Supporting Information (SI)

Mechanism of protection of drug extrusion by brain capillary endothelial cells via lysosomal drug trapping and disposal by neutrophils

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Other supplementary materials for this manuscript include the following:

Movie S1

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SI Materials and Methods

Cell Culture

Human cerebral microvascular endothelial cells (hCMEC/D3 [RRID: CVCL_U985]; for authentication see Fischer et al.[1]) were kindly provided by Dr. Pierre-Oliver Couraud (Institute COCHIN, Paris, France). This cell line was developed by immortalization of primary human brain capillary endothelial cells (BCECs; obtained from neurosurgery) through co-expression of the human telomerase reverse transcriptase (hTERT) and Simian Vacuolating Virus 40 (SV40) large T antigen, via a lentiviral vector system[2]. The advantage of using hCMEC/D3 cells for the present experiments is that they model the human BBB, express a number of properties of the in situ BBB, maintain those properties reliably over several passage numbers, and mimic the changes in permeability across the BBB that occur in situ in response to leucocytes and inflammatory cytokines[3]. The doxycycline-inducible Pgp-EGFP expressing hCMEC/D3 cells (hCMEC/D3-MDR1-EGFP) used in the present experiments were previously generated by our laboratory as described before[4].

hCMEC/D3 cells (passages 15-28) were cultured in 100 mm culture dishes coated with collagen type I (100 µg/mL; Gibco/Life Technologies, Carlsbad, CA, USA). For a heterogeneous BBB phenotype, hCMEC/D3 wildtype (WT) cells ($8x10^5$) were cocultured to equal amounts with hCMEC/D3-MDR1-EGFP cells ($8x10^5$) as described recently[5]. Cells were maintained in endothelial cell basal medium-2 (EBM-2, Lonza, Cologne, Germany) supplemented with 5% fetal calf serum (FCS, PAA Laboratories, Cölbe, Germany), 1% penicillin (100 U/mL), streptomycin (100 µg/mL) (Invitrogen, Karlsruhe, Germany), 1.4 µM hydrocortisone (Sigma-Aldrich, Munich, Germany), 5µg/mL ascorbic acid (Sigma-Aldrich), 1% lipid concentrate (Invitrogen), 10 mM HEPES (Invitrogen) and 1 mg/mL basic FGF (Sigma-Aldrich). Pgp-EGFP expression was induced by culturing cells in growth medium supplemented with 1 µg/mL doxycycline (Biochrom, Berlin, Germany). As previously described[2], hCMEC/D3 cells showed a morphology and size like primary cultures of brain

endothelial cells, with monolayers of tightly packed elongated cells (see SI Appendix, Fig. S9) that exhibited contact inhibition at confluence.

For our experiments hCMEC/D3 cultures were treated with one of two fluorescent Pgp substrates, eFluxx-ID Gold acetoxymethyl ester (EFIG-AM; 2 µL/mL, according to the supplier's instructions; ENZO Life Sciences, Lörrach, Germany) or doxorubicin (10 µM; Alexis Biochemicals, San Diego, CA, USA) in phenol red free Opti-MEM medium (Invitrogen) at 37°C for 30 min. For quantification of barrier bodies, cells and barrier bodies from doxorubicin (n=4) or EFIG-AM (n=6) treated hCMEC/D3 cultures were counted on 10 randomly captured fluorescent micrographs.

The human promyelocytic leukemia cell line HL-60 was maintained as suspension culture in RPMI 1640 medium (PAA, Freiburg, Germany) supplemented with 10% FCS and 1% penicillin/streptomycin. A neutrophil-like phenotype of HL-60 cells was induced by a treatment with 1.25% DMSO for 3 days without medium change. All cells were maintained at 37°C and 5% CO_2 by serial passaging. Experiments were performed one day post-confluency if not differently indicated.

Primary cultures of porcine brain capillary endothelial cells

Primary porcine brain capillary endothelial cells (pBCECs) were isolated as described earlier[6], with slight modifications. In brief, fresh porcine brain hemispheres from *Sus scrofa domestica* (domestic pig) were kindly provided by the local slaughterhouse in Gleidingen, Hannover, Germany and stored on ice for transport. First, meninges and large blood vessels were removed from the cerebral cortex under sterile conditions. After roughly separating white from gray matter, the gray matter was minced and sequentially treated with digestion enzymes, density gradient centrifugation, filtration and erythrocyte lysing as described earlier[6]. Purified pBCECs were seeded on collagen IV-coated (Sigma-Aldrich) glass coverslips in 24-well plates or in 100 mm culture dishes at 37°C and 5% CO_2 in a density of

3.6×10^5 cells/cm^2 in medium 199 (Gibco/Life Technologies) supplemented with 10% newborn calf serum (Biochrom), 0.7 mM L-glutamine (Gibco/Life Technologies), 1% penicillin/streptomycin, and 1% gentamicin (Gibco/Life Technologies). Medium was exchanged to remove debris from attached cells after 1 h in culture. The next day 4 μg/mL puromycin (Sigma-Aldrich) was added to the cultures for the following 2 days to prevent growth of non-endothelial cells. Experiments were performed after 5-7 days in culture. Treatment of the pBCECs with the Pgp substrates EFIG-AM or doxorubicin as well as incubation with porcine neutrophils were conducted in phenol red free Opti-MEM. To determine subtoxic concentrations of doxorubicin, pBCECs were subjected to a range of different doxorubicin concentrations (10, 15, 20, 30, 40, 50 μM). After 30 min, cell viability was analyzed by light microscopy compared to controls. First signs of cell death could be observed in the supernatant of cells treated with 40 μM DOXO. A subtoxic concentration of 10 μM DOXO was chosen for all subsequent experiments.

For phase-contrast micrographs, pBCECs (or hCMEC/D3) were cultured in 100 mm culture dishes until 1-2 days post-confluency as described before. To assess cell morphology, the cell cultures were analyzed using an inverted fluorescence microscope (Olympus IX-70, Hamburg, Germany) with 10x magnification. Barrier body quantification was done by counting cells and barrier bodies in 25 randomly captured fluorescent micrographs of different pBCEC cultures treated with either doxorubicin (n=14) or EFIG-AM (n=11).

Isolation and characterization of human blood-derived neutrophils

Human neutrophils were isolated from whole blood, freshly drawn from healthy donors by venipuncture (n = 6; 3 males and 3 females; age range 26 to 40 years). Blood was obtained in agreement with the local ethical board. The neutrophil isolation was performed by density gradient centrifugation at 500 x g using PolymorphPrep (Axis-Shield, Oslo, Norway) as described previously[7]. Isolated human neutrophils were finally suspended in RPMI 1640

medium. Cell count of viable neutrophils was performed after trypan blue staining in a Neubauer chamber. The cell count was adjusted to $2x10^7$ neutrophils/mL. High purity of neutrophils was confirmed by analysis with neutrophil specific markers and flow cytometry. After isolation, neutrophils were directly used for experiments.

Isolation and purification of porcine blood-derived neutrophils

Blood collection from healthy piglets was approved by the authors' institutional review board (LAVES) and registered under no. 33.9-42502-05-18A302. Purification of porcine neutrophils was conducted from heparinized blood as previously described with Ficoll Hypaque 1077 (Biochrom, Berlin, Germany) and hypotonic lysis of erythrocytes[8]. After isolation, neutrophils were directly used for experiments with pBCECs.

Fluorescent labeling of neutrophils for confocal microscopy

Human neutrophils were labeled by nuclear staining with 5 mM bisbenzimide H (Hoechst 33342) (Sigma-Aldrich) diluted in phenol red free Opti-MEM medium for 5 min at 37°C. Alternatively neutrophils were labeled by Cell Proliferation Dye eFluor670 (eBioscience, Frankfurt, Germany) which binds to any cellular protein, containing primary amines. eFluor670 was applied as recommended by the manufacturer. Fluorescent labeled neutrophils were maintained in Opti-MEM medium at 37°C.

Western blot analysis

Brain capillary endothelial cells were solubilized on ice in a buffer containing 25 mM Tris-HCL pH 8, 50 mM NaCl, 0.5% (w/v) sodium deoxycholate (DOC) and 0.5% (w/v) Triton X-100, supplemented with complete protease inhibitor (Roche, Mannheim, Germany) (referred to as lysis buffer). Protein concentration of whole cell extracts was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Bonn, Germany) according to the

manufacturer's protocol and equal amounts of total protein were either resolved on 12% polyacrylamide gels for caspase-3 or on 8% gels for Pgp detection. Proteins were transferred to PVDF membranes that were subsequently immunostained using an anti-caspase-3 antibody (# 9662; Cell Signaling Technology, Leiden, Netherlands, 1:1000) and a secondary horseradish peroxidase (HRP)-coupled anti-rabbit antibody (#P0448; Dako, Hamburg, Germany, 1:1000) diluted in PBS-T (0.05% tween), for 1 h respectively. Pgp and β-actin were detected using an anti-Pgp antibody (C219; #SIG-38710; (Signet Laboratories, Dedham, MA, USA, 1:200) and an anti-actin antibody (#A2066; Sigma, 1:5000), respectively. Protein bands were visualized by enhanced chemiluminescence (ECL) using SuperSignal West Femto Chemiluminescent substrate (Thermo Fisher Scientific) and documented with a ChemiDoc XRS system (Bio-Rad, Munich, Germany) and Quantity One software (Bio-Rad).

Confocal fluorescence microscopy

For confocal microscopy, hCMEC/D3 or pBCECs were seeded on collagen-coated glass coverslips inserted to cell culture dishes and either fixed and immunostained or used for live cell imaging. Prior to fixation cells were treated with EFIG-AM or doxorubicin as described above and washed with PBS. Fixation was performed with 4% paraformaldehyde (PFA) in PBS for 30 min at RT, followed by permeabilization with 0.2% Triton X-100 in PBS for 30 min at RT. As indicated, some samples were fixed with aceton-methanol (1:1) for 10 min on ice, dependent on the primary antibody used for immunofluorescent staining. Blocking and incubation of the cells with primary and secondary-fluorescent labeled antibodies were performed in PBS containing 1% BSA, 0.5% saponin and 0.1% Triton X-100. Lysosomes were vizualized by staining for the lysosomal associated membrane protein-2 (LAMP-2) using a primary anti-LAMP-2/CD107b antibody (#555803; BD Biosciences, San Jose, CA, USA, 1:25), followed by incubation with an Alexa Fluor 633 secondary antibody (#A-21052; Invitrogen, 1:500) for 1 h at RT, respectively. Accordingly, indirect staining for Pgp or CD31

in pBCECs was performed by using a primary anti-Pgp (C219; # MA1-26528, 1:100) or CD31/PECAM1 antibody (#AP15436PU-M; Acris (OriGene), Herford, Germany; 1:100), respectively, and a secondary Alexa Fluor 488 antibody (#A11001; Thermo Fisher Scientific, 1:500). After washing, coverslips were finally mounted in Prolong Gold antifade (Carl Roth GmbH, Karlsruhe, Germany) including 4',6-diamidino-2-phenylindol (DAPI) as a nuclear counterstain. Prior to live cell imaging and dependent on the experiment cells were either treated with LysoTracker Blue DND-22 (Invitrogen) for labeling of lysosomes or bisbenzimide H for visualization of cell nuclei. LysoTracker Blue was dissolved in phenol red free Opti-MEM medium to a final concentration of 75 nM and added to the cells for 1 h at 37°C. Bisbenzimide H staining was performed as described above. Cells were carefully washed to remove excess LysoTracker or bisbenzimide H and incubated with Pgp substrate as described above. The glass coverslips were then inserted into a PeCon open chamber (PeCon, Erbach, Germany) and cells were maintained in fresh phenol red free Opti-MEM medium at 37°C. For neutrophil experiments $1x10^6$ eFluor670 or bisbenzimide H labeled human neutrophils were added to the cell culture. Cell cultures were examined by time-lapse imaging. Similarly, $1x10^6$ eFluor labeled porcine neutrophils were used for incubation with pBCEC cultures grown on glass coverslips in 24-well plates before removal of supernatant and fixation. All samples were examined with a Leica TCS SP5 II fluorescence microscope (Leica Microsystems, Bensheim, Germany) and an HCXPL APO 63x lambda blue 1.4 oil or 63x1.2 water immersion objective. Excitation wavelength of 405 nm (bisbenzimide H, DAPI or LysoTracker), 488 nm (Pgp-EGFP), 530 nm (EFIG), 476 nm (doxorubicin) and 633 nm (eFluor670 or secondary Alexa Fluor 633 antibody) were used. Images (1024 x 1024 pixels, frame size: 246.03 µm x 246.03 µm) were acquired using a conventional galvanometer scanner of the Leica SP5 II tandem scanning system with a scan speed of 400 Hz (line frequency) by sequential scanning of the different channels. Apart from images S1a (lower panel), c and S9f, all other images are shown in a xy plane. Images were analyzed using Leica

LAS AF software (version 2.7.3). The draw counter function in LAS AF was used to count cells and barrier bodies. Sizes of cellular structures were determined by scale bars in the images. Confocal microscopic images were acquired under consideration of image guidelines as proposed by Cromey[9]. All experiments were performed with appropriate controls ± treatment and microscopic settings (e.g. gain) were adjusted to respective negative controls to exclude background fluorescence. If applicable, brightness and contrast adjustments as well as cropping of images to draw attention to objects of interest in an image were performed. In case the signal intensity was increased, a linear enhancement was applied to the whole file. In all images no non-linear changes were made. Acquisition of images to be compared (± treatment) was performed with identical conditions (see Table S1). Additionally, images for quantification were randomly captured to reduce observers' bias. To correctly represent the variability within a sample, representative images were captured after screening an entire specimen. When treated with Pgp substrates, cells all over the sample showed barrier body formation in contrast to untreated controls (no barrier body formation). To correctly reflect the sample appearance, 5 to 25 images were acquired (see details in text and figure legends).

Fluorescence microscopy of hCMEC/D3 cultures grown on 6-well filter inserts

hCMEC/D3 cultures were grown on 6-well permeable supports (ThinCert^{TM}) from Greiner Bio-One (translucent PET membrane, 0.4 μm pore size, $1x10^8$ pores/cm^2) until day 9 of post-confluency. After substrate treatment (addition to both compartments, 30 min) and incubation with neutrophils ($4.5x10^6$ per well, 1 h, addition to upper compartment) cells were fixed with 4% paraformaldehyde. For microscopic analysis filters were cut from their inserts and mounted on glass microscopy slides, cells facing up by usage of Prolong Gold antifade reagent containing DAPI.

3D reconstruction and depth coding

In order to gain insights into the spatial localization of barrier bodies the EFIG-AM treated hCMEC/D3 cocultures were analyzed by three dimensional (3D) reconstructions. The 3D images were obtained from a maximum projection image, which was in turn created from a z-stack of 40 serial optical sections through the sample. The step size (micrometer increasement) was set to 0.42 µm. In total, a stack of 16.8 µm was analyzed. In a depth coding reconstruction of the confocal z-stack focal planes (z-height) were assigned to a color code ranging from blue (0 µm in the stack) to red (16 µm in the stack). Leica Application Suite (LASX, version 1.9.0.13747) was used for 3D reconstructions of the data stack and depth coding analysis.

Scanning electron microscopy

The hCMEC/D3 cocultures or pBCECs were seeded on collagen coated coverslips in 24-well plates and allowed to grow for 3 days (hCMEC/D3) or 5 days (pBCECs). Cells were treated with doxorubicin (10 µM) for 30 min. Untreated cells were used as control. The cells were washed with PBS and fixed with 2.5% glutaraldehyde in 1% sodium cacodylate buffer. Then the samples were dehydrated in a series of graded ethanol, dried and coated in a sputter-coater (SCD 040; Oerlikon Balzers, Balzers, Liechtenstein) with gold. A digital scanning electron microscope (DSM 940, Carl Zeiss Jena GmbH) was used for visualization.

Treatment of hCMEC/D3 cultures with cytochalasin D, nocodazole or elacridar and barrier body quantification.

hCMEC/D3 cocultures were grown on glass coverslips until 3 days post-confluency. Then, the cultures were pre-incubated with Opti-MEM either containing 10 µM cytochalasin D (CytD; 15 min, Sigma-Aldrich), 10 µM nocodazole (Noc; 30 min, Sigma-Aldrich) or 0.2 µM elacridar (30 min, Sigma-Aldrich) followed by an incubation with the respective agent in combination with either EFIG-AM or doxorubicin for another 30 min. Samples were fixed

with 4% paraformaldehyde and barrier body formation was analyzed by confocal microscopy in comparison to untreated controls (- CytD, - Noc, - Ela, + DOXO or EFIG-AM). Barrier body formation was quantified by counting barrier bodies and cells on 5-8 randomly captured images. Counting bias was reduced as much as possible by ensuring the microscope counts were done blind to the treatment of the samples. A total of 785-1008 cells were counted per treatment (Ctr-1: 785 cells [157 ± 11 cells per image, n = 5 images]; CytD: 911 [114 ± 10, n = 8]; Noc: 862 [123 ± 8, n = 7]; Ctr-2: 1008 [168 ± 10, n = 6]; Ela: 911 [130 ± 9, n =7]. Barrier bodies were identified by the following morphological criteria and a specific staining: vesicular aggregates on the cell layer exhibiting green (Pgp-EGFP) and red (doxorubicin or EFIG) but no blue (DAPI, DNA) fluorescence. A tubulin staining in cultures ± Noc was conducted with an anti-tubulin-antibody (#T6793; Sigma-Aldrich, 1:500) followed by incubation with a secondary Alexa Fluor 568 antibody (#A21124; Thermo Fisher Scientific, 1:500). Staining of F-actin in cultures ± CytD was performed with Alexa Fluor 568 phalloidin (#A12380; Thermo Fisher Scientific, 1:100).

Isolation of extracellular Pgp/EFIG positive vesicles by differential centrifugation and cell sorting

Cocultures of hCMEC/D3-MDR1-EGFP cells and hCMEC/D3 WT cells were grown on collagen type I coated cell culture dishes (100 mm), in the presence of doxycycline as described above. Prior to seeding hCMEC/D3 WT cells were labeled with eFluor670 to differentiate if extracellular Pgp/EFIG vesicles originate from WT or Pgp-EGFP overexpressing cells. Cellular DNA of hCMEC/D3 cells were stained with bisbenzimide H as described above, allowing evaluating a possible apoptotic origin, by presence or absence of genetic material within the isolated extracellular vesicles. Cocultures were treated with EFIG-AM for 30 min at 37°C as described. The substrate containing supernatant was discarded and cells were detached using trypsin/EDTA (Biochrom). Differential centrifugation was

performed to get rid of unwanted material: 500 x g for 10 min at RT for elimination of viable cells, 2000 x g for 20 min at 4°C to remove cell debris and bulky apoptotic bodies and finally 10,000 x g for 30 min at 4°C for pelleting of extracellular target vesicles. The pellet of the 10,000 x g fraction was resuspended in 1 mL PBS and vesicles were isolated at a FACSAria IIu cell sorter (Becton Dickinson, Heidelberg, Germany). Vesicles were analyzed with the parameters forward scatter (FSC) and side scatter (SSC), bisbenzimide H (DAPI channel), EFIG (PE channel), eFluor670 (APC channel) and EGFP (FITC channel). Vesicles positive for Pgp-EGFP and EFIG and negative for bisbenzimide H were collected in PBS.

Flow cytometric analysis

Uptake of Pgp-EGFP and the Pgp substrate EFIG by human primary neutrophils was analyzed using flow cytometry. Cocultures of hCMEC/D3-MDR1-EGFP cells and hCMEC/D3 WT cells were grown in 6-well culture dishes and treated with EFIG-AM as before. Freshly isolated neutrophils were labeled with eFluor670 and subsequently incubated with hCMEC/D3 cocultures at a cell density of $2.7x10^6$ neutrophils per well, supplied with Opti-MEM medium. Control neutrophils were maintained in a cell incubator without any contact to other cells. After 24 h supernatants from cocultures, containing the neutrophils, were collected. Neutrophils were centrifuged at 500 x g, resuspended in 100 µl PBS and analyzed by a flow cytometer (FACSCanto; BD Biosciences, Heidelberg, Germany) compared to the controls. Cells were measured with the parameters forward scatter (FSC) and side scatter (SSC), EFIG (PE channel), eFluor670 (APC channel) and EGFP (FITC channel). All experiments were performed in triplicate with the measurement of a minimum of 10,000 individual events.

Dot blotting

Pgp/EFIG positive vesicles isolated by differential centrifugation and FACS were further

characterized by dot blot assay for LAMP-2, Pgp, Rho A, flotillin-2 and actin. As described above, LAMP-2 was used as a lysosomal marker; Rho A was shown to be involved in vesicle shedding at the cellular outer plasma membrane[10] and lipid rafts are described to be present in extracellular vesicle biogenesis[11]. Isolated vesicles were solubilized in the lysis buffer described above and 2 µg of the lysate was applied to a nitrocellulose membrane (Whatman, Dassel, Germany). Membranes were blocked with 5% milk in PBS-T for 1 h. After blocking, membranes were incubated with primary antibodies anti-Pgp (C219; #SIG-38710; Signet Laboratories, 1:200), anti-LAMP-2/CD107b (#PA 1-655; Thermo Fisher Scientific, 1:1000), anti-Rho A (26C4; #sc-418; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:1000), anti-flotillin-2 (B-6; #sc-28320; Santa Cruz Biotechnology; 1:1000) or anti-actin antibody (#A2066; Sigma, 1:1000) for 1 h. Subsequently membranes were incubated with HRP-coupled anti-rabbit (#P0448; Dako, Hamburg, Germany, 1:1000) or anti-mouse (#P0260; Dako; 1:1000) antibodies for 45 min. Membranes were developed using SuperSignal West Femto Chemiluminescent substrate (Thermo Fisher Scientific) and visualized by ChemiDoc XRS system and Quantity One software.

LL-37 and IL-8 ELISA from cell culture supernatant

hCMEC/D3 cocultures exposed to doxorubicin or EFIG-AM (as described above) were grown in 100 mm cell culture dishes and cell culture supernatant was analyzed for LL-37 or interleukin-8 (IL-8) by a LL-37 ELISA (Human LL-37 ELISA kit, Hycult Biotech, Uden, The Netherlands) or a human IL-8 coated ELISA kit (Thermo Fisher Scientific) according to the manufacturer's instructions in triplicates. The release of LL-37 or IL-8 into the supernatant was measured over a period of 30 min.

Statistics

Quantification of barrier bodies in images of hCMEC/D3 and pBCEC cell cultures was

independently performed by two investigators in a blinded fashion. Differences between groups were calculated by one-way ANOVA and Dunnett's multiple comparison test. P-values of $P<0.05$ were considered significant. For all statistical analysis, the Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA) was used.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Supplemental figures

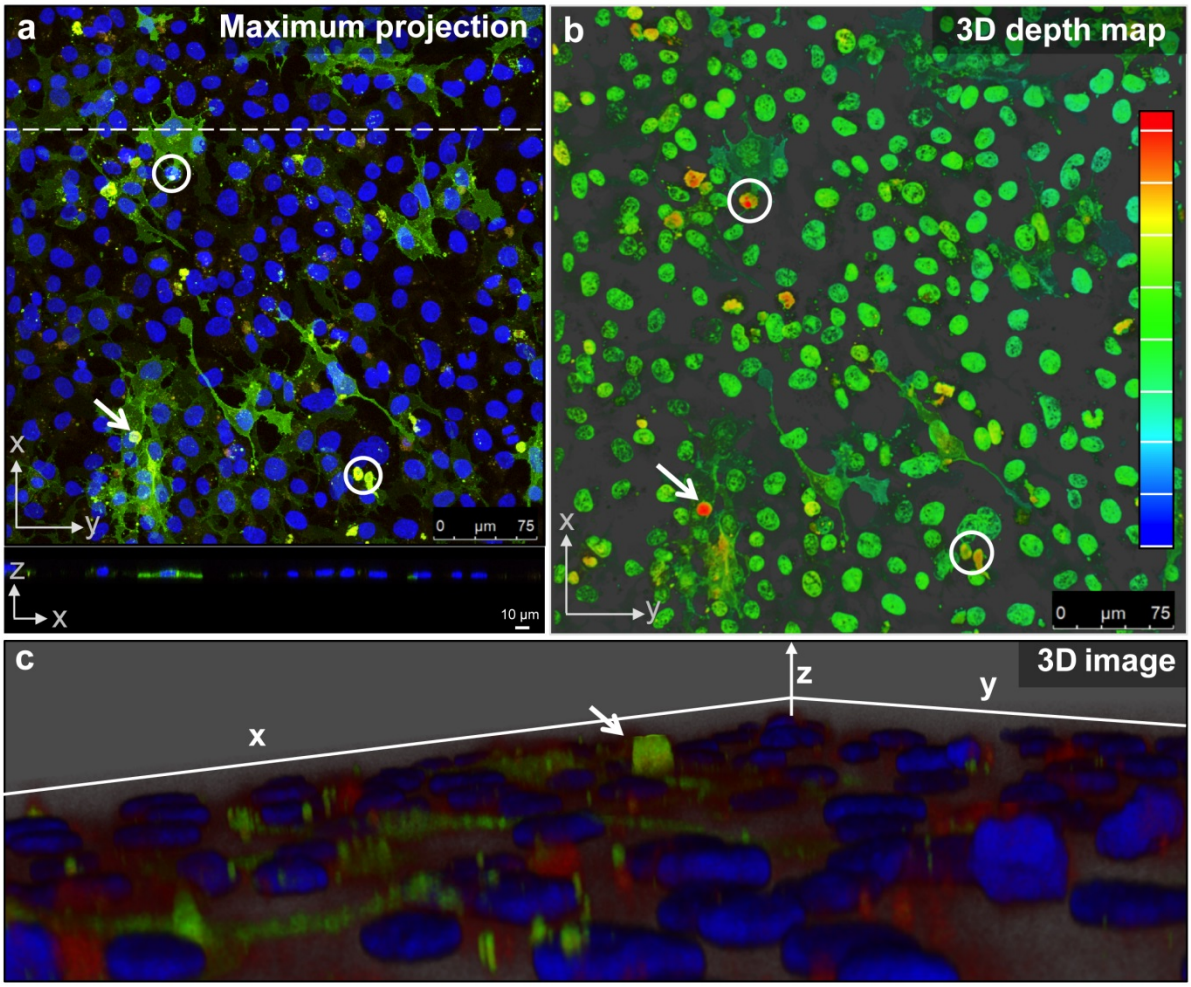

Fig. S1. Barrier bodies at the upper cell surface of brain capillary endothelial cells. hCMEC/D3 cocultures were grown on coverslips to equal amounts in the presence of doxycycline (see Methods). After treatment with eFluxx-ID Gold acetoxymethyl ester (EFIG-AM; 30 min), cells were fixed by acetone-methanol and coverslips were embedded in DAPI containing mounting medium for nuclear counterstain (blue). Samples were analyzed at a confocal fluorescence microscope by serial optical z-sectioning, taking a total of 40 images from various focal planes. (a) Barrier bodies are detected in the maximum projection (xy view) as yellow fluorescent structures (colocalization of EFIG and Pgp-EGFP). Representatively barrier bodies are highlighted by white circles. The lower panel shows an xz view of the cells. Position of the xz scan is indicated by a dashed line in the upper image. (b) Depth coding reconstruction (in z-height) of the confocal z-stack revealed an elevated

localization of the barrier bodies on the upper surface of the cells. Z-height difference is assigned to a color code ranging from 0 µm (blue) to 16 µm (red) allowing to recognize the spatial localization of barrier bodies within the stack. A representative barrier body is indicated by an arrow. (c) The 3D reconstruction and rotation of the data stack (x: 83.90°, y: -36.75°, z: -171.25°; distance: 0.21) shows a magnification of the selected barrier body on top of the cell layer.

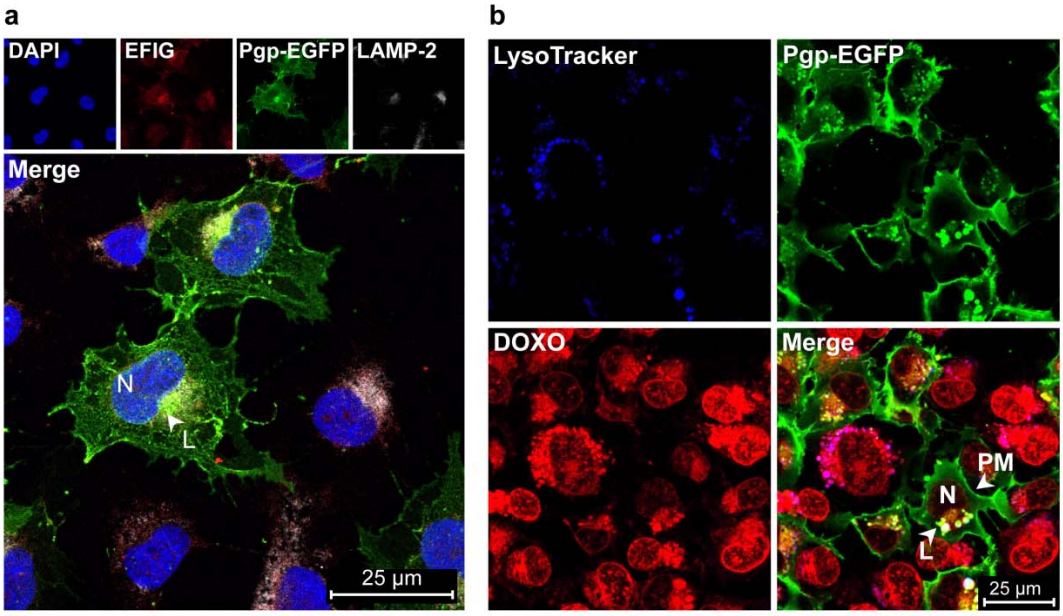

Fig. S2. Identification of intracellular Pgp/Pgp substrate vesicles as lysosomes.

hCMEC/D3 cocultures were either treated with (a) EFIG-AM or (b) DOXO. (a) Cells were fixed with PFA and lysosomes were visualized by indirect immunofluorescence using a primary antibody against LAMP-2 (gray). EFIG sequesters within Pgp-enriched lysosomes (L). (b) hCMEC/D3 lysosomes were labeled by LysoTracker treatment (75 nM, 1 h), afterwards cells were incubated with DOXO and analyzed via live cell imaging and confocal microscopy. DOXO localizes with Pgp-EGFP in lysosomes. PM: Plasma membrane, L: Lysosome, N: Nucleus.

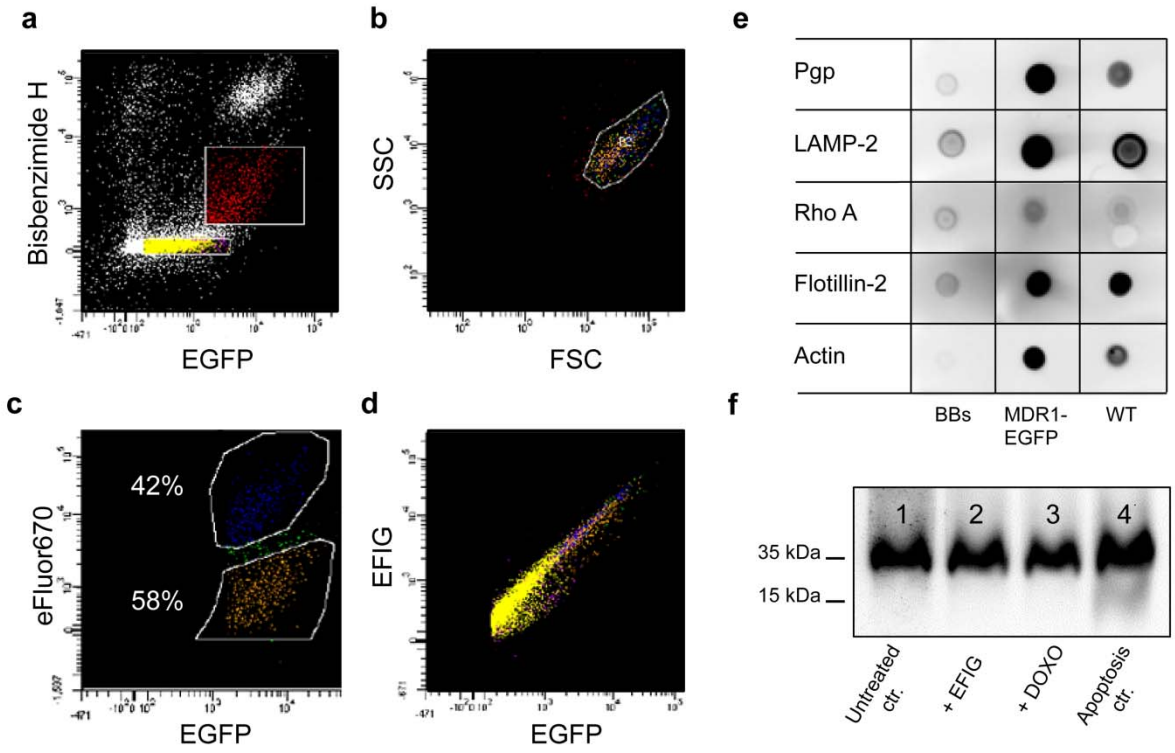

Fig. S3. Isolated barrier bodies are positive for lysosomal marker LAMP-2 and not a consequence of apoptosis. a-e: Single barrier body vesicles were isolated from EFIG-AM treated hCMEC/D3 cocultures by differential centrifugation and FACS analysis of the 10,000 x g fraction for Pgp-EGFP/EFIG positive and bisbenzimide H negative vesicles. Before the experiment bisbenzimide H was added to the culture medium for fluorescence staining of DNA. Additionally, prior to coculturing WT cells were eFluor670 labeled to differentiate from which cell type barrier bodies originate. (a) Scatter plot of bisbenzimide H versus Pgp-EGFP fluorescence indicating the total number of events. Clearly bisbenzimide negative vesicles (lower gate) could be differentiated from strong EGFP positive vesicles with a diffuse bisbenzimide H signal (upper gate). This population in the upper gate was selected according to barrier body characteristics defined before by microscopy. Another ungated population in the upper right corner of the plot (EGFP highly positive and bisbenzimide H highly positive) likely corresponds to smaller apoptotic bodies that were not excluded by centrifugation. This

population was not further investigated. (b) Analysis of the barrier body population (upper EGFP-positive gate in a) for FSC/SSC properties revealed a morphologically homogenous population of vesicles. (c) A scatter plot of this population for eFluor670 versus Pgp-EGFP revealed the cellular origin of these vesicles (WT origin: 42%, Pgp-EGFP transfected cell origin: 58%). (d) Vesicles from the morphologically homogeneous population had strong signal for both Pgp-EGFP and EFIG. Scatter plots are representative of five independent preparations. Fluorescence filters were compensated with appropriate controls. (e) For subsequent barrier body (BB) characterization by protein blotting only vesicles from the morphologically homogenous population (SSC/FSC in b) with a strong signal for EGFP and EFIG were used. The vesicles were solubilized in lysis buffer and 2 µg of lysates were applied for dot blotting with the indicated protein markers. Lysates of hCMEC/D3 WT and hCMEC/D3-MDR1-EGFP (MDR1-EGFP) cells served as controls. FSC: Forward scatter, SSC: Side scatter. (f) Analysis for caspase-3 dependent apoptosis in brain capillary endothelial cells after treatment with EFIG-AM or doxorubicin (DOXO). Western blot analysis of lysates from cocultured hCMEC/D3 cells, for caspase-3 cleavage as apoptosis marker is shown. Cocultures are untreated or treated with 10 µM doxorubicin (DOXO) or EFIG-AM for 30 min (according to the manufacturer's instructions). Cell lysate of a hCMEC/D3 coculture treated with 20 µM DOXO for 24 hours was used as apoptosis control. Detection of the 17 kDa fragment of caspase-3 resulting from cleavage indicates apoptosis (lane 4). In cocultures treated with DOXO or EFIG-AM for 30 minutes (lanes 2, 3) no apoptosis is detectable as only the full length caspase-3 (35 kDa) protein band is visible. For Western blot analysis 30 µg of total protein of each sample was subjected to SDS-PAGE.

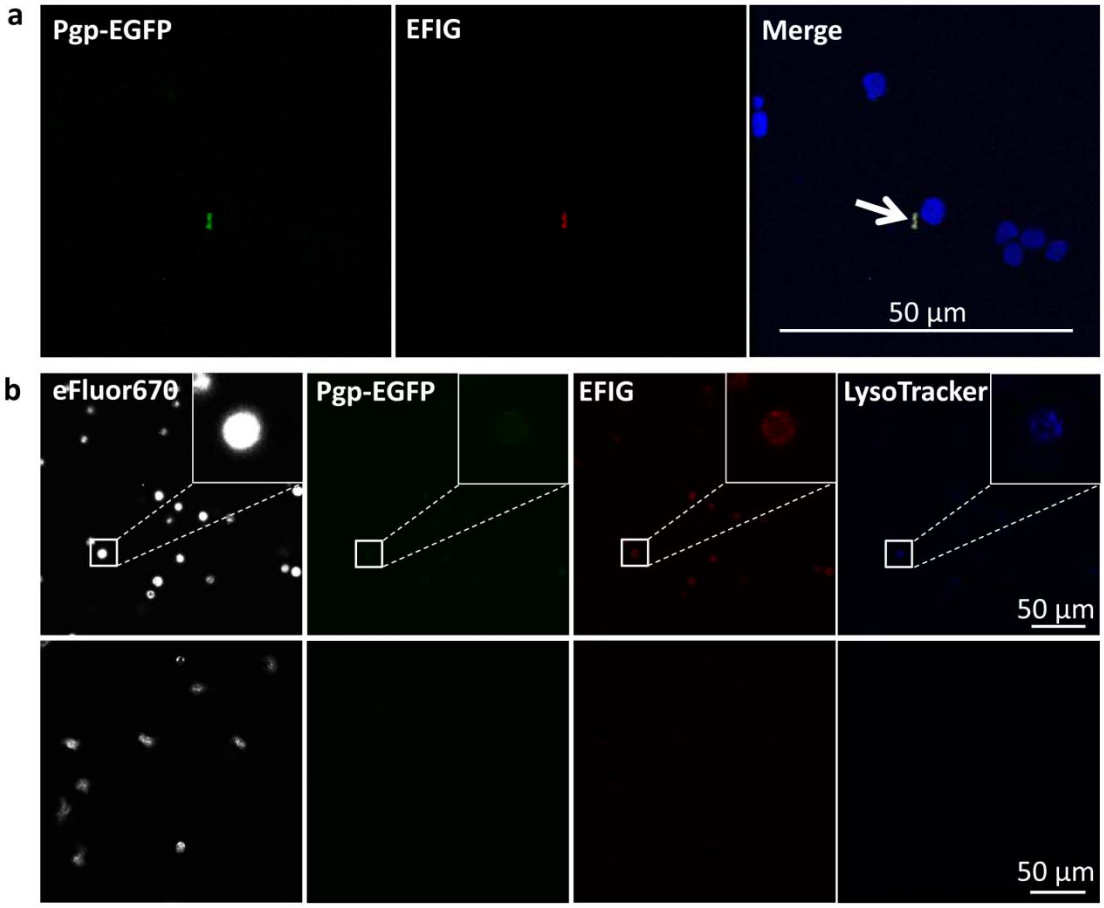

Fig. S4. Ingestion of isolated barrier bodies by HL-60 cells and human neutrophils. Barrier bodies were isolated from EFIG-AM treated hCMEC/D3 cocultures by differential centrifugation and cell sorting. (a) Subsequently, isolated barrier bodies were incubated with nuclear stained (blue) HL-60 cells, which were differentiated to a phagocytic phenotype. After an incubation period of 1 h at 37°C cells were carefully washed and examined. Confocal live cell imaging showed a perinuclear localization of isolated barrier bodies (positive for Pgp: green, EFIG: red) (arrow), indicating an ingestion of vesicles. (b) Additionally, isolated barrier bodies were incubated with eFluor670 labeled (white) primary neutrophils and analyzed at a confocal microscope. Fluorescence signals that were used for sorting of barrier bodies (Pgp-EGFP: green, EFIG: red) could be retrieved in neutrophil cells, preferably in punctuate patterns (magnification, white frames). Furthermore, a blue fluorescence signal of neutrophils was visible when hCMEC/D3 cocultures were treated with LysoTracker (blue; 75

nM, 1 h) prior to barrier body isolation (upper panel). Lower panel (negative control): In the absence of barrier bodies, no autofluorescence of eFluor670 labeled neutrophils was observed, using excitations of 488 nm (Pgp-EGFP), 530 nm (EFIG), 405 nm (LysoTracker).

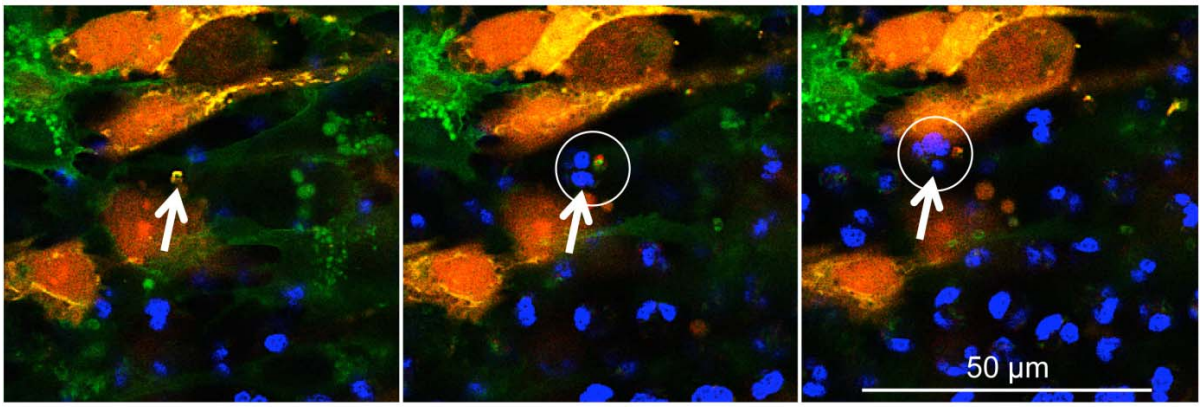

Fig. S5. Pgp/EFIG-vesicle ingestion by a neutrophil. Cocultures of hCMEC/D3 cells were treated with EFIG-AM. Primary neutrophils were nuclear stained by bisbenzimide H and incubated with hCMEC/D3 cocultures at 37°C. Time-lapse imaging was performed at a confocal fluorescence microscope to examine uptake of extracellular vesicles by neutrophils. The three images were taken at 5, 16, and 22 min after adding the neutrophils to the coculture. Over time neutrophils showed an increase in punctuate structures, characterized by a Pgp-EGFP (green) and an EFIG (red) fluorescence signal. Furthermore, the ingestion of extracellular vesicles by a single neutrophil is indicated (arrow). The migrating neutrophil (visible by nuclear stain; blue) that ingests the extracellular vesicle is marked by a white circle.

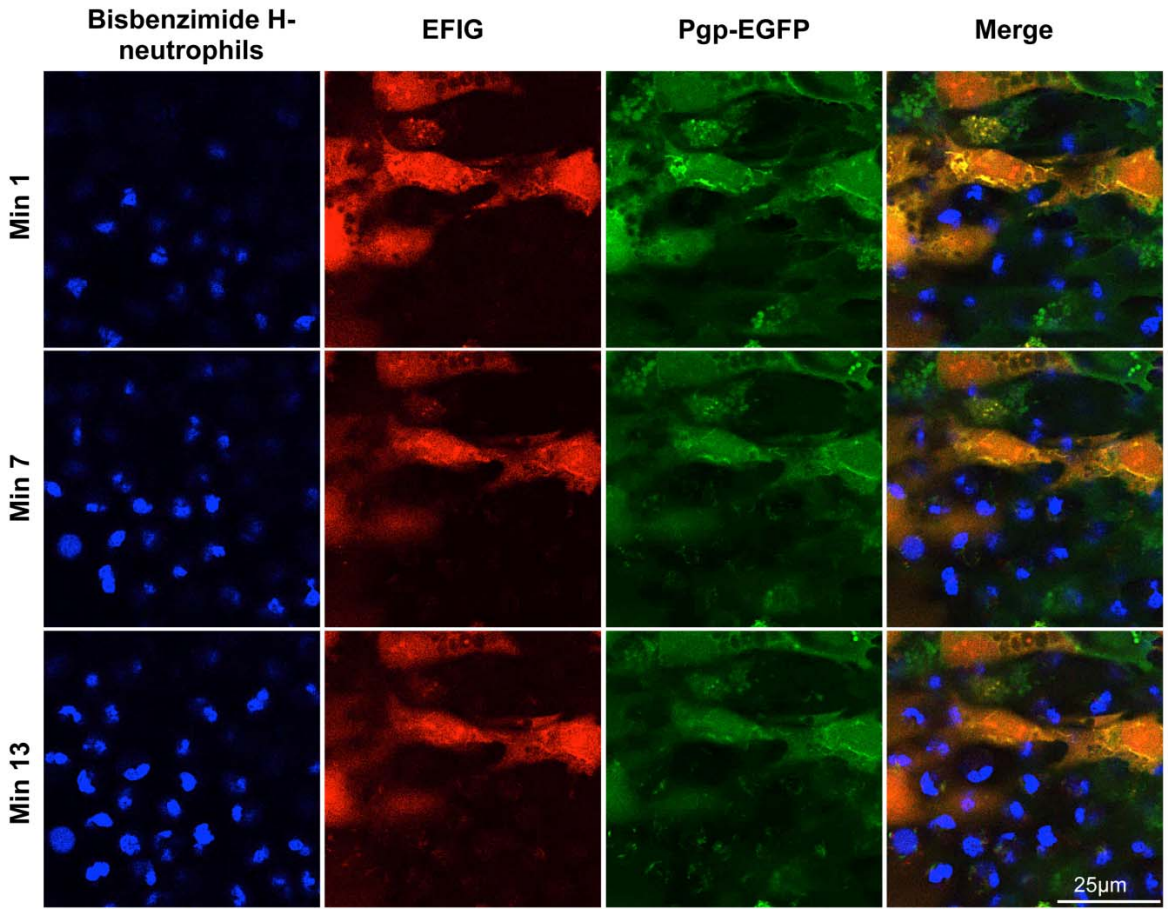

Fig. S6. Ingestion of barrier bodies by nuclear stained neutrophils incubated with brain capillary endothelial cells. Primary neutrophils were visualized by nuclear bisbenzimide H staining (blue), alternatively to eFluor670 staining that labels whole cytoplasm. Subsequently, neutrophils were incubated with EFIG-AM-treated hCMEC/D3 cocultures. Time-lapse imaging with a confocal microscope revealed uptake of yellow fluorescent vesicular structures, containing Pgp-EGFP (green) and Pgp-substrate EFIG (red) by neutrophils. These structures localize in a perinuclear manner (merged images) and increase in number over time.

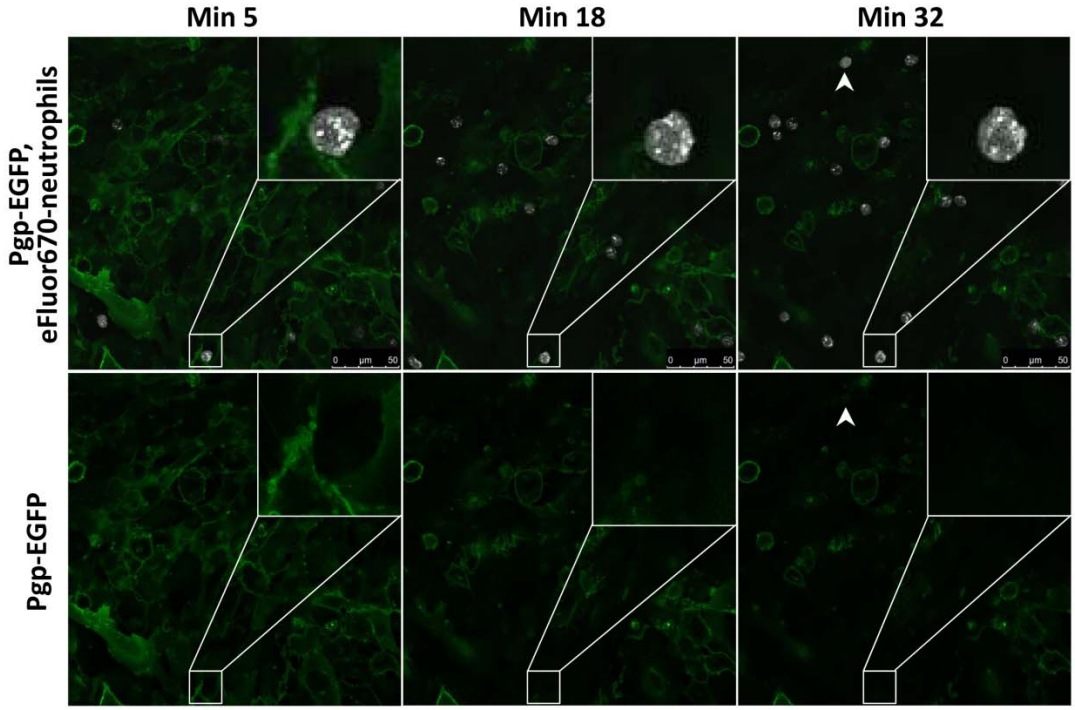

Fig. S7. Lack of Pgp uptake by neutrophils without exposure of brain capillary endothelial cells to Pgp substrate. Examination of possible Pgp-EGFP (green) uptake by human eFluor670 labeled neutrophils (white) incubated with hCMEC/D3-MDR1-EGFP cells in absence of Pgp substrate. Analysis was performed by live cell imaging and confocal microscopy at 37°C. Images are at indicated timepoints after coculturing with neutrophils. Absent green fluorescent signal of neutrophils indicate lack of Pgp uptake. Only one neutrophil showed a weak green coloration (arrow).

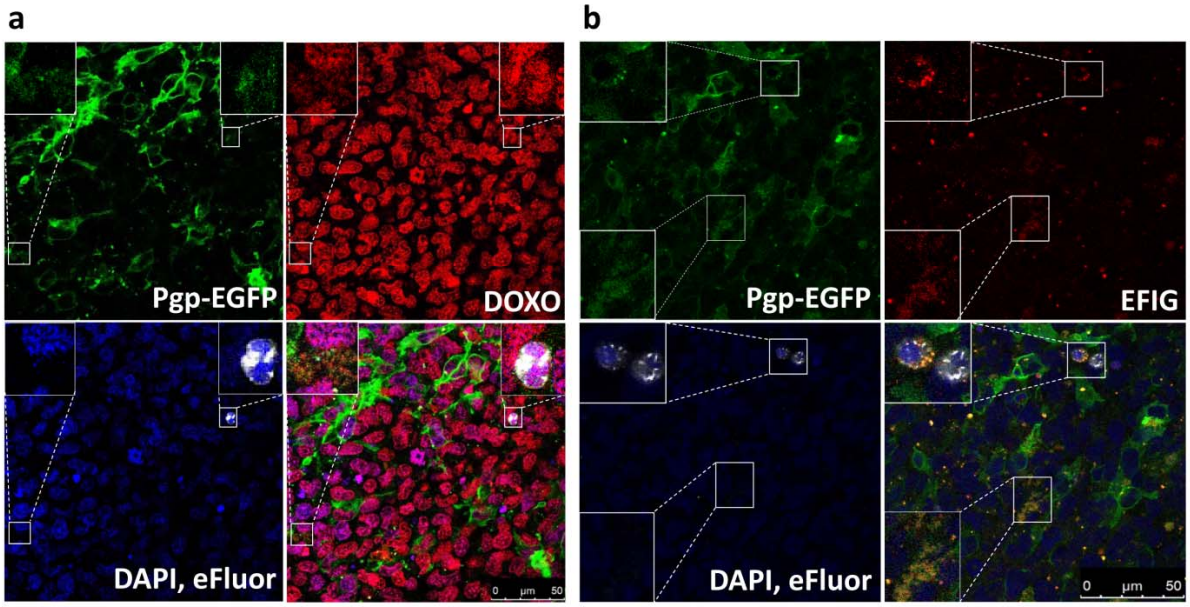

Fig. S8. Barrier body formation by post-confluent hCMEC/D3 monolayers grown on filter inserts and uptake by human neutrophils. hCMEC/D3 cocultures were grown on 6-well filter inserts until 9 days post-confluency. After treatment with either EFIG-AM (30 min) or DOXO (10 µM, 30 min) freshly isolated and eFluor670-labeled human neutrophils were incubated with hCMEC cells in the apical compartment (1 h, 37 C°). Cells were fixed with paraformaldehyde; filters were cut from the inserts and mounted with Prolong Gold antifade reagent with DAPI (blue). Samples were subsequently analyzed by confocal microscopy. (a) Confocal micrograph of DOXO (red) treated cultures incubated with eFluor670-labeled neutrophils (white). Part of the neutrophil population showed staining for Pgp-EGFP (green) and DOXO. Magnified image of a representative Pgp-EGFP- and DOXO-positive neutrophil is shown in the upper right corner, magnification of a barrier body in the upper left corner. (b) Similarly, some of the neutrophils incubated with EFIG-AM treated cultures showed a punctate staining for EFIG (red) and Pgp-EGFP. A magnified barrier body is shown in the lower left corner.

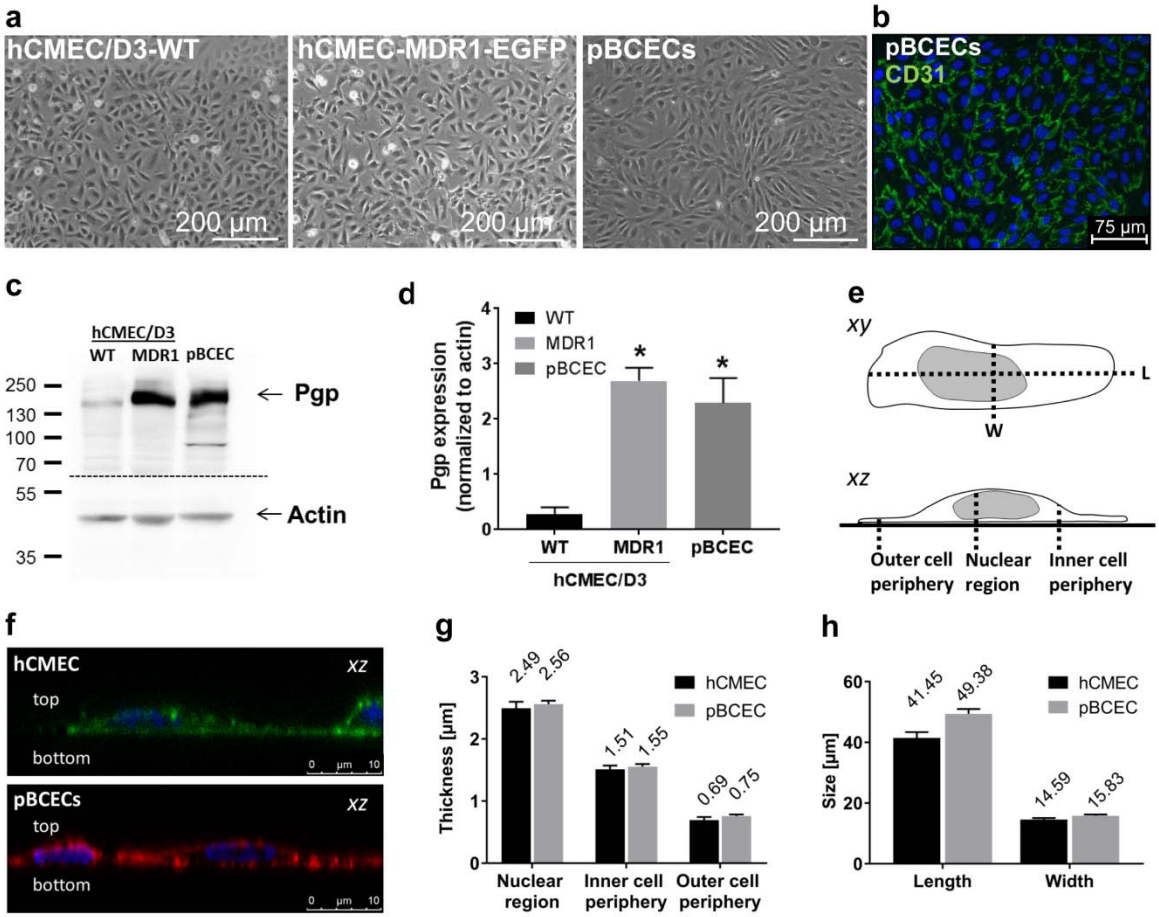

Fig. S9. Morphological characterization of hCMEC and pBCECs and comparison of Pgp expression. (a) Phase contrast micrographs of hCMEC/D3-WT, hCMEC/D3-MDR1-EGFP and primary pBCEC cultures 1-2 days post-confluency. All 3 cell types show elongated cell morphology. (b) Purity of pBCEC isolation was evaluated by fluorescent staining for the endothelial cell marker CD31 (green). Confocal fluorescent microscopy was performed after 6 days of culturing on glass coverslips followed by paraformaldehyde fixation. Cell nuclei were counterstained with DAPI (blue). Green staining of the cells for CD31 demonstrates a high purity of the culture for endothelial cells. (c) Representative Western blot showing Pgp expression in hCMEC/D3-WT, hCMEC/D3-MDR1-EGFP and pBCEC cultures. Cells were solubilized in lysis buffer and 30 µg of total protein was subjected to SDS-PAGE (8% stacking gel). β-actin was used as a loading control. (d) Quantification of Western blot bands

and normalization of Pgp expression to β-actin. Comparable Pgp expression in hCMEC/D3-MDR1-EGFP and primary pBCEC cultures and about 9-fold lower Pgp expression in hCMEC/D3-WT cells that only express endogenous Pgp. pBCEC cells were 5 days in culture at time of Pgp determination, at which Pgp expression is ~70% lower than in freshly isolated pBCECs[12]. Data are represented as mean ± SEM of n=3 independent experiments. A one-way ANOVA was used for statistical analyses. *P<0.05 was considered statistically significant. (e) Schematic drawing depicting xz and xy view of a cell. Cell dimensions for comparison between hCMEC and pBCEC cultures are labeled. W: width, L: length, xy: top view of a cell, xz: side view of a cell. (f) Confocal xz-scan of hCMEC cocultures and primary pBCECs 3-5 days after seeding on glass coverslips and fixation with paraformaldehyde. hCMEC cocultures were visualized by Pgp staining (green, upper panel) and pBCECs were stained with phalloidin against F-actin (red, lower panel). Cell nuclei were counterstained with DAPI (blue). (g) Thickness of hCMEC and pBCECs was determined by confocal optical xz sectioning of Pgp and F-actin stained cultures. The height of ≥40 cells was measured in 3 different cellular regions (nuclear region, inner and outer cell periphery), respectively. In total, ≥25 microscopic images of hCMEC and pBCEC cultures were analyzed. Both hCMEC and pBCECs showed a comparable cell thickness, highest in the nuclear regions and lowest in the cell periphery (means are displayed above the bars). Note that cell shape in culture may change depending on cell confluency. (h) Length and width of hCMEC and pBCECs was measured in ≥25 xy scans of cultures stained for Pgp and F-actin. Dimensions were measured in ≥40 cells, respectively.

Legend for Supporting Movie 1

Movie S1.Time-lapse imaging of neutrophils incubated with EFIG-AM-treated brain capillary endothelial cells. Lysosomes of cocultured hCMEC/D3-MDR1-EGFP and hCMEC/D3 WT cells were labeled by LysoTracker (blue) and cells were subsequently treated with EFIG-AM (EFIG; red) after excess LysoTracker was removed by washing. The cocultures were then incubated with freshly isolated blood-derived human neutrophils that were labeled by eFluor670 (white) before. The triple-culture was examined with a confocal microscope and time-lapse imaging at 37°C. The initially white eFluor670 labeled neutrophils show over time a punctate colocalizing staining for Pgp and EFIG as well as for LysoTracker. The neutrophil highlighted by arrow 1 is shown as detailed view in Fig. 7. Neutrophil extend pseudopods towards surface of hCMEC/D3 cell likely scanning for target antigens (arrow 2).

Table S1. Microscope settings for acquisition of confocal laser scanning images.

Fig.	Sequential mode	Zoom	Scan mode	Scan speed	Pinhole (airy)	PMT
1	yes	1	xyz	400 Hz	1	PMT1 (blue, bisbenzimide H), collected emission range: 427 nm-469 nm, Gain: 885, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 501nm-540 nm, Gain: 1050, Offset:-5 PMT3 (red, EFIG), collected emission range: 542 nm-557 nm, Gain: 915, Offset: 0 PMT4 (white, eFluor670), collected emission range: 568 nm-607 nm, Gain: 1018, Offset: 0
2	yes	1	xyz	400 Hz	1	PMT1 (blue, bisbenzimide H), collected emission range: 412 nm-480 nm, Gain: 774, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 788, Offset:-2 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 915, Offset: 0
3a	yes	1.5	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 825, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-527 nm, Gain: 800, Offset: 0 PMT3 (white, LAMP-2), collected emission range: 640 nm-695 nm, Gain: 708, Offset: 0
3b	yes	1.0	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 414 nm-474 nm, Gain: 774, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 788, Offset:-2
3c	yes	1.2	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 731, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-547 nm, Gain: 781, Offset: 0 PMT3 (red, EFIG), collected emission range: 560 nm-630 nm, Gain: 808, Offset: 0
3d	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 825, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 800, Offset: 0 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 709, Offset: 0
5	yes	1	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 412 nm-480 nm, Gain: 911, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-597 nm, Gain: 839, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0
6a1	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 760, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 869, Offset: -2 PMT3 (red, tubulin; Alexa Fluor 568), collected emission range: 570 nm-620 nm, Gain: 729, Offset: -3
6a2	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 760, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 869, Offset: -2 PMT3 (red, tubulin; Alexa Fluor 568), collected emission range: 570 nm-620 nm, Gain: 729, Offset: -3
6a3	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 824, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-533 nm, Gain: 816, Offset: 0 PMT3 (red, actin; Alexa Fluor 568 phalloidin), collected emission range: 570 nm-630 nm, Gain: 863, Offset: 0
6a4	yes	1	xyz	400 Hz	29 1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 761, Offset: 0

						PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-533 nm, Gain: 915, Offset: 0 PMT3 (red, actin; phalloidin 568), collected emission range: 570 nm-630 nm, Gain: 825, Offset: 0
6b1	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 646, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 781, Offset: 0 PMT3 (DOXO), collected emission range: 570 nm-630 nm, Gain: 734, Offset: 0
6b2	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 646, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 781, Offset: 0 PMT3 (DOXO), collected emission range: 570 nm-630 nm, Gain: 734, Offset: 0
6b3	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 646, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 781, Offset: 0 PMT3 (DOXO), collected emission range: 570 nm-630 nm, Gain: 734, Offset: 0
7	yes	1	xyt	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 412 nm-480 nm, Gain: 911, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 710, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0 PMT4 (white, eFluor670), collected emission range: 655 nm-695 nm, Gain: 721, Offset: 0
9a	yes	1	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 412 nm-480 nm, Gain: 873, Offset: 0 PMT2 (green, Pgp; Alexa Fluor 488), collected emission range: 495 nm-535 nm, Gain: 922, Offset: 0 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 868, Offset: 0 PMT4 (white, eFluor670), collected emission range: 640 nm-695 nm, Gain: 708, Offset: 0
9b	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 561, Offset: 0 PMT2 (green, Pgp; Alexa Fluor 488), collected emission range: 495 nm-535 nm, Gain: 912, Offset: 0 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 868, Offset: 0 PMT4 (white, eFluor670), collected emission range: 640 nm-695 nm, Gain: 708, Offset: 0
9c	yes	1	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 412 nm-480 nm, Gain: 832, Offset: 0 PMT2 (green, Pgp; Alexa Fluor 488), collected emission range: 495 nm-557 nm, Gain: 934, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0 PMT4 (white, eFluor670), collected emission range: 655 nm-695 nm, Gain: 756, Offset: 0
S1 (Maximum projection out of 40 z-stacks; micrometer increasement 0.42 µm)	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 427 nm-469 nm, Gain: 752, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 496 nm-532 nm, Gain: 965, Offset:-5 PMT3 (red, EFIG), collected emission range: 535 nm-568 nm, Gain: 844, Offset: 0 PMT4 (white, eFluor670), collected emission range: 650 nm-696 nm, Gain: 747, Offset: 0
S2a	yes	2	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 635, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-539 nm, Gain: 781, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0

						PMT4 (white, LAMP-2), collected emission range: 640 nm-695 nm, Gain: 708, Offset: 0
S2b	yes	2.4	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 414 nm-474 nm, Gain: 774, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 788, Offset: -2 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 814, Offset: -3
S4a	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 427 nm-469 nm, Gain: 1250, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 496 nm-532 nm, Gain: 813, Offset: -5 PMT3 (red, EFIG), collected emission range: 535 nm-568 nm, Gain: 1051, Offset: 0
S4b upper and lower panel	yes	1	xyz	400 Hz	1	PMT1 (blue, LysoTracker), collected emission range: 412 nm-480 nm, Gain: 683, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 710, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0 PMT4 (white, eFluor670), collected emission range: 655 nm-695 nm, Gain: 708, Offset: 0
S5, S6	yes	1	xyt	400 Hz	1	PMT1 (blue, bisbenzimide H), collected emission range: 412 nm-480 nm, Gain: 683, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 710, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 733, Offset: 0
S7	yes	1	xyt	400 Hz	1	PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 710, Offset: 0 PMT4 (white, eFluor670), collected emission range: 655 nm-695 nm, Gain: 721, Offset: 0
S8a	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 746, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 918, Offset: 0 PMT3 (red, DOXO), collected emission range: 570 nm-620 nm, Gain: 863, Offset: 0 PMT4 (white, eFluor670), collected emission range: 640 nm-695 nm, Gain: 638, Offset: 0
S8b	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 683, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-557 nm, Gain: 767, Offset: 0 PMT3 (red, EFIG), collected emission range: 570 nm-630 nm, Gain: 773, Offset: 0 PMT4 (white, eFluor670), collected emission range: 655 nm-695 nm, Gain: 584, Offset: 0
S9b	yes	1	xyz	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 777, Offset: 0 PMT2 (green, CD31), collected emission range: 495 nm-535 nm, Gain: 973, Offset: 0
S9f, upper panel	yes	4.9	xzy	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 701, Offset: 0 PMT2 (green, Pgp-EGFP), collected emission range: 495 nm-535 nm, Gain: 1026, Offset: -2
S9f, lower panel	yes	4.9	xzy	400 Hz	1	PMT1 (blue, DAPI), collected emission range: 412 nm-480 nm, Gain: 609, Offset: 0 PMT2 (red, Phalloidin-568), collected emission range: 570 nm-630 nm, Gain: 726, Offset: 0