigma Aldrich	Cat. # 33539
L-Glutamine	PAN-Biotech	Cat. # P04-80100
Methanol	Sigma Aldrich	Cat. # 32213
Methyl acetate	Sigma Aldrich	Cat. # 8.09711.100
Methyl- α -cyclodextrin	AraChem	Cat. # CDexA-076/BR
Non-essential amino acids	Gibco	Cat. # 11140-050
NuPAGE 4-12% BisTris gels	Thermo Fisher Scientific	Cat. # NP032
Paraformaldehyde 4%	VWR Chemicals	Cat. # 20910.294
Penicillin-Streptomycin	PAN-Biotech	Cat. # P06-07050
Phosphoserine	Selleckchem	Cat. # S5137
Phospholipase D from <i>Streptomyces c.</i>	Sigma Aldrich	Cat. # P0065
PS decarboxylase His6- Δ 34PkPSD	Voelker lab	Choi et al., 2012 ⁵⁵
Serine	Sigma Aldrich	Cat. # S4500
Sodium chloride	Sigma Aldrich	Cat. # 1.06404
Sodium hydroxide	Sigma Aldrich	Cat. # 415413
Sodium pyruvate	Gibco	Cat. # 11360-070
Sulfuric acid	Merck	Cat. # 1007311000
TMB substrate	SeraCare	Cat. # 5120-0078
TopFluor® PC	Avanti Polar Lipids	Cat. # 810281C
Tris(2-carboxyethyl)phosphine	Sigma Aldrich	Cat. # 646547
Triton X-100	Sigma Aldrich	Cat. # T9284
Trypsin-EDTA	PAN-Biotech	Cat. # P10-023100
Trypsin-EDTA	Gibco	Cat. # 25200-072
Tween-20	Sigma Aldrich	Cat. # P7949
Critical commercial assays		
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	Cat. # G7571
MACSPlex Exosome Kit, human	Miltenyi Biotec	Cat. # 130-108-813
Pierce™ Rapid Gold BCA Protein Assay Kit	Thermo Fisher Scientific	Cat. # A53225
TaqMan™ Fast Virus 1-Step Master Mix	Thermo Fisher Scientific	Cat. # 4444436
LysoTracker™ Green DND-26	Thermo Fisher Scientific	Cat. # L7526

Experimental models: Cell lines		
BHK-21	ATCC	Cat. # CCL-10
Calu-3	ATCC	Cat. # HTB-55
ELVIS™	ATCC	Proffitt <i>et al.</i> , 1995 ⁵⁶
HEK293T	ATCC	Cat# CRL3216
HFF	J. von Einem, Ulm University Medical Center	N/A
Huh-7	R. Bartenschlager, Heidelberg University	N/A
TZM-bl	NIBSC	Cat. # ARP5011
Vero E6	ATCC	Cat. # CRL-1586
Experimental models: Organisms/strains		
CHIKV	G. Simmons, UCSF	Tsetsarkin <i>et al.</i> , 2006 ⁵⁷
DENV-R2a	N/A	Fischl <i>et al.</i> , 2013 ⁵⁸
EBOV-trVLP	Poehlmann Lab, GPC Göttingen	Watt <i>et al.</i> , 2014 ⁵⁹
<i>Ex vivo</i> vaginal tissue	OB/GYN Clinic, Ulm University Medical Center	N/A
HCMV (TB40/E strain)	J. von Einem, Ulm University Medical Center	Sinzger <i>et al.</i> , 2008 ⁶⁰
HCV (Jc1 ^{p7-GLuc-2A-NS2})	N/A	Hofmann <i>et al.</i> , 2018 ⁶¹
HIV-1 92TH014	A. Papkalla, University of Erlangen	Papkalla <i>et al.</i> , 2002 ⁶²
HSV-1 (F strain eGFP)	B. Kaufer, FU Berlin	N/A
HSV-2 (333 strain eGFP)	P.G. Spear, NU Chicago	N/A
SARS-CoV-2 B.1.617.2	H. Streeck, University of Cologne	Weil <i>et al.</i> , 2023 ⁶³
VSV	K.K. Conzelmann (Ludwig-Maximilians- University of Munich) and S. Whelan (Washington University St. Louis)	N/A
ZIKV (FB-GWUH-2016 strain)	O. Vapalahti, University of Helsinki	N/A
ZIKV (MR766 strain)	J. Schmidt-Chanasit, Bernhard-Nocht-Institute Hamburg	N/A

Oligonucleotides		
ZIKV-F 5'-ACGGCYCTYGCTGGAGC-3'	Biomers.net	N/A
ZIKV-R 5'- GGAATATGACACRCCCTTCAAYCTAAG-3'	Biomers.net	N/A
ZIKV-P FAM-AGGCTGAGATGGATGGTGCAAAGGG- BNQ535	Biomers.net	N/A
Software and algorithms		
GraphPad Prism 9.5.1 and 10.0.2	GraphPad Software, Inc	www.graphpad.com
FIJI Version 2.3.0	N/A	www.imageJ.net/Fiji
CytExpert Version 2.3.0.84	Beckman Coulter, Inc	www.beckman.com
Huygens Professional 19.10	Scientific Volume Imaging	www.svi.nl
Other		
Extruder Set	Avanti Polar Lipids	Cat. # 610000
TFF hollow-fibre module 300 kDa MWCO	Repligen	Cat. # T06-E300-05-S
CaptoCore 700 BE-SEC	Cytiva	Cat. # 17548115
Vivaspin 20 100 kDa MWCO	Sartorius	Cat. # VS2042
Amicon Ultra-15 100 kDa MWCO	Sigma Aldrich	Cat. # UFC9110096
Amicon Ultra-0.5 10 kDa MWCO	Sigma Aldrich	Cat. # UFC501024
0.2 µm syringe filter	Merck Millipore	Cat. # SLGP033RB
Stericup 0.22 µm PVDF	Merck Millipore	Cat. # S2GVU05RE
HPTLC (High Performance TLC) Glass Plates	Sigma Aldrich	Cat. # Z740222
Hirschmann microcapillary pipette 1-5 µl	Sigma Aldrich	Cat. # Z611239