ELECTRONIC SUPPORTING INFORMATION (ESI)

H-Ferritin Nanoparticle-Mediated Delivery of Antibodies across a BBB in Vitro Model for Treatment of Brain Malignancies†

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Supplementary Materials and Methods

HFn nanocage design: HFn was produced as recombinant protein made of only H-chain subunits in E. coli BL21(DE3)/pET30b and purified following our established protocol.[1]

Cell cultures: U-87 MG and T98 cell lines were used as EGFR+ models of glioblastoma primary brain cancer, while SKBR3 cell line was used as $HER2^+$ model of metastatic breast cancer. Rat brain microvascular endothelial cells (RBMEC) and human cerebral microvascular endothelial cells (hCMEC/D3) were used as representative rat and human BBB models, respectively. U-87 MG and T98 were cultured in Dulbecco's Modified Eagle's Medium (DMEM), whereas SKBR3 cells were cultured in 50% DMEM and 50% Ham's F12. Both media were supplemented with 10% FBS, 2 mM L-glutamine, penicillin (50 UI mL^{-1}) and streptomycin (50 µg mL^{-1}). RBMEC cells were purchase from Innoprot (Spain) and sub-cultured in ECM supplemented with 5% FBS, 1% endothelial cell growth supplement, 1% penicillin–streptomycin (Innoprot). hCMEC/D3 cells were provided by Dr S. Bourdoulous (Institut Cochin, Inserm, Paris, France) and grown on tissue culture flasks, pre-treated with rat tail collagen type I (0.05 mg/ml). hCMEC/D3 cells were cultured in EBM-2 medium (Lonza, Basel, Switzerland) supplemented with 5% FBS, 1% penicillin–streptomycin (P/S), 1.4 µM hydrocortisone, 5 µg mL^{-1} ascorbic acid, 1% chemically defined lipid concentrate (CDLC), 10 mM HEPES and 1 ng mL^{-1} basal FGF. All cell lines were maintained at 37 °C in humidified atmosphere containing 5% CO_2 and sub-cultured prior to confluence using trypsin/EDTA.

Cell binding assay: 5×10^5 SKBR3, RBMEC, U-87 MG and T98 cells were seeded in a 6-wells plate. The day after, cells were incubated 1 h at 37 °C in culture medium supplemented with 10 and 100 µg mL^{-1} of FITC-labeled HFnF-TZ and HFnF-IgG (for SKBR3 and RBMEC cells) or with 50 µg mL^{-1} of FITC-labeled HFn conjugated with CTX (HFnF-CTX) (for U-87 MG and T98 cells). After incubations cells were washed thrice with PBS, detached from plate and analyzed by CytoFLEX flow cytometer (Beckman Coulter). 20,000 events were acquired for each analysis, after gating on viable cells and

on singlets. Samples of untreated cells were used to draw the appropriate gates and set the positivity region. Data were analyzed by FlowLogic and Kaluza software.

Competition assay: SKBR3, U-87 MG and T98 cells (5×10^5) were transferred in FACS tubes and washed twice with PBS. Then, cells were incubated with 10 μg mL^{-1} of HFnF-TZ and HFnF-IgG with or without TZ as competitor (580 μg mL^{-1}) (for SKBR3 cells) or with 50 μg mL^{-1} of HFnF-CTX with or without CTX as competitor (140 μg mL^{-1}) (for U-87 MG and T98 cells) in PBS supplemented with 0.3 % BSA at 37 °C for 30 min. Cells were washed thrice, resuspended in PBS (500 μL) and analyzed by CytoFLEX flow cytometer (Beckman Coulter). 10,000 events were acquired for each analysis, after gating on viable cells and on singlets. Samples of untreated cells were used to set the positivity region. Data were analyzed by FlowLogic and Kaluza software.

Cell viability assay: 5×10^3 SKBR3 cells were seeded in a 96-well plate and incubated at 37 °C in starvation medium with TZ, HFn-TZ or HFn-IgG (1, 10 μg mL^{-1}). Untreated cells were used as controls. After 3, 5, 7 days of treatment, cells were washed with PBS and incubated for 3 h at 37 °C with 0.1 mL of a stock solution of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine ethosulfate (PES) previously diluted 1:10 in DMEM medium without phenol red (CellTiter 96® AQueous One Solution Reagent; Promega). Absorbance was measured by EnSight™ multimode plate reader (Perkin Elmer, Waltham, MA, USA) setting absorbance wavelength at 490 nm. Results were normalized on viability of untreated samples and expressed as means ± s.e.

TZ-mediated HER2 phosphorylation in Y1248: 2×10^5 cells were seeded in a 12-wells plate, and serum-starved in medium containing 0.1% FBS overnight at 37 °C. Then, cells were treated for 1 h with 4 μg mL^{-1} of TZ free or nanoformulated (HFn-TZ) or with HFn-IgG as a control, in starvation medium. Negative control was represented by untreated cells. At the end of incubation, cells were washed twice with ice cold PBS, lysed with 130 μL lysis buffer (20 mM Tris HCl pH 7.6, 150 mM NaCl,

1 mM EDTA, 1% Triton X-100, 1% glycerol, 1 mM Na_3VO_4, 10 mM NaF, 4% Protease Inhibitor Cocktail, 1 mM PMSF) and cleared at 13,000 rpm for 20 min at 4 °C. The protein content was quantified using the Coomassie Plus Protein Assay Reagent (ThermoFisher Scientific) with BSA as standard protein. Approximately 35 µg of protein from each sample were dissolved in sample buffer 5× (125 mM Tris HCl pH 6.8, 10% SDS, 20% glycerol, 0.02% Bromophenol blue, 5% β-mercaptoethanol), denatured 5 min at 95 °C, separated by SDS-PAGE and transferred onto PVDF membrane (Immobilon-P, EMD Millipore Corpora-tion, MA, USA). The membrane was blocked in 5% BSA or 5% skim milk in TBS with 0.1 % Tween 20 for 1 h and incubated with appropriate primary antibodies: anti-phospho-HER2/ErbB2 Tyr1248 (Cell Signaling Technology, Inc.), anti-HER2/ErbB2 (clone 29D8, Cell Signaling Technology, Inc.), or anti-α-tubulin (Sigma-Aldrich) used as loading control. Antibodies conjugated to horseradish peroxidase (Abcam) were used as secondary antibodies, chemiluminescence reaction was developed with the ECL star kit (Euroclone) and the signal was detected using the Chemidoc System (Biorad). Densitometric analysis of protein bands was performed with ImageJ sofware.

Cell cycle analysis: 2.5×10^5 U-87 MG cells were seeded in a 12-well plate and incubated overnight at 37 °C. Then, cells were treated with 10 µg mL^{-1} of CTX or HFn-CTX in complete medium for 24, 48 and 72 h. Untreated cells were used as negative control. At each time point, cells were collected in FACS tubes and fixed with cold ethanol 90% for 1 h. Then cellular DNA was stained with a solution of propidium iodide (10 µg mL^{-1}) and RNase A (20 µg mL^{-1}). 10,000 events were acquired for each sample, using flow cytometry equipped with a doublet discriminator module (GalliosTM Flow Cytometer - Beckman Coulter Inc.) and the DNA content was analyzed by FlowLogic software.

Effector cells isolation: Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on Ficoll-Paque Plus (GE Healthcare) of blood samples from healthy donor (30 min at 1,500 rpm without brakes). At the end the PBMCs layer was carefully transferred to a new 50 mL

tube, diluted with PBS and centrifuged for 6 min at 1,400 rpm. Then supernatant was eliminated to remove platelets and the procedure was repeated for four times decreasing the centrifugation speed up to 1,000 rpm. Washed PBMCs were resuspended in RPMI-1640 medium with 10% of decomplemented FBS and 1,000 U mL^{-1} IL-2 (BioLegend, San Diego, CA, USA) for 24 h at 37 °C. The described procedure was approved by Ethical Committee of the University of Milano-Bicocca (prot.#351, 13th November 2017) after submission of the project together with informed consent by the healthy volunteers.

ADCC assay: ADCC was performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega Corporation, Madison, WI, USA). Firstly, target cells, plated at a density of 5×10^3 in 96-well, were coated with CTX or HFn-CTX (10 µg mL^{-1}) and with TZ or HFn-TZ (2 µg mL^{-1}) for 30 min at 4 °C in RPMI-1640 medium. Before running the experiment, PBMCs were incubated 24 h with IL-2 to allow their activation. Then, activated PBMCs were added onto target cells at an E:T ratio of 40:1 and incubated 4 h at 37 °C. The LDH released from target cells was measured by EnSight™ multimode plate reader (Perkin Elmer, Waltham, MA, USA) setting absorbance wavelength at 490 nm. Percentage of ADCC was calculated following protocol instructions.

Confocal live image microscopy of ADCC: T98 cells were stained with *CellTrace™ Far Red* (ThermoFisher Scientific, USA) diluted 1:1000 in PBS for 20 min at 37 °C and seeded (2×10^5 cells) in a 35 mm petri dish. Cells nuclei were stained with Hoechst (ThermoFisher Scientific, USA), while membranes with WGA-AlexaFluor® 488 Conjugate (ThermoFisher Scientific, USA). After, cells were incubated 30 min at 4 °C with 10 µg mL^{-1} of HFn-CTX. WGA-AlexaFluor® 488 Conjugate pre-stained effector cells were added to the petri dish (E:T ratio of 40:1). Images were acquired every 15 min for 3 h using a Nikon confocal microscope (A1 series).

CD16 shedding assay: 1.5×10^4 SKBR3, U-87 MG and T98 cells were seeded in a 48-well plate. The day after, medium was removed and cells were incubated 30 min at 4 °C in RPMI culture medium

without FBS with 5 and 10 μg mL^{-1} of HFn-CTX or 0.2 and 2 μg mL^{-1} of HFn-TZ. Then, IL-2-activated PMBCs were added at an E:T ratio of 40:1. After 4 h incubation at 37 °C, 300 μL of the medium containing PBMCs were collected and transferred in FACS tubes. Effector cells were centrifuged for 5 min at 1,300 rpm and washed once with PBS before adding the AlexaFluor® 488 anti-human CD16 antibody (clone 3G8; BioLegend®) with a dilution of 1:100. The antibody was incubated 10 min in the dark. The amount of the not cleaved CD16 on effector cells was evaluated by FACS analysis. 10,000 events were acquired for each sample, using flow cytometry equipped with a doublet discriminator module (GalliosTM Flow Cytometer - Beckman Coulter Inc.) and data were analyzed by FlowLogic software.

Co-cultured BBB model: hCMEC/D3 were used to set up an *in vitro* BBB model.[2] Briefly, 5×10^4 cells (passage 25-35) were seeded onto collagen-coated (40 μg/cm^2 rat tail collagen type 1; Invitrogen) transwell filters (polyester 12-well, pore size 0.4 μm, translucent membrane insert 1.12 cm^2; Costar) to establish a polarized monolayer. After 48 h, 4.6×10^4 U-87 MG cells were seeded in the lower compartment, whereas the apical one was filled with 0.5 mL of EBM-2 medium supplemented with 5% FBS, 1% CDLC, 1% P/S, 10 mM Hepes, 5 μg mL^{-1} ascorbic acid, 1.4 μM hydrocortisone,10 mM LiCl. The basolateral chamber was filled with 1 mL of U-87 MG culture medium. Medium was changed every 2 days. Trans-endothelial electrical resistance (TEER) was measured at day 1, 3 and 5 of co-culture to monitor the formation of tight junctions between endothelial cells by means of EVOM2 meter, STX2 electrode (World Precision Instruments, Sarasota, FL,USA), as previously described.[3] After 5 days of co-culture, the quality of the BBB model was assessed by measuring the paracellular permeability of [^{14}C]-sucrose and the transcellular permeability of [^{3}H]-propranolol across the hCMEC/D3 monolayer. [^{14}C]-sucrose (0.5 μCi, 3.52 μM) and [^{3}H]-propranolol (0.5 μCi, 76 nM) were incubated in the apical compartment of the transwell system for up to 120 min. Every 30 min, 100 μL of samples were collected from the basolateral side

with replenishment using fresh culture medium and the radioactivity was measured by Tri-Carb 2200 CA Liquid Scintillation Analyzer (Packard). The endothelial permeability (EP) of the radiolabelled molecules and the EP coefficients ratio (EPpropranolol:EPsucrose), indicating the discrimination level of the BBB model between transcellular and paracellular probes, were calculated as previously described.[4,5] HFn-CTX and HFn-TZ ability to cross the BBB model was evaluated by measuring the EP of the nanoformulations across hCMEC/D3 monolayer. At day 7 of culture, fluorescent-labeled HFn-CTX or HFn-TZ was incubated in the apical compartment of the transwell system (the concentration of each protein species in the complex was HFn/TZ = 0.35 mg mL^{-1} and CTX/TZ = 0.1 mg mL^{-1}). Every 30 min, samples were collected from the basolateral chamber for up to 3 h and the fluorescence was measured by spectrofluorometer. The uptake of AF660-labeled CTX/TZ or HFn-CTX/HFn-TZ by hCMEC/D3 cells was quantified 3 and 24 h after the incubation in the apical chamber of the transwell system by spectrofluorometer. Briefly, cells were trypsinized, collected and centrifuged at 2,000 rpm for 5 min. Cells were resuspended in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% v/v Triton X-100, 0.1% SDS, 1 mM DTT) and incubated for 30 min while rotating in the dark and then centrifuged at 10,000 rpm for 5 min. Supernatants were measured by spectrofluorometer. The uptake was expressed as a percentage of the initial fluorescence incubated in the apical chamber of the transwell system.

Statistical analysis: Statistical analyses were conducted using two-tailed Student's *t*-test. All plots show mean values ± standard error (s.e.). All tests assumed normal distribution and the statistical significance threshold was set at $P<0.05$.

Supplementary details to Figure 2. Due to the different expression of TfR1 in the SKBR3 and glioblastoma cell lines, the binding experiments were conducted using low and high concentration of HFn conjugates respectively. Using SKBR3 the percentage of positive cells were considered,

whereas for glioblastoma we saturate the positive cells and analyzed the MFI. Both experiments settings provide information of binding efficiency of HFn-mAb towards specific cell lines.

Supplementary Figures

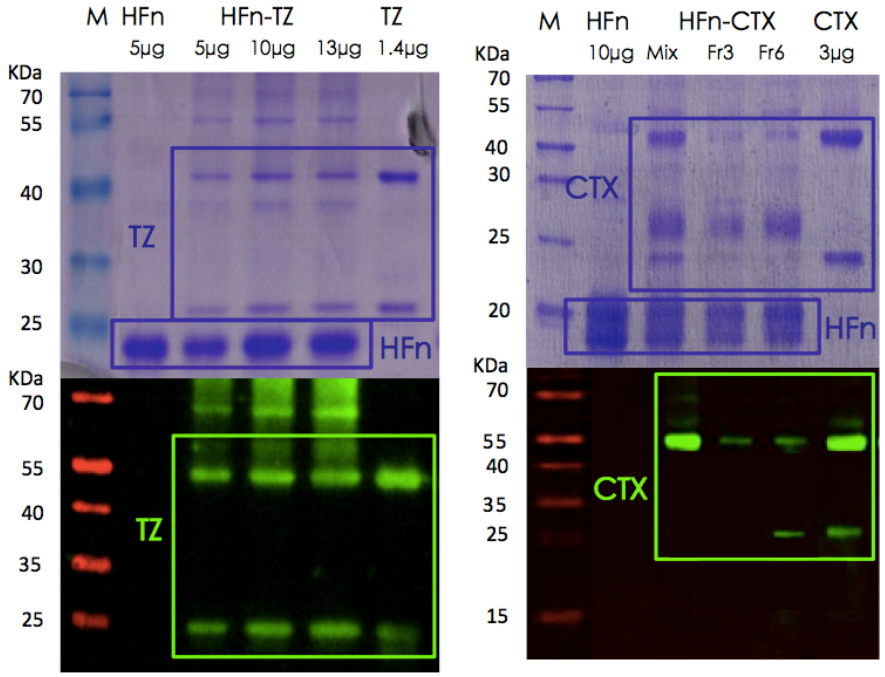


Figure S1. Qualitative evaluation of the presence of both HFn and mAbs in the nanoconjugate, after FPLC elution. The presence of HFn, TZ and CTX in the nanoformulations was qualitatively checked via 12% SDS-PAGE separation. Starting from the left, the protein marker is in the first lane, then standards of HFn and mAb were loaded in second and last lane, respectively. Different samples of HFn-mAb complex were loaded in the central lanes. For WB analysis membranes were incubated over night with rabbit-monoclonal antibody against ferritin heavy chain (EPR3005Y; Abcam) at 1:1000 dilution in 5% BSA in TBS with 0.1% tween 20. After, membranes were washed thrice with TBS with 0.1% tween 20 and reacted 1 h with rabbit polyclonal anti-human IgG secondary antibody (A80-118P; Bethyl) at 1:20000 dilution in 5% BSA in TBS with 0.1% tween 20. Membranes were washed thrice with TBS with 0.1% tween 20 and the bound antibodies were revealed using ECL star

reagent (Euroclone) and the chemiluminescence signal was detected using Odyssey Fc Imaging System (Licor).

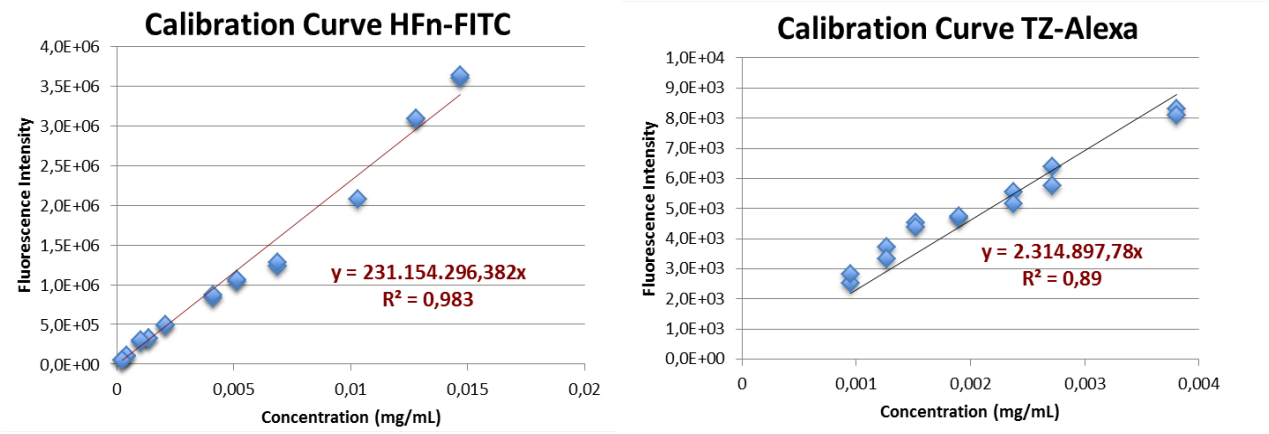


Figure S2. Quantification of protein species in the nanoformulation using double-labeled samples. HFn and mAbs were labeled using Fluorescein Isotiochianate (FITC) and AlexaFluor660®, respectively and calibration curves were obtained for each of them before the reaction took place. After the reaction, the fluorescence intensity of each dye was recorded and used to calculate the protein concentration. Thanks to this quantification we obtained that HFn was functionalized with one monoclonal antibody on the surface (HFn:mAb ratio of 1.2).

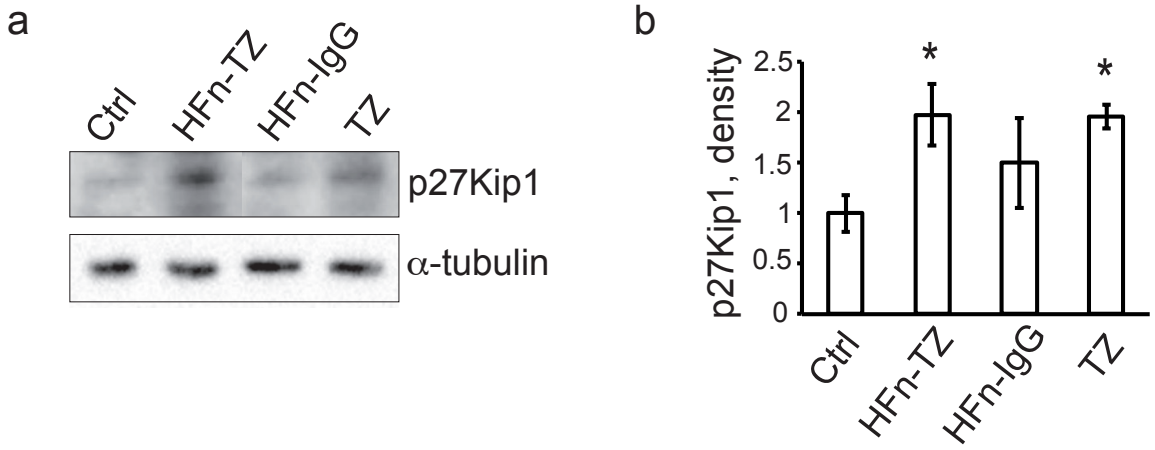


Figure S3. (a) Western blot analysis of p27Kip1 expression in SKBR3 cells treated for 24 h with 10 μg mL^{-1} of free or nanoformulated TZ, or with equal concentrations of HFn-IgG. (b) Densitometric

quantification of p27Kip1 levels relative to α-tubulin. Mean ± SE (n=3). *p<0.05 *vs.* untreated cells (Ctrl).

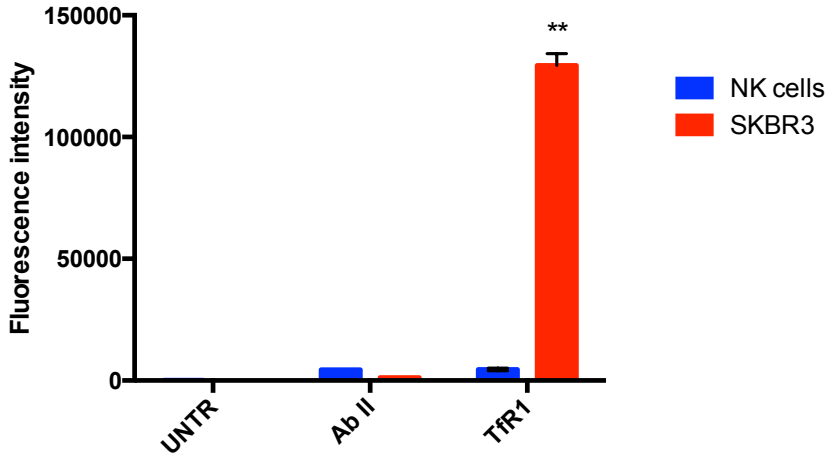
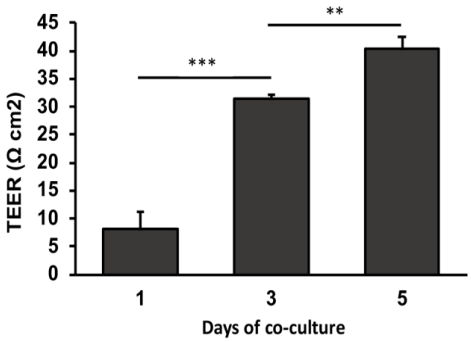


Figure S4. TfR1 expression level in NK and SKBR3 cells. The expression level of TfR1 was determined by flow cytometry analysis after incubating cells with an anti-human TfR1 antibody. Reported values are means of 3 replicates ± se. ** = $p < 0.001$ vs. TfR1 expression on NK cells (Student's t-test).

TfR1 expression. 5×10^5 SKBR3 and freshly isolated NK cells were incubated 30 min at 4 °C in blocking buffer (PBS, 2% BSA, 2% goat serum); then the anti-human TfR1 antibody was added (0.25 µg for 5×10^5 cells, ab38171, Abcam) and cells were incubated for further 30 min at 4 °C. Cells were washed thrice with PBS and incubated with the AlexaFluor® 488-conjugated goat anti-mouse IgG secondary antibody (Life Technologies) with a dilution of 1:1000 in blocking buffer for 30 min at 4 °C in the dark. After incubation, cells were washed and analyzed using flow cytometry Gallios™ Flow Cytometer (Beckman Coulter Inc.). 10,000 events were acquired for each sample, after gating on viable cells, and isotype-control antibodies were used to set the appropriate gates. Data were analyzed by FlowLogic software.

a



b

Permeability coefficient of [^{14}C]-sucrose	$1.60 \pm 0.13 \times 10^{-5}$ cm sec^{-1}
Permeability coefficient of [^{3}H]-propranolol	$2.59 \pm 0.36 \times 10^{-5}$ cm sec^{-1}
Ratio	1.62

Figure S5. Bioelectrical and functional properties of the co-cultured BBB model. To establish a co-culture transwell model, hCMEC/D3 cells were seeded on the apical side of the transwell filter. After 48 h of culture, U-87 MG cells were seeded on the bottom of the lower compartment. (a) To assess the formation of tight junctions between hCMEC/D3 cells, the trans-endothelial electrical resistance (TEER) was monitored at day 1, 3 and 5 of co-culture. Reported values are means of 3 replicates ± se. ** = $p < 0.01$; *** = $p < 0.001$ (Student's t-test). (b) After 5 days of co-culture, the integrity of the BBB model was also assessed by measuring the paracellular permeability of [^{14}C]-sucrose and the transcellular permeability of [^{3}H]-propranolol. The permeability coefficients were calculated as above described (see supplementary materials and methods).

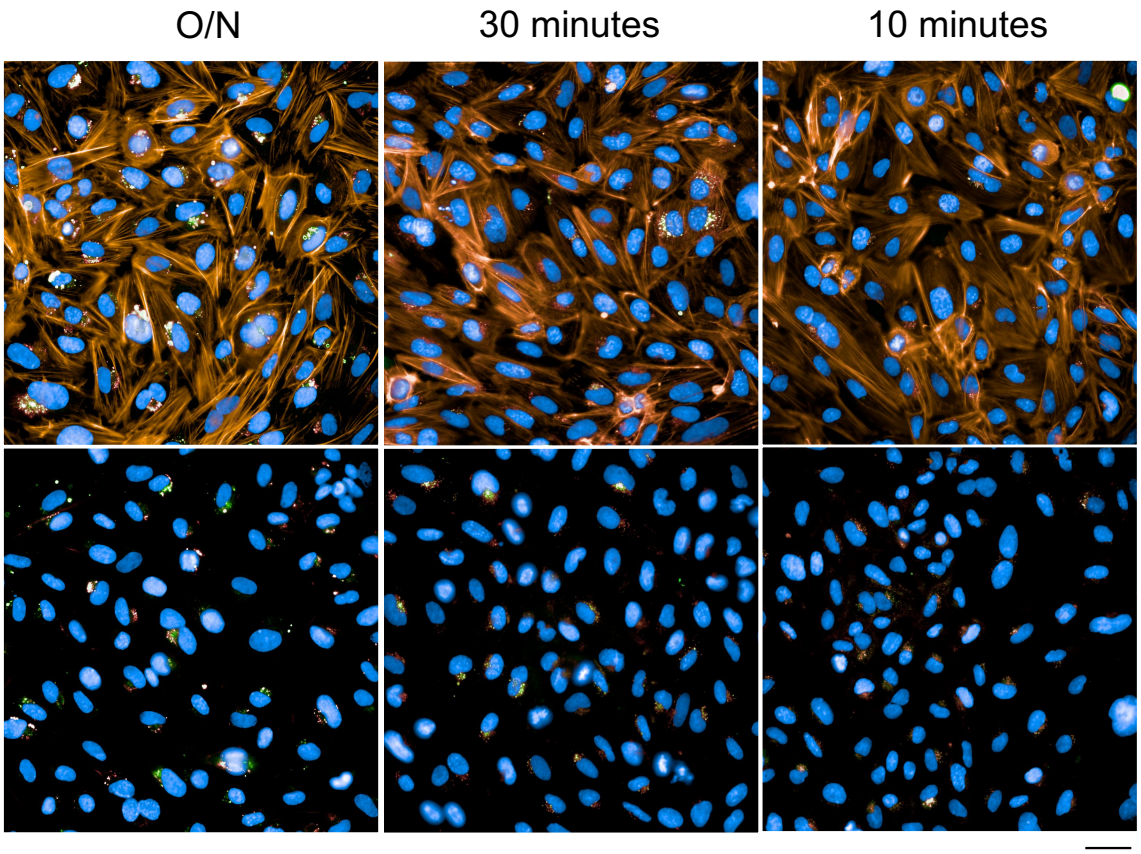


Figure S6. Confocal images of HFn-CTX uptake by hCMEC/D3. hCMEC/D3 cells were treated with HFn-CTX at the concentration of 0.1 mg mL^{-1}. Images were acquired after 10 mins, 30 mins and over night (O/N) using Operetta high content system and show that NPs uptake is very fast and time dependent. Nuclei were stained with Dapi (blue); Cytoskeleton was stained with Phalloidin Texas-Red (orange); CTX was labeled with AF660 (red); HFn was labeled with FITC (green). Scale bar = 50 µm.

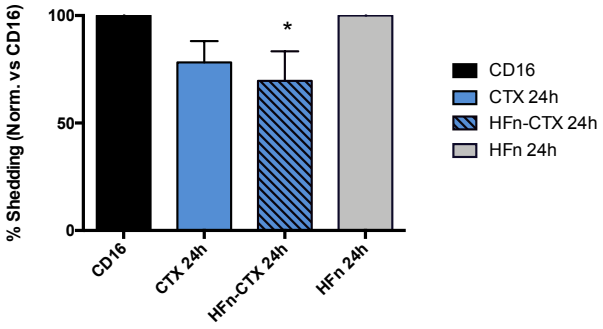


Figure S7. CD16 Shedding assays after 24 h transwell incubation. Data confirmed that, after crossing the BBB, HFn-conjugated CTX maintain the capability to recruit and activate immune cells. Reported values are means of 3 replicates ± se. * = $p < 0.1$ *vs.* UNTR (CD16) (Student's *t*-test). Untreated cells were used to set the positive region and the singlet gate in the shedding analysis.

ADCC movie (Attached file SV1). Live image confocal microscopy. T98 cells were stained with *CellTrace™ Far Red* (red signal) before seeding them. Cells nuclei were stained with Hoechst (blue signal) while membranes were stained with WGA-AlexaFluor® 488 (green signal). After, cells were incubated 30 min at 4 °C with 10 µg mL−1 of HFn-CTX. WGA-AlexaFluor® 488 pre-stained effector cells were added to the petri dish and images were acquired every 15 min for 3 h. During this period of time, activation of ADCC was observed, which led to cancer cell death.

References

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