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Supplemental Information

Equid Herpesvirus-1 Exploits the Extracellular Matrix of Mononuclear Cells to Ensure Transport to Target Cells

Mohamed Kamel, Selvaraj Pavulraj, Beatrix Fauler, Thorsten Mielke, and Walid Azab

Supplemental Information

Supplemental figures and legends

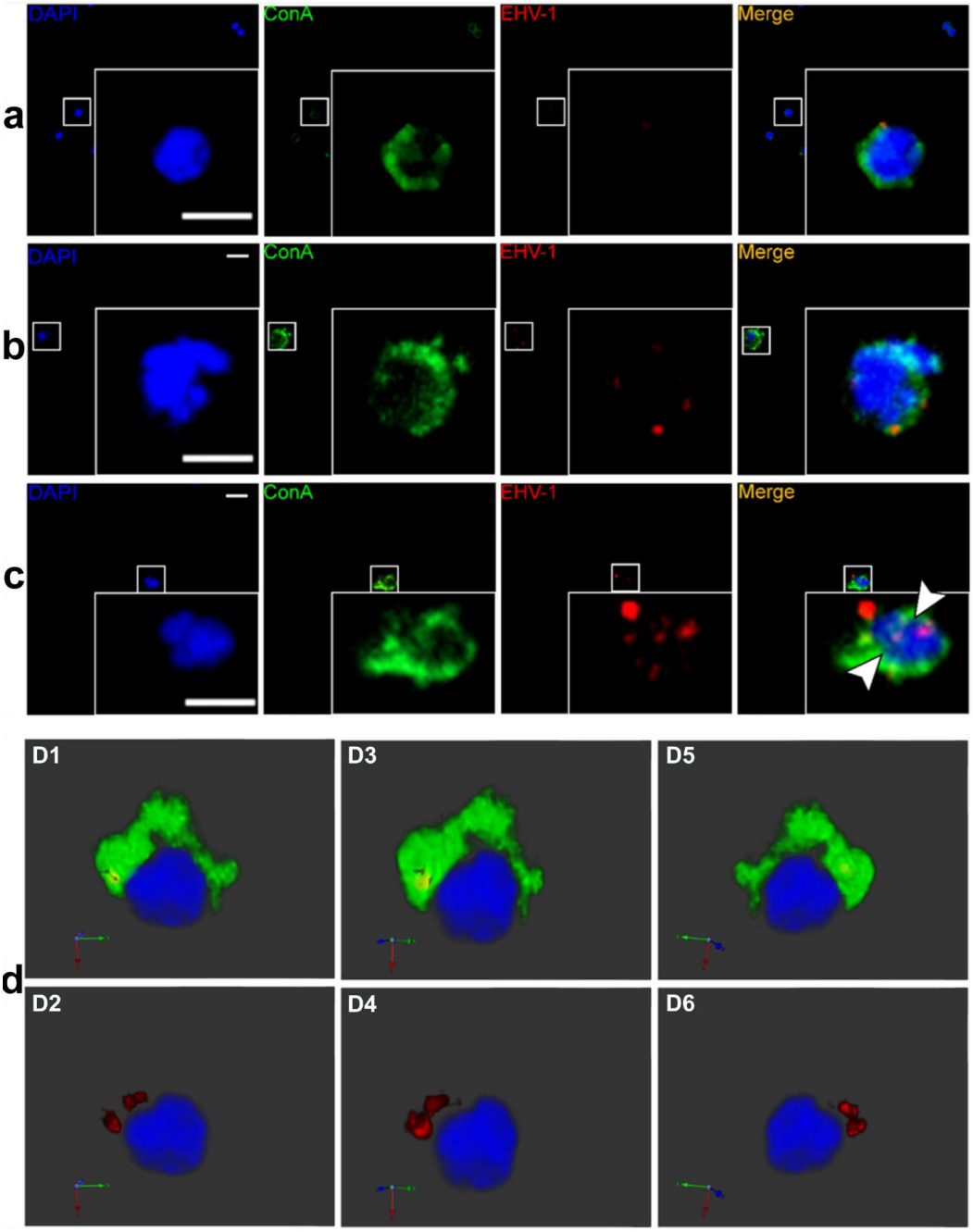

Figure S1. Colocalization of virus particles with the carbohydrate-rich extracellular matrix, Related to Figure 1. PBMC were infected with EHV-1^{RFP} (red; MOI=0.5) for 1 hr (a) and 24 hr (b and c). Cell surface glycoproteins of the ECM were stained green with FITC-labeled ConA. PBMC nucleus was stained with DAPI (blue). After 24 hr, EHV^{RFP} viral particles were detected outside the cells colocalizing with ECM (b) as well as inside the PBMC (c, arrowheads). Data are representatives of three independent experiments. Scale bar = 10 µm and scale bar of magnification = 7 µm. Image stacks (number of stacks = 17 with 0.75 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks. (d) 3D overview of embedded viruses in the ECM. Red: RFP-labeled virus particles; green: FITC-labeled ConA. (D1 and D2) are the front overviews, (D3 and D4) are the side overviews and (D5 and D6) are the back overviews. The 3D images were reconstituted from total projection of stack series image planes (number of stacks = 17) with 0.75 µm z-stack step size.

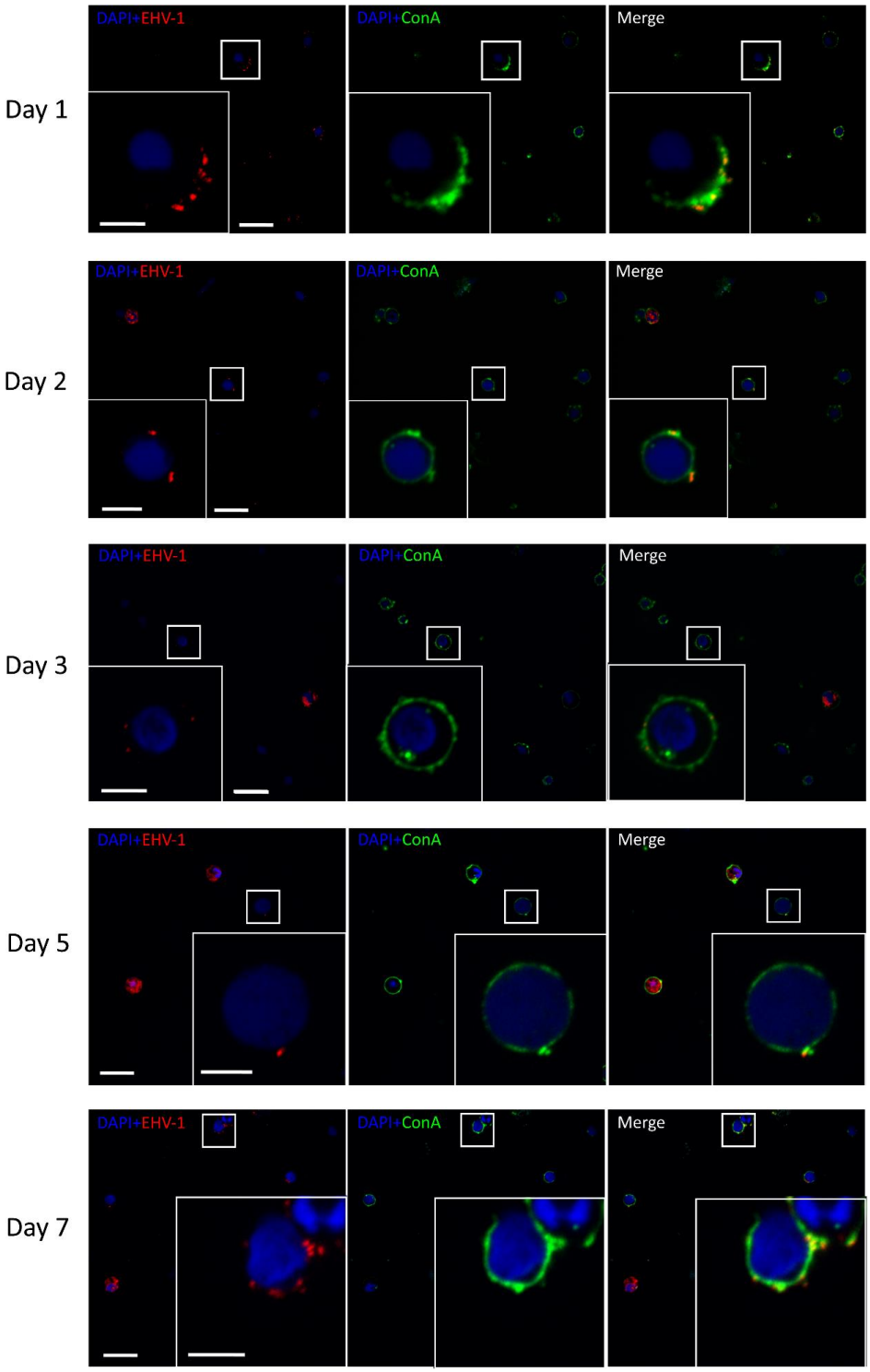

Figure S2. Colocalization of virus particles with the carbohydrate-rich extracellular matrix after 7 days of infection, Related to Figure 1. PBMC were infected with EHV-1^{RFP} (red; MOI=0.5) for 24 hr and up to 7 days. At different time points (day 1, 2, 3, 5 and 7) PBMC surface glycoproteins of the ECM were stained green with FITC-labelled ConA. Nucleus was stained with DAPI (blue). Data are representatives of three independent experiments. Scale bar = 30 µm and scale bar of the magnification = 5 µm. Image stacks (number of stacks = 15 with 0.5 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

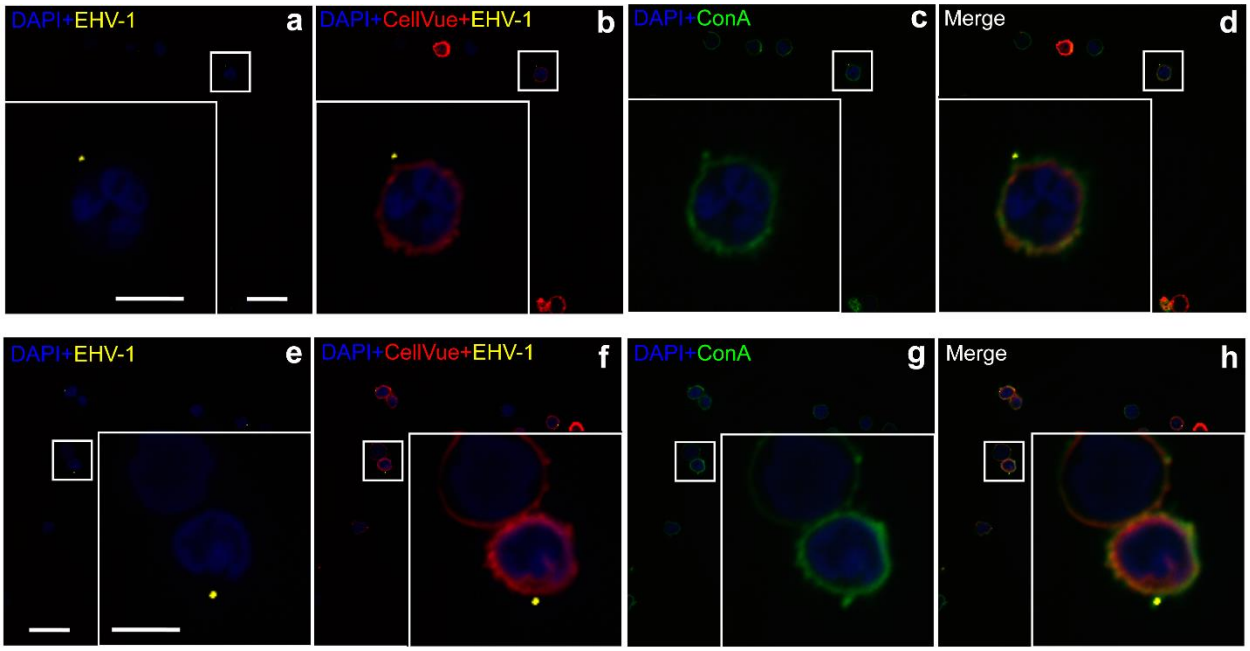

Figure S3. Colocalization of virus particles with the carbohydrate-rich extracellular matrix, Related to Figure 1. PBMC were infected with EHV-1^{RFP} (MOI=0.5) for 5 min. Cell surface glycoproteins of the ECM were stained green with FITC-labelled ConA. Plasma membrane was stained with CellVue® Claret Far Red Fluorescent dye (red) and nucleus was stained with DAPI (blue). Two independent representative experiments are shown (**a-d** and **e-f**). EHV-1^{RFP} viral particles (yellow) were detected outside cells colocalizing with ECM (**c** and **g**), but not with plasma membrane (**b** and **f**). Scale bar = 30 µm and scale bar of the magnification = 5 µm. Image stacks (number of stacks = 15 with 0.5 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

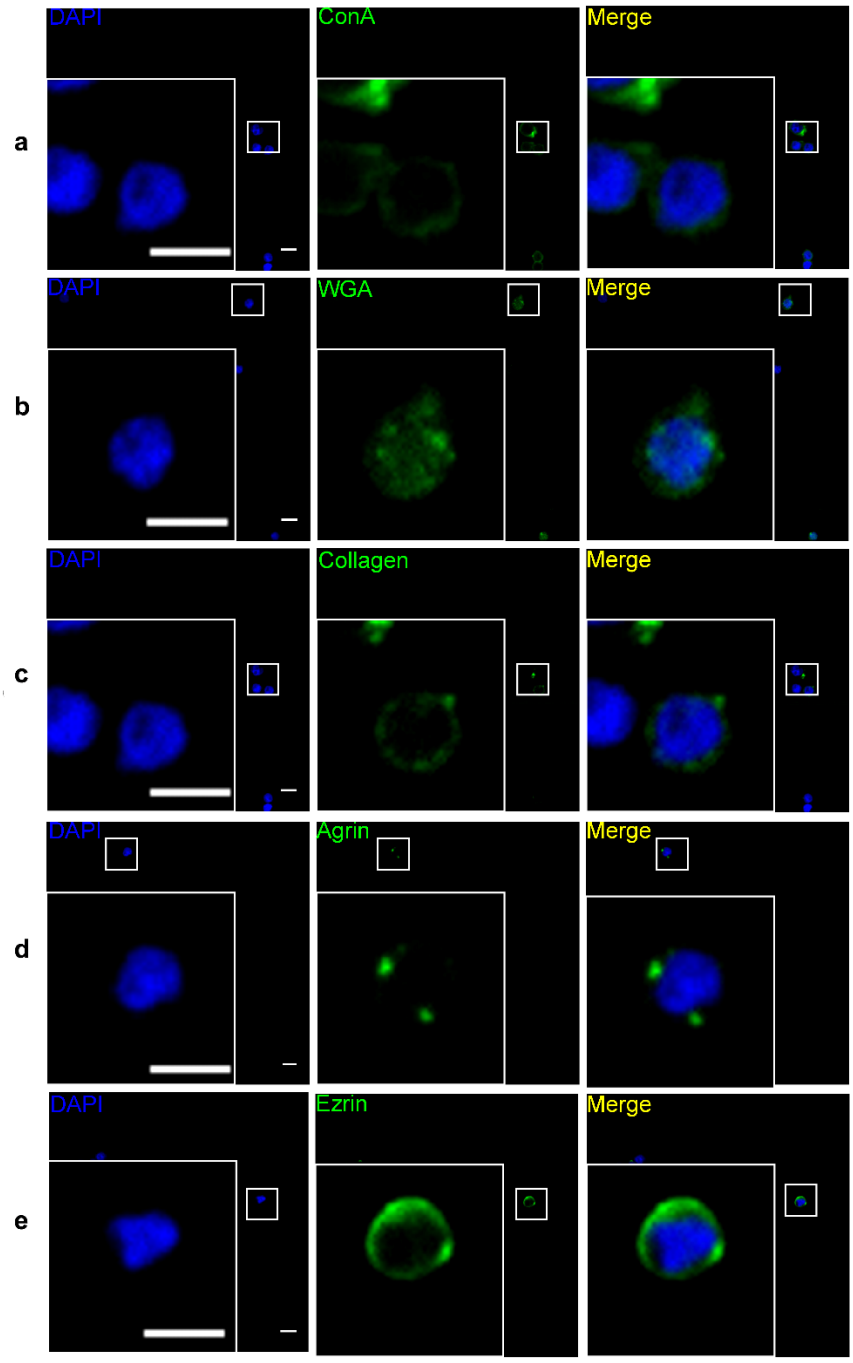

Figure S4. Extracellular matrix components of non-infected PBMC, Related to Figure 1. Cell surface glycoproteins of the non-infected PBMC stained green with FITC-labeled ConA (**a**), lectin from Triticum vulgaris (WGA; **b**), anti-collagen (**c**), anti-agrin (**d**), or anti-ezrin (**e**). PBMC nucleus was stained with DAPI (blue). Data are representatives of three independent experiments. Scale bar = 10 μm and scale bar of magnification = 7 μm. Image stacks (number of stacks = 17 with 0.75 μm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

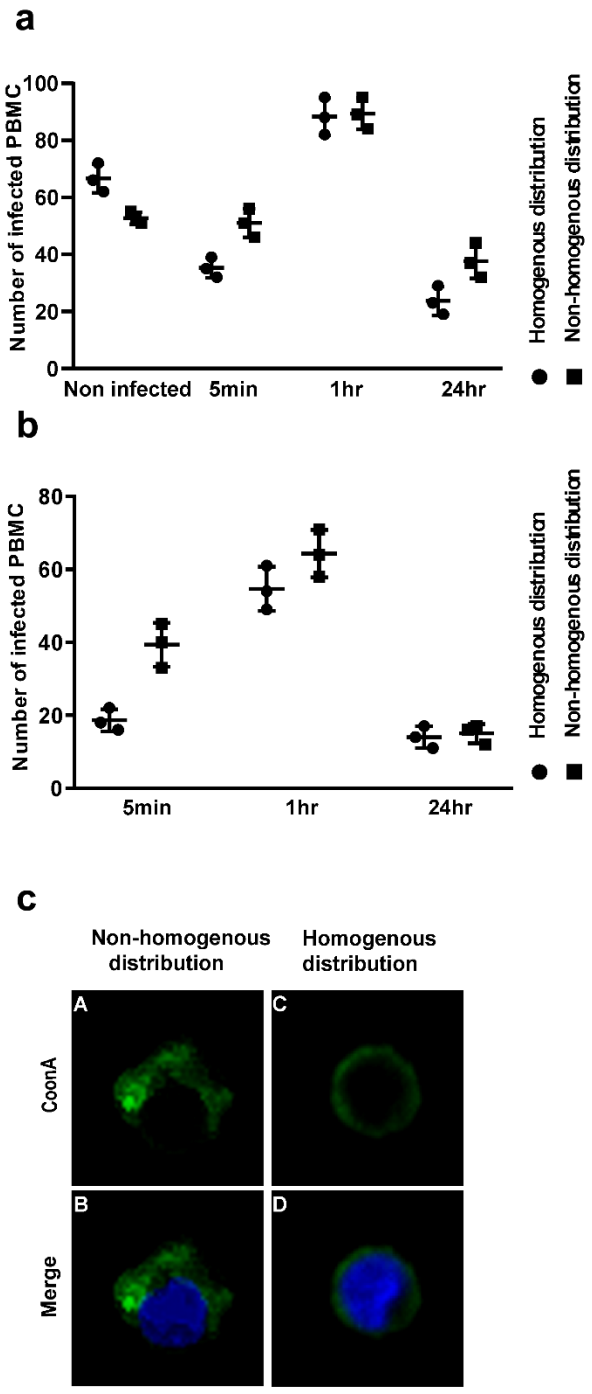

Figure S5. Modulation of ECM on the surface of infected and non-infected PBMC, Related to Figure 1. PBMC were either mock-infected or infected with EHV-1^{RFP} (MOI=0.5) for 5 min, 1 hr, or 24 hr. The cells were stained green with FITC-labeled ConA (**a**) or lectin from Triticum vulgaris (WGA; **b**). The experiment was performed three independent times in blinded fashion. The y-axis indicates the total number of cells involved in the assay. Kruskal–Wallis with Dunns test was used to compare modulation of ECM on infected cells at different time points to the non-infected cells; no significant difference was detected. (**c**) Confocal images showing non-homogenous (A and B) and homogenous (C and D) distribution of ECM stained with ConA (green). Nucleus was stained with DAPI (blue). Image stacks (number of stacks = 17 with 0.75 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

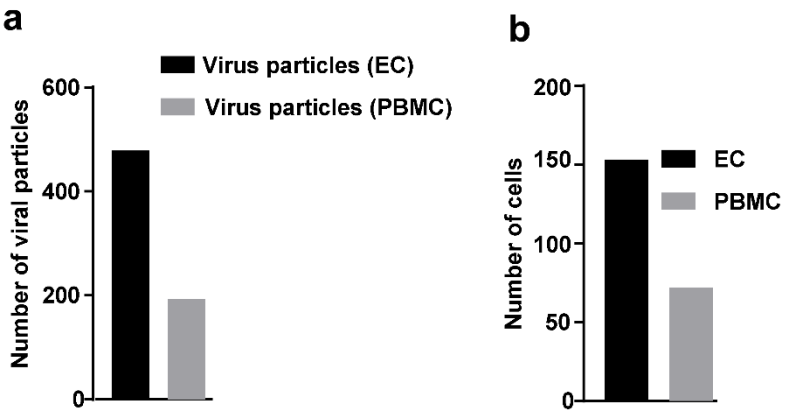

Figure S6. Virus transmission from infected PBMC to EC, Related to Figure 2. Number of counted viral particles embedded in the ECM of PBMC and transferred to EC is shown. PBMC were infected with EHV-1^{RFP} (MOI=0.5) for 5 min and added to endothelial cells for 2 hr followed by fixation with PFA 4% and image stacks (number of stacks = 17 with 0.75 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. (**a**) Number of counted viral particles on the surface of PBMC and EC. (**b**) Number of PBMC and EC on which virus particles were present. The experiment was conducted one time in triplicate.

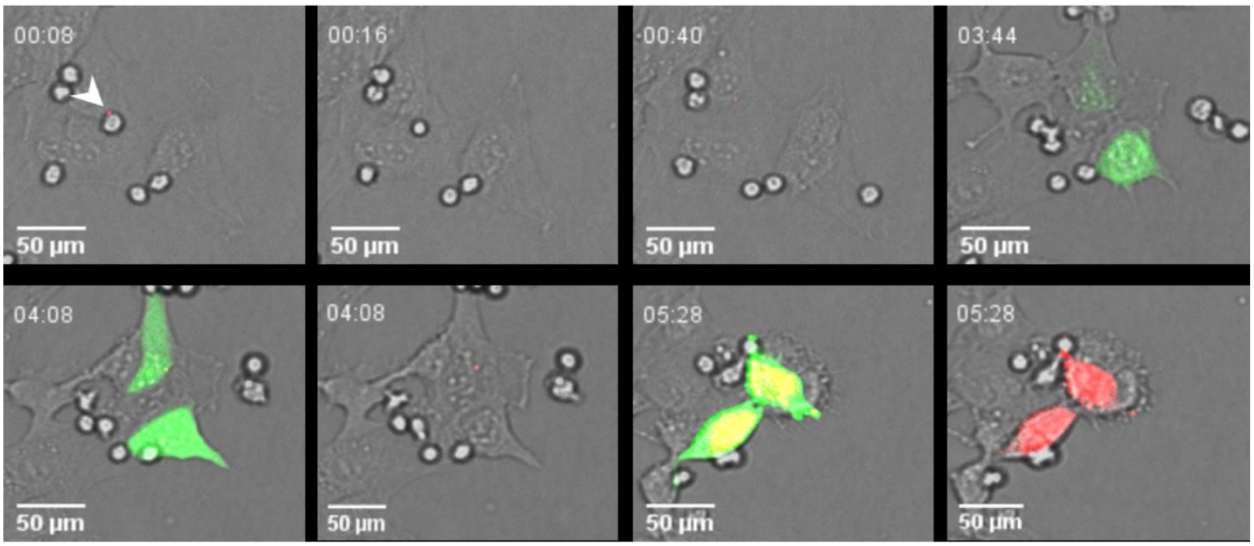

Figure S7. Live cell imaging of virus transmission from infected PBMC to endothelial cells, Related to Figure 2. Time-lapse live cell imaging confocal microscopy showing EHV-1$^{RFP+GFP}$ viral particles transfer from the overlaid 5-min-infected-PBMC on endothelial cells. Viral particles (red, arrowhead) were visualized moving from infected PBMC to endothelial cells. Virus replication in EC is indicated by GFP expression, which is integrated in the viral genome (green) and production of new progenies RFP-labeled virus (red) in the infected cells. At time points 04:08 and 05:28 min, the green pseudocolor was removed to clearly expose virus-RFP signals. Image stacks (number of stacks = 15 with 0.5 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

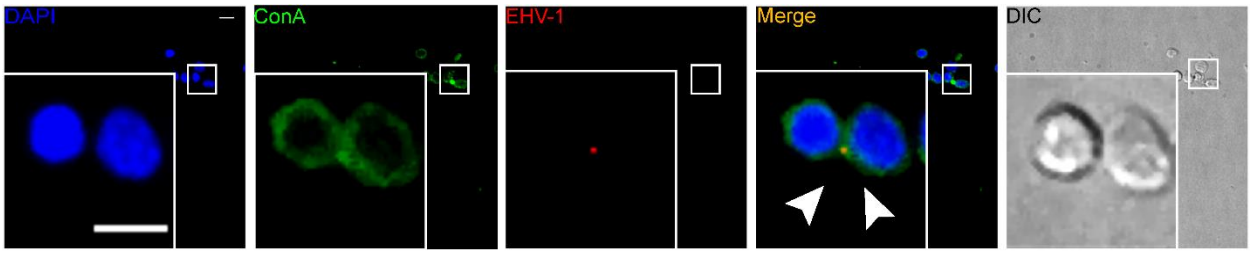

Figure S8. EHV-1 transfer between PBMC, Related to Figure 2. PBMC infected with EHV-1^{RFP} (red; MOI=0.5) for 1 hr and stained with ConA (green) display aggregates of two PBMC with the likelihood of virus transfer in between. Data are representatives of three independent experiments. Scale bar = 10 μm and scale bar of magnification = 7 μm. Image stacks (number of stacks = 17 with 0.75 μm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

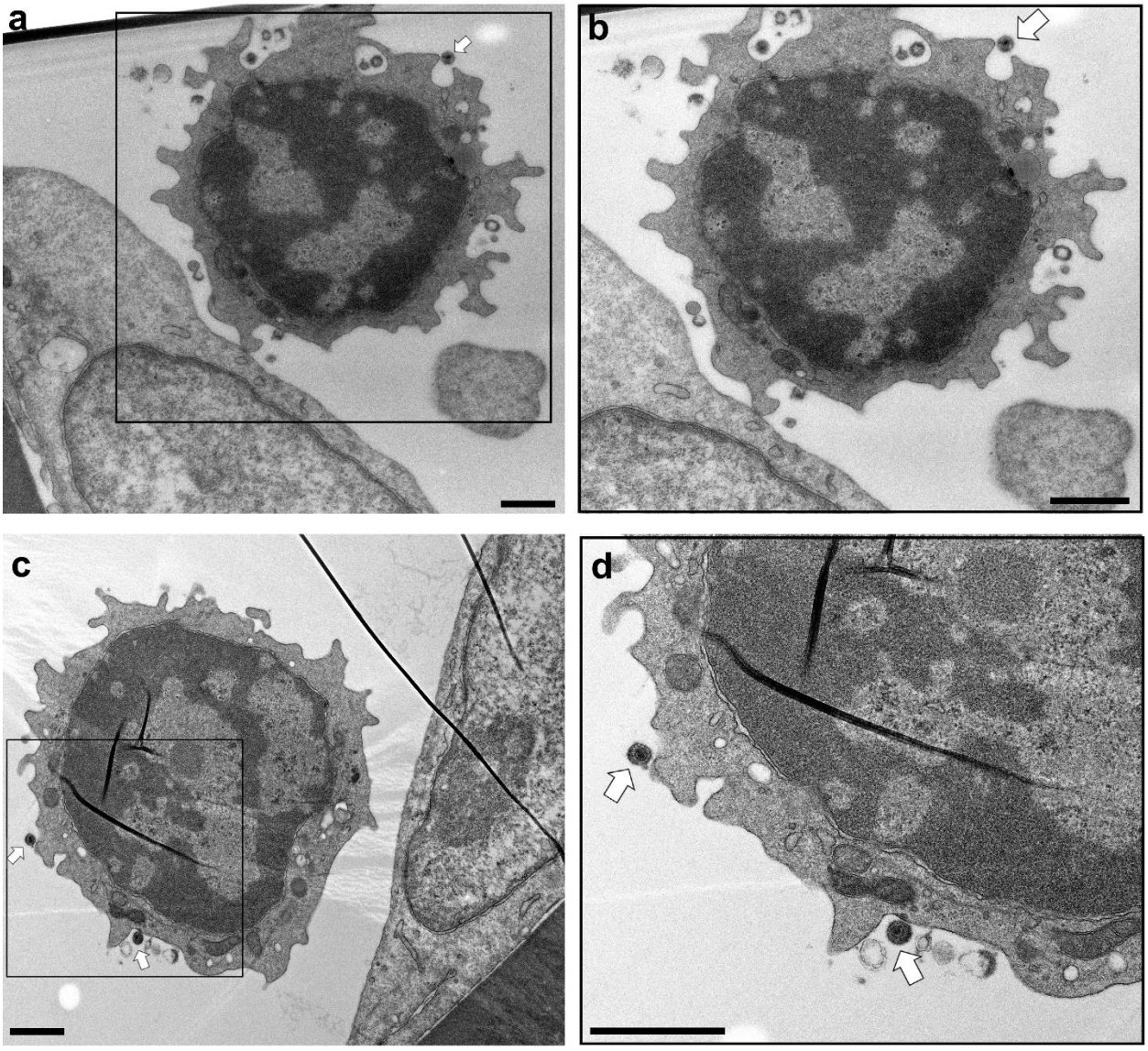

Figure S9. Virus particles on the surface of infected-PBMC, Related to Figure 3. Transmission electron microscopy (TEM) of EC grown on gridded coverslips and overlaid with EHV-1^{RFP} infected-PBMCs. Infected cells were observed either interacting with (**a** and **b**) or in close proximity (**c** and **d**) to EC. TEM images of the targeted PBMC show virus particles (white arrows) on the surface of infected PBMC. Marked parts with rectangles in (**a** and **c**) are magnified in (**b** and **d**, respectively). Scale bar = 1 µm.

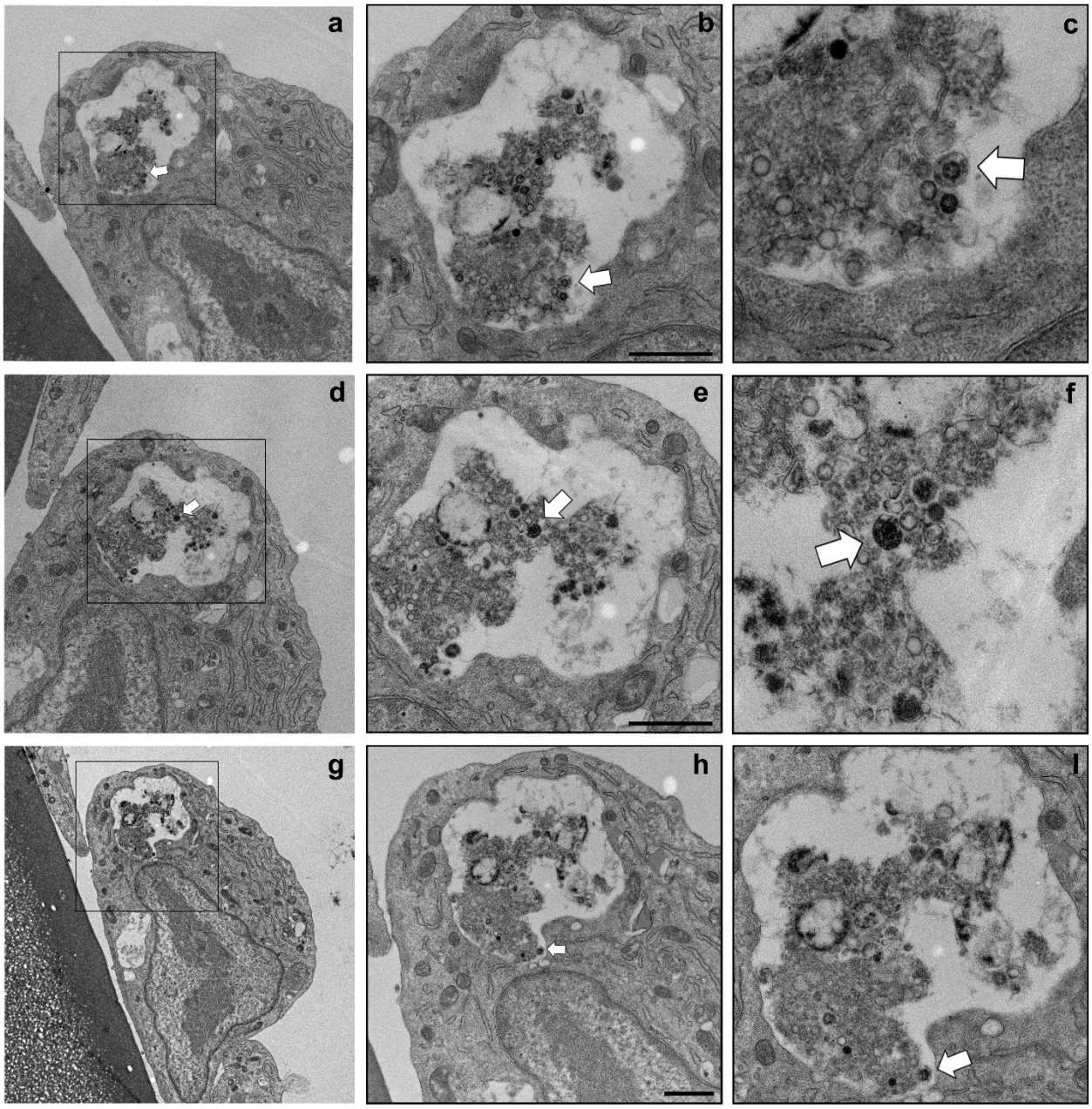

Figure S10. Transcellular migration of infected-PBMC through endothelial cells, Related to Figure 5. Ultrathin cross sections of PBMC crossing endothelial cells. PBMC were infected for 5 min with EHV-1^{RFP} and incubated over adherent EC for 3 hr. Partially digested infected-PBMC (at different stacks) is shown (**a, d, g**) and marked with a rectangle, which is magnified (**b-c, e-f, h-i**). Virus particles attached to migrating PBMC is marked with white arrows. Scale bar =1 μm.

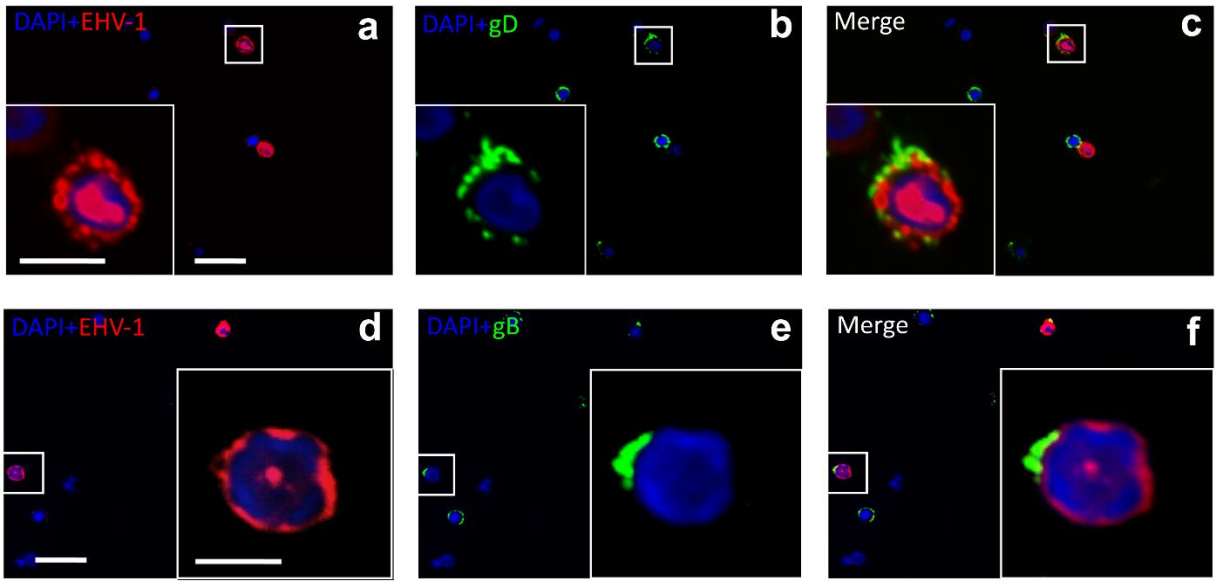

Figure S11. Expression of viral envelope glycoproteins (gD and gB) on the surface of infected PBMC, Related to Figure 6. PBMC were infected with EHV-1^{RFP} (red; MOI=0.5) for 24 hr and probed with antibody against EHV-1 gD (**a-c**) or gB (**d-f**) followed by anti-mouse Alexa Fluor-488 (green). Nucleus was stained with DAPI (blue). Red signals on cell surface and within the cytoplasm or the nucleus of the infected PBMC indicates RFP-labelled virus particles and expression of viral capsid proteins. Data are representatives of three independent experiments. Scale bar = 30 µm and scale bar of the magnification = 5 µm. Image stacks (number of stacks = 15 with 0.5 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks.

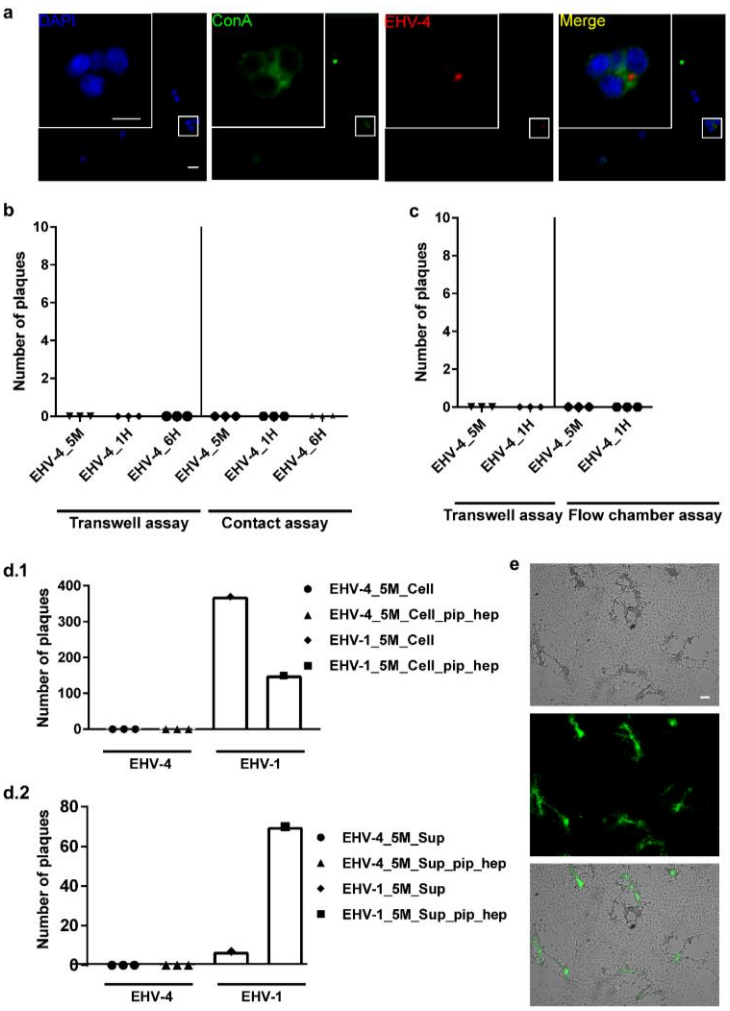

Figure S12. Colocalization of EHV-4 with the carbohydrate-rich extracellular matrix, Related to Figure 7. (**a**) PBMC were infected with EHV-4^{GFP} (MOI=0.5) for 5 min. Cell surface glycoproteins of the ECM were stained with FITC-Labeled ConA (green). EHV-4 particles were stained with anti-glycoprotein D monoclonal antibodies (red). PBMC nucleus was stained with DAPI (blue). Data are representatives of three independent experiments. Scale bar = 10 μm and scale bar of magnification = 7 μm. Image stacks (number of stacks = 17 with 0.75 μm z-stack step size) were photographed using VisiScope Confocal FRAP microscope. Presented here is a single optical section of the stacks. (**b** and **c**) EHV-4 transmission from infected PBMC to EC under "static" and "dynamic" states. PBMC were infected with EHV-4^{GFP} for 5 min. Infected PBMC were added to EC under "static" conditions (**b**) or allowed to flow over EC "dynamic" (**c**) in the presence of neutralizing antibodies. After 24 hr, virus spread was assessed by counting the plaques on EC. As a control, infected PBMC were placed into a transwell insert without direct contact between EC and PBMC "no contact". The data represent the mean ± standard deviation of three independent and blinded experiments. (**d**) PBMC were infected with EHV-4^{GFP} (MOI=0.1) for 5 min. The ECM was disrupted with both mechanical (extensive pipetting) and chemical (heparin treatment) together (EHV-4_5M_Cell_pip_hep). The disrupted cells (**d**.1) or the supernatant (EHV-4_5M_Sup_pip_hep; **d**.2) were added to EC. As a control, the ECM was left undisrupted (EHV-4_5M_Cell or EHV-4_5M_Sup). As a control of pipetting and heparin treatment, EHV-1 was used. The data represent the mean ± standard deviation of three independent and blinded experiments with EHV-4. Cells were infected with EHV-1 once to confirm virus spread and infection (see Fig 4a and b). (**e**) Endothelial cells are permissive for EHV-4 cell-free viruses. EC were infected with EHV-4^{GFP} (MOI 0.1) for 1 hr. Virus and media were removed and the cells were overlaid with methylcellulose media 1.5% for 3 days. EHV-4 GFP-expressing viral plaques were imaged with Carl Zeiss AXIO imager microscope equipped with a Zeiss Axiocam. Scale bar = 70 μm.

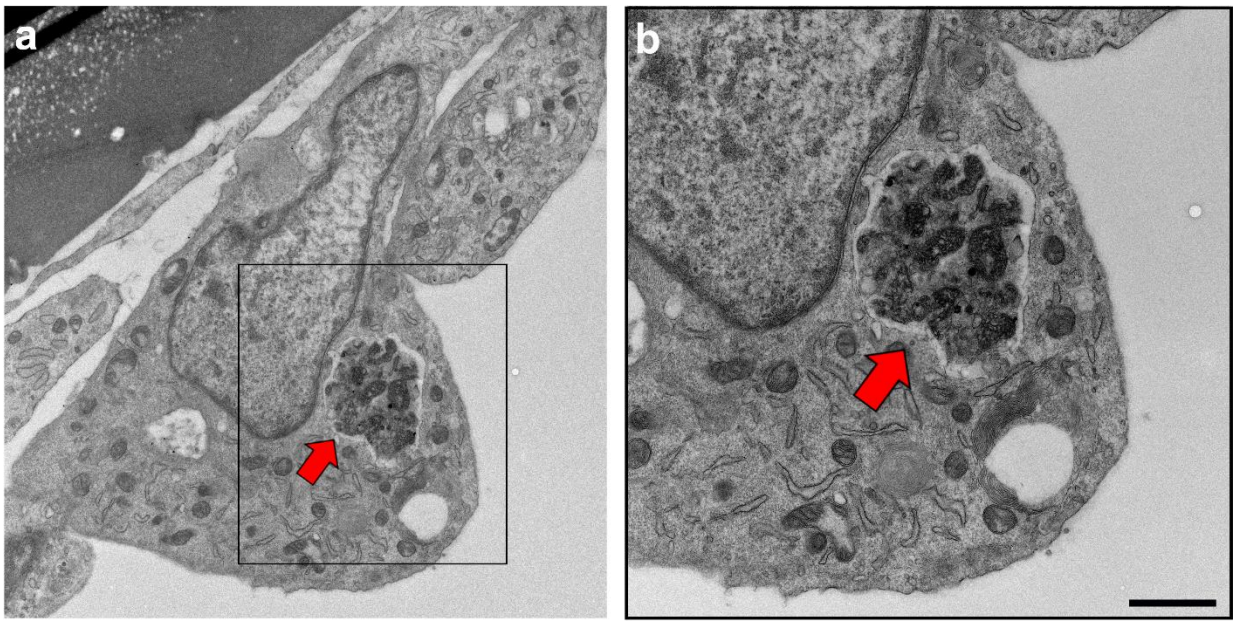

Figure S13. Transcellular migration of PBMC through endothelial cells, Related to Figure 5.
Ultrathin cross section of non-infected PBMC crossing endothelial cells. Partially digested naïve PBMC is shown within a cytoplasmic vacuole of EC. The migrating PBMC marked with a rectangle in (**a**, red arrow) is magnified in (**b**, red arrow). Scale bar = 1 µm.

Transparent Methods

Methods

Viruses. All viruses used in this study were produced and recovered from infectious bacterial artificial chromosome (BAC) clones. Those were BACs of EHV-1 (EHV-1^{GFP}) strain Ab4 (Goodman et al., 2007), EHV-4 (EHV-4^{GFP}) strain TH20p(Azab et al., 2009), recombinant EHV-1_gB4 and EHV-4_gB (Spiesschaert et al., 2015b), and EHV-1_gD4 and EHV-4_gD1 (Azab and Osterrieder, 2012). Viruses were grown on equine dermal (ED) cells (CCLV-RIE 1222, Federal Research Institute for Animal health, Germany) (Spiesschaert et al., 2015b). All recombinant viruses express the enhanced green fluorescent protein (EGFP; encoded in the mini-F sequence under the control of the immediate-early CMV promotor) for efficient identification of infected cells. All virus stocks were prepared by infecting ED cells until having 100 % cytopathic effect (CPE). Infected cultures (supernatant and cells) were collected and centrifuged at 6000 x g for 5 min to get rid of cellular debris. Virus-rich supernatants were collected and stored at -80 °C.

mRFP1-labeled viruses. Insertion of monomeric red fluorescent protein (mRFP1) into VP26 of EHV-1 Ab4 strain was performed as described before (Azab et al., 2013b; Tischer et al., 2006). Briefly, mRFP1 was amplified by PCR using pEPmRFP1-in (Tischer et al., 2006) as a template; all used primers are referenced in (Azab et al., 2013b). The resulting PCR products were electroporated into GS1783 (a kind gift from Dr. Greg Smith, Northwestern University, Chicago, IL) harboring the corresponding BACs (EHV-1 with and without GFP; to give rise of EHV-$1^{RFP+\ GFP}$ and EHV-1^{RFP}, respectively). Kanamycin-resistant colonies were purified and screened by PCR and restriction fragment length polymorphism (RFLP) analyses using EcoRV and BamHI. Positive clones were subjected to a second round of Red recombination to obtain the final constructs after excision of kanamycin gene. The final clones were confirmed by PCR, RFLP and sequencing (data not shown). All viruses (EHV-$1^{RFP+\ GFP}$ and EHV-1^{RFP}) were reconstituted as described before (Azab et al., 2013b).

Cells. Primary equine carotid artery endothelial cells (a kind gift from Prof. Dr. Johanna Plendl, Freie Universität Berlin, Institut für Veterinär-Anatomie) were propagated in Dulbecco's modified Eagle's medium (DMEM) (PAN-Biotech Ltd, Germany) supplemented with 20% fetal bovine serum (FBS) (PAN-Biotech Ltd, Germany), 1% nonessential amino acids (Biochrom, Germany), and 1% penicillin-streptomycin. Endothelial cells were obtained according to the rules of Institutional Animal Care and Committee of Berlin (Landesamt für Gesundheit und Sociales, L 0089/14). Equine PBMC were isolated from heparinized blood collected from healthy horses by density gradient centrifugation over Biocoll L 6715 (Biochrom, Germany), following the manufacturer's instructions. After two washing steps, cells were resuspended in RPMI-1640 (Pan Biotech, Germany) supplemented with 10% FBS, 0.3 mg/ml glutamine, nonessential amino acids, and 1% penicillin-streptomycin. Blood collection was performed according to the rules of Institutional Animal Care and Committee of Berlin (Landesamt für Gesundheit und Sociales, L 0294/13). PBMC were negative for both EHV-1 and EHV-4 as tested by qPCR (data not shown). PBMC were either used fresh after isolation or preserved in liquid-nitrogen and reconstituted when needed.

Plasmids. For assessing fusion assay by luciferase activity measurement, we used pT7EMCLuc plasmid, which expresses firefly luciferase reporter gene under the control of T7 promoter, and pCAGT7 plasmid, which expresses T7 RNA polymerase. Both plasmids were kindly provided by Dr. Richard Longnecker, Northwestern University. A plasmid expressing mRFP under the control of Human CMV promoter, pEP-CMVmRFP-in (Campbell et al., 2002; Tischer et al., 2006), was used for fusion assay. pEPmRFP1-in carrying kanamycin mRFP cassette had been used as a shuttle plasmid for EHV-1 strain Ab4-mRFP-VP26 construction.

Antibodies. FITC-labeled ConA (C7642), Triticum vulgaris (WGA; L4895) were from Sigma. Mouse anti-agrin antibody (D-2: sc-374117) and mouse anti-ezrin antibody (3C12: sc-58758) were from Santa Cruz Biotechnology. Rabbit anti-human Collagen I/II/III/IV/V was from (Biorad: 2150-2206). Mouse anti-EHV-4 gD Mab was kindly provided by Jules Minke, Merial: 105E2K (Azab et al., 2013a). Mouse Anti-EHV-1 gD 19-mer polyclonal antibodies (Allen and Yeargan, 1987) were kindly provided by Dr. Dennis O'Callaghan, Louisiana State University Health Sciences Center, Shreveport, LA. Mouse EHV-1 anti-gB monoclonal antibodies (mAb) (Wellington et al., 1996) were kindly provided by Dr. Udeni B. R. Balasuriya, Gluck equine research center, University of Kentucky, KY.

Confocal microscopy and immunofluorescence. For detection of the extracellular carbohydrate-rich matrix, $2x10^6$ PBMC (in suspension) were infected with EHV-1^{RFP} or EHV-4^{GFP} (MOI=0.5) for 5 min, 1 hr, 24 hr, 2 days, 3 days, 5 days, and/or 7 days. Cells were centrifuged at 400 x g for 5 min and treated with ice cold citrate buffer (pH 3) for 1.5 min. The cells were washed three times with PBS and fixed with 4% Paraformaldehyde (PFA) for 20 min. For EHV-4, virus particles were stained with mouse anti-EHV-4 gD monoclonal antibody followed by Alexa Fluor 568-labeled goat anti-mouse IgG (1:1000; Invitrogen 1841756). The carbohydrate-rich extracellular matrix was stained with FITC-labelled ConA and WGA (dilutions were 1:250 and 1:1000, respectively). Collagen, agrin and ezrin were stained by rabbit anti-human Collagen I/II/III/IV/V, mouse anti-agrin antibody, and mouse anti-ezrin antibody with dilutions of 1:100, 1:50, and 1:50, respectively, followed by Alexa Fluor 488-labeled goat anti-rabbit (1:1000; Invitrogen: 1966932) and anti-mouse IgG (1:1000; Invitrogen: 2015565). Plasma membrane was stained with CellVue® Claret Far Red Fluorescent dye (Sigma-Aldrich). DAPI (Life Technologies) was used to stain the nucleus. Image stacks (number of stacks = 15-17 with 0.5-0.75 µm z-stack step size) were photographed using VisiScope Confocal FRAP microscope (×40 magnification) and equipped with ANDOR iXon Ultra 888 camera (Visitron Systems GmbH, Germany). The images were processed and analyzed by using Image J software (https://imagej.nih.gov/ij/) and Metamorph software 7.8 (Molecular Devices).
In another experiment, $1x10^6$ PBMC were infected with EHV-1^{RFP} (MOI=0.5). All infected cells were treated with ice-cold citrate buffer and virus-neutralizing antibodies as described above. The cells were added to endothelial cell monolayers for 2 hr. The cells were fixed and stained with FITC-labeled ConA and WGA. Image acquisition, processing and analysis were performed as mentioned above.

Flow chamber assay. The assay was performed as previously described (Spiesschaert et al., 2015b). Briefly, PBMC ($1x10^6$) were infected with EHV-1^{GFP} or EHV-4^{GFP} (MOI=0.5) for different time points (5 min, 1 hr, 6 hr). PBMC were treated with ice-cold citrate buffer (pH 3). The infected cells were washed two times and resuspended in medium containing 1:100 dilution of virus neutralizing antibody (titer: 1:2028 as determined by serum neutralization test (Azab et al., 2019)) at 37°C for 30 min (Goehring et al., 2011; Spiesschaert et al., 2015a). Endothelial cells were grown to confluency in 0.4 collagen IV-coated cell flow chambers (Ibidi Integrated BioDiagnostics) that were connected to a perfusion system by Luer locks (Ibidi Integrated BioDiagnostics). The flow chamber set was incubated at either 4°C or 37°C. Infected PBMC were introduced at a flow rate of 0.5 mm/s, which is within the mammalian physiological range (0.34 to 3.15 mm/s) (Hudetz et al., 1996). The velocity was calculated according to the size of the chamber and the velocity in mammalian brain capillaries and was generated by a NE-4000 double-syringe pump (New Era Pump System). After 24 hr, GFP-viral plaques on the EC monolayer were counted (excluding the inlet/outlet areas) using an inverted fluorescence microscope and equipped with AxioCam MRc camera (Zeiss Axiovert 100).
To evaluate virus transfer from different PBMC subpopulations (i.e. T lymphocyte, B lymphocyte and monocytes) to EC, each PBMC subpopulation was sorted out and flow chamber assay was performed. Briefly, PBMC were stained with (1:200 diluted) primary mouse antibodies against equine CD3 (T lymphocyte), IgM (B lymphocyte) and CD14 (monocyte). The antibodies were kindly provided by Dr. Bettina Wagner, Cornell University, Ithaca, NY, USA. PBMC were then labelled with secondary Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen) and sorted using FACSAria (BD Bioscience™). Sorted T- lymphocytes, B-lymphocytes and monocytes ($1x10^6$) were infected with EHV-1^{GFP} (MOI=0.5) for 5 min at 37 ºC. After incubation, cells were treated with citrate buffer, resuspended in EHV-1 neutralizing antibodies and flow chamber experiment was performed as described above.

Contact assay. PBMC ($5x10^5$) were infected with EHV-1^{GFP} or EHV-4^{GFP} at MOI of 0.1 at different time points (5min, 1 hr, and 6 hr). Infected cells were treated with ice-cold citrate buffer and added to EC for 2 hr in the presence of virus neutralizing antibody as described above. Non-adherent cells were then removed by extensive and gentle washing with PBS. In the “no-contact” setup, virus-infected PBMC were placed into a transwell insert (with a membrane pore size of 0.4 µm; Corning Transwell support system) without direct contact with EC. Methylcellulose 1.5% was added and cells were incubated for 24 hr. Virus transfer was assessed by counting the green fluorescent viral plaques using an inverted fluorescence microscope (Zeiss Axiovert 100).

Disruption of extracellular virus assemblies. PBMC ($5x10^5$) were infected with EHV-$1^{RFP+GFP}$ or EHV-4^{GFP} at MOI of 0.1 for 5 min as described above. The cells were subjected to vigorous pipetting, treated with heparin, or both. Heparin (50µg/ml; Sigma-Aldrich) was added to the cells for 30 min. PBMC (cells or supernatant) were added to EC for 2 hr. After washing, methylcellulose 1.5% was added to the cells. Viral plaques on EC were counted after 24 hr using an inverted fluorescence microscope. For confocal microscopy, EHV-1-infected PBMC were stained with FITC-labeled ConA and then treated with heparinase III (10 U ml^{-1}) for 1 hr or heparin (50µg/ml) for 30 min at 37°C in serum-free medium. Image stacks were photographed using VisiScope Confocal FRAP microscope. Image acquisition, processing and analysis were performed as mentioned above.

Fusion assays. Luciferase assay. Day 1: PBMC were nucleofected with 2µg pCAGT7 plasmid using Amaxa® Cell Line Nucleofector® Kit V (Lonza) according to the manufacturer instructions. Day 2: PBMC were infected with EHV-1^{GFP} at MOI of 1. Endothelial cells were nucleofected with 2µg pT7EMCLuc and seeded in collagen-coated 24 well plate. Day 3: Nucleofected-infected-PBMC were washed and overlaid on the nucleofected endothelial cells. Day 4: Activation of the reporter luciferase gene resulted from fusion of nucleofected infected PBMC to nucleofected endothelial cells was measured by Luciferase Assay System kit (E1500, Promega, USA) using TriStar LB 941 Multimodal plate reader (Berthold Technologies) for luminescence measurement according to the manufacturer's instructions (Promega). Briefly, endothelial cells were washed to remove non-fused PBMC and lysed with the lysis buffer. The lysed cells were left on a shaker for 15 min and 20 µl of the cell lysates were used for the reading. As negative controls, only nucleofected PBMC or only infected PBMC were overlaid on the nucleofected endothelial. As a positive control, PBMC were nucleofected with both plasmids (pCAGT7 and pT7EMCLuc).
In another assay, PBMC were nucleofected with 2 µg pEP-CMVmRFP-in as described above. After 24 hr, nucleofected PBMC were infected with EHV-1^{GFP} at MOI of 1. After 24 hr, nucleofected-infected PBMC were added to endothelial cells for 3.5 hr. Endothelial cells were washed and fixed with 4% PFA and analyzed using VisiScope Confocal FRAP microscope (×40 magnification). Images were pseudocolored according to their respective emission wavelengths, overlaid and processed using Image J and Metamorph softwares.
To determine the expression of fusogenic viral glycoproteins (gB and gD) on the surface of infected PBMC, cells were infected with EHV-1^{RFP} (MOI=0.5) for 24 hr. Infected cells were fixed with 4% PFA for 20 min and stained with primary mouse antibody against EHV-1 gB and gD (dilution of 1:250) for 1 hr. Goat anti-mouse Alexa Fluor-488 was used as secondary antibody and nucleus was stained with DAPI. Cells were imaged using VisiScope Confocal FRAP microscope as described above.

Live cell imaging. PBMC were infected with EHV-$1^{RFP+GFP}$ (MOI=3) for 5 min then the cells were treated with ice-cold citrate buffer and virus neutralizing antibody. The infected cells were added to endothelial cells grown on 8-wells ibidi slide coated with collagen (Ibidi Integrated BioDiagnostics). Cells were imaged in non-phenol red DMEM cell culture media (Pan Biotech, Germany) supplemented with 20% FBS, 1% penicillin and streptomycin, and 1% non-essential amino acid. The temperature on the microscope stage was held stable during time-lapse sessions using an electronic temperature-controlled airstream incubator. The field area was chosen displaying EHV-$1^{RFP+GFP}$ viral particles (red signals) on the surface of PBMC overlaying the EC. Images were captured in time-lapse every 8 min time series using a VisiScope Confocal FRAP microscope (×20 magnification). Images and movies were generated and analyzed using ImageJ and Metamorph softwares. Long time-lapse experiments were carried out using the autofocusing function integrated into the advanced time series macro set.

Correlative fluorescent and electron microscopy. For TEM analysis, endothelial cells were grown on correlative microscopy gridded coverslips (LCMC34A, Plano GmbH, Germany). PBMC were infected with EHV-1^{RFP} (MOI=5) for 5 min then the cells were treated with ice-cold citrate buffer and virus neutralizing antibody. The infected-PBMC were overlaid on EC and incubated at 37°C for 1 or 3 hr. Cells were fixed with 4% PFA for 30 min at room temperature. Cell nuclei were counter-stained with 2µg/ml Hoechst 33342 (Thermo Fisher Scientific, Germany) in PBS for 10 min at room temperature and kept in PBS after staining. Cells covering the region of one grid square on the coverslip were imaged using a CellDiscoverer 7 microscope (Carl Zeiss, Germany) at 20x magnification. For TEM, cells were then fixed on the coverslips in 2.5% glutaraldehyde (Grade I, Sigma, Germany) freshly prepared in PBS from a 25%

stock solution (overnight at 4°C). After washing 3 times in PBS, coverslips were incubated in 0.5 % (v/v) osmium tetroxide (Science Services GmbH, Germany) in PBS for 1 hr at room temperature, washed 4 times for 20 min in distilled water, incubated for 30 min in 100 mM Hepes buffer containing 0.1 % (w/v) tannic acid (Science Services, Germany), washed 3 times for 10 min in distilled water and then again incubated in 2 % (w/v) uranyl acetate (Sigma-Aldrich, Merck, Germany) for 1.5 hr at room temperature. After washing once in distilled water, samples were then dehydrated through a series of increasing ethanol concentrations (5 min in 30 %, 10 min in 50 %, 15 min each in 70%, 90%, 96% and finally 3 times 10 min in absolute ethanol, respectively). Samples were then incubated overnight at 4°C in 100 % SPURR resin (Low Viscosity Spurr Kit, Ted Pella, CA, USA). After exchanging fresh SPURR resin 2 times (2 hr at room temperature), coverslips were mounted between two pieces of Aclar Embedding Film (Science Services, Germany) for polymerization (24 hr at 60°C). For TEM imaging, areas (comprising approximately 3x3 squares from the grid pattern) exhibiting 2-4 PBMC carrying a virus load closely attached to EC were cut out, mounted on a preformed resin dummy and embedded again in SUPRR resin in order to cut target cells with an orientation perpendicular to coverslip support. After polymerization, sample blocks were carefully trimmed close to the selected cells using a EM Trim2 specimen trimmer (Leica Microsystems, Germany) followed by cutting 70 nm sections using a UC7 ultra-microtome (Leica Microsystems, Germany) equipped with a 3 mm diamond knife (Diatome, Biel, Switzerland). Sections were placed on 3.05 mm carbon-coated Formvar copper slot grids (Plano GmbH, Germany) and post-contrasted using 2% (w/v) uranyl acetate and Reynolds lead citrate. TEM images were taken at 1100x - 6500x nominal magnification using a Tecnai Spirit transmission electron microscope (FEI) operated at 120 kV and equipped with a 4k x 4k F416 CMOS camera (Tietz Video and Image Processing Systems GmbH; TVIPS).

Statistical analyses. Statistics were performed using SPSS 23 and GraphPad Prism 7. Normally distributed data sets, determined by the Shapiro-Wilk test, were analyzed with one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Data sets that were not normally distributed were analyzed with Kruskal-Wallis test followed by Dunns test for multiple comparison tests. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

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