Supplemental Information for

Quantifying CEACAM targeted liposome delivery using imaging flow cytometry

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A.

Opa(HV-) sequence:

CATATGGCAAGTGAAGACGGCGGCCGCGGCCCGTATGTGCAGGCGGATTTAGCC
TACGCCTACGAACACATTACCCACGATTATCCGGAACCAACCGCTCCAAACAAG
AACAAAATAAGCACGGTAAGCGATTATTTCAGAAACATCCGCACCCGCTCCGTC
CACCCCCGGGTGTCGGTCGGCTACGACTTCGGCGGCTGGAGGATAGCGGCAGAT
TATGCCCGTTACAGAAAATGGGGATCAGGATCAGGATCAGGAACGGAAAATCAG
GAAAACGGTACGTTCCACGCCGTCTCCTCACTCGGCTTATCCGCTATTTACGATTT
CCAAATAAACGATAAATTCAAACCCTATATCGGCGCGCGCGTCGCCTACGGACA
CGTCGGATCAGGATCAGGATCAGGACGCGTGGGTCTCGGTGTCATCGCCGGCGT
CGGTTTCGACATCACGCCCAAGCTGACCCTGGACGCCGGGTATCGCTACCACAAC
TGGGGACGCTTGGAAAACACCCGCTTCAAAACCCACGAAGCCTCATTGGGCGTG
CGCTACCGCTTCTAAGGATCC

Length (bp): 564

Vector: pET-28b

5' site: NdeI

3' site: BamHI

B.

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Opa60     1    ASEDGGRGPYVQADLAYAYEHITHDYPEPTAPNKNKISTVSDYFRNIRTRSVHPRVSVGY  60
Opa(HV-)  1    ASEDGGRGPYVQADLAYAYEHITHDYPEPTAPNKNKISTVSDYFRNIRTRSVHPRVSVGY  60

Opa60     61   DFGGWRIAADYARYRKWNNNKYSVNIENVRIRKENGIRIDRKTENQENGTFHAVSSLGLS  120
Opa(HV-)  61   DFGGWRIAADYARYRKWGSGSGS-----------------GTENQENGTFHAVSSLGLS  102

Opa60     121  AIYDFQINDKFKPYIGARVAYGHVRHSIDSTKKTIEVTTVPSNAPNGAVTTYNTDPKTQN  180
Opa(HV-)  103  AIYDFQINDKFKPYIGARVAYGHVGSGSGSGRVGLG-----------------------  138

Opa60     181  DYQSNSIRRVGLGVIAGVGFDITPKLTLDAGYRYHNWGRLENTRFKTHEASLGVRYRF  238
Opa(HV-)  139  -------------VIAGVGFDITPKLTLDAGYRYHNWGRLENTRFKTHEASLGVRYRF  183
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Figure S1. Opa(HV-) genetic sequence and amino acid sequence alignment. (A) Nucleotide sequence of Opa(HV-) inserted into pET-28b vector MCR using NdeI and BamH1 restriction sites. (B) Opa_{60} and Opa(HV-) sequence alignment. Removal of HV1 and HV2 in Opa(HV-) results in a smaller protein than Opa_{60}.

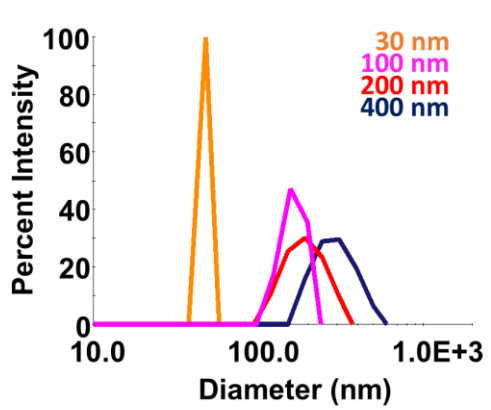


Figure S2. Dynamic Light Scattering (DLS) of extruded liposomes. DLS data is shown for liposomes that were extruded through polycarbonate membranes containing pore sizes of 30 nm (yellow), 100 nm (magenta), 200 nm (red), and 300 nm (blue). The corresponding liposome diameters and percent polydispersity is given in Table S1.

Table S1. Liposome sizes		
Membrane pore size	Liposome diameter (nm)	Polydispersity (%)
0.4 µm	298	28
0.2 µm	197	28
0.1 µm	165	16
0.03 µm	48	--

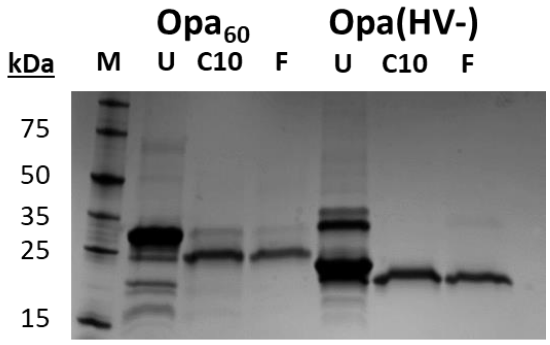


Figure S3. Opa$_{60}$ and Opa(HV-) folding gel. SDS-PAGE of Opa$_{60}$ and Opa(HV-) refolded into fluorescent DMPC liposomes after folding for 4 days at 37°C. Shown are unfolded Opa (U), Opa folded into C10-PC lipids (C10) and Opa folded into final liposomes (F) for both Opa$_{60}$ and Opa(HV-) proteins, as well as the molecular weight marker (M). The fold of Opa proteins is largely SDS resistant and can be monitored using SDS-PAGE gel electrophoresis. Following folding, Opa proteins run at lower apparent molecular weights compared to unfolded Opa, which is seen in folded proteins as an apparent size decrease of approximately 5 kDa.

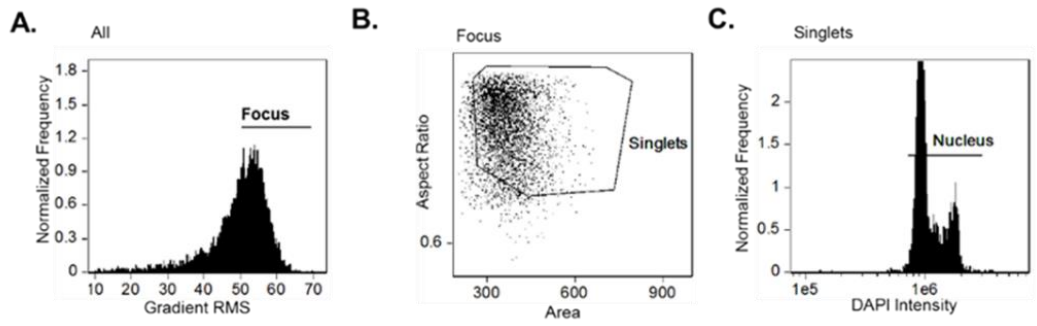
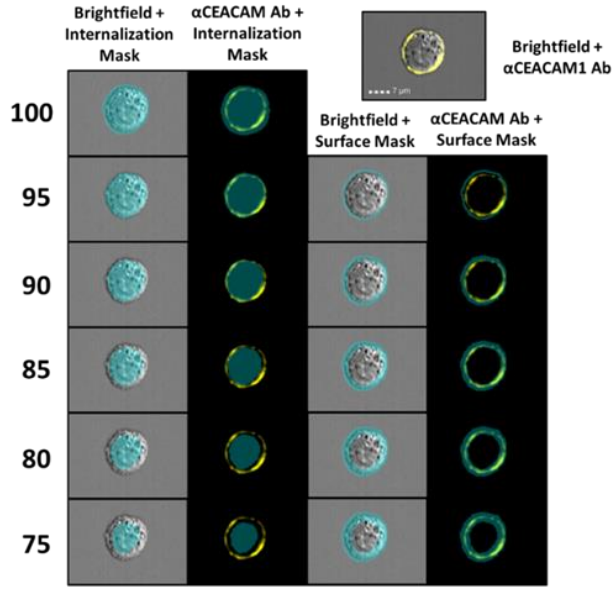


Figure S4. Selecting in-focus, single cells. Fluorescence located within or at the surface of single HeLa cells can be calculated. HeLa cells were imaged using the Imagestream®^{X} MarkII and data was processed in IDEAS software. (A) Gradient RMS selects for cells with high edge contrast in brightfield, enabling gating on cells in focus. (B) Area and aspect ratio allow gating on single HeLa cells, while (C) DAPI intensity enables selection of nucleated cells.

A.



B.

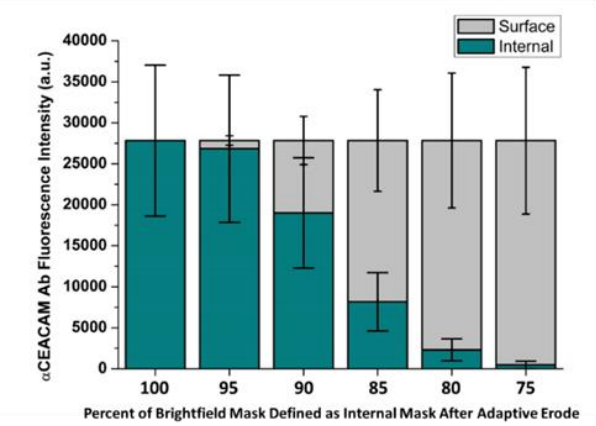


Figure S5. Developing internal and surface cell masks for cell surface antibody fluorescence. Fluorescence values of surface antibody fluorescence were determined using internal and surface masks applied to stained CEACAM1 expressing HeLa cells. (A) CEACAM1 HeLa cells were stained with a pan-CEACAM antibody (yellow). A surface or internalization mask was used to determine surface versus internalized fluorescence for different Adaptive Erode percent values (95-75%) of the full brightfield mask (100%). (B) When used to quantify internal and surface antibody fluorescence in cells, our method reported high surface and low internal fluorescence when an Adaptive Erode mask was set to 75% of the full brightfield mask. Error bars represent 95% C.I.

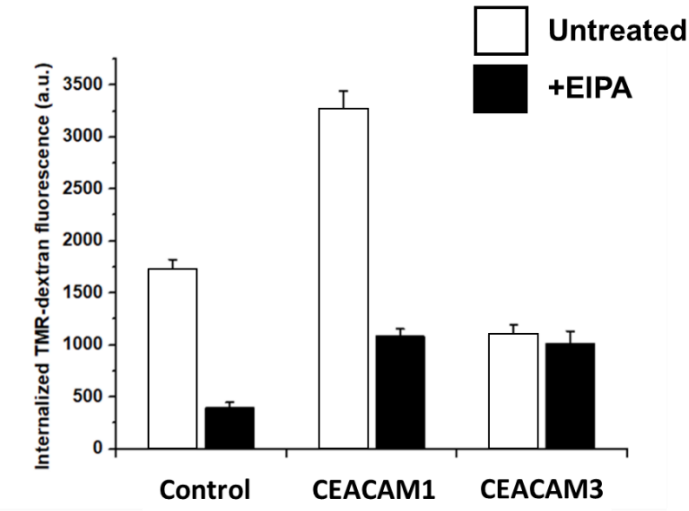


Figure S6. CEACAM1 HeLa cells internalize higher levels of 70 kDa TMR-dextran than control or CEACAM3 HeLa cells. CEACAM1 cells internalize 70 kDa dextran (white bars), a fluid-phase marker for macropinocytosis[1], showing that CEACAM1 cells may internalize particles non-specifically at higher levels than control or CEACAM3 cells. EIPA inhibition of dextran (black bars) uptake into CEACAM1 cells supports this being a macropinocytic-driven process. It is noteworthy that CEACAM3, which is not an adhesion CEACAM, did not promote 70 kDa dextran uptake. Error bars represent 99% C.I.

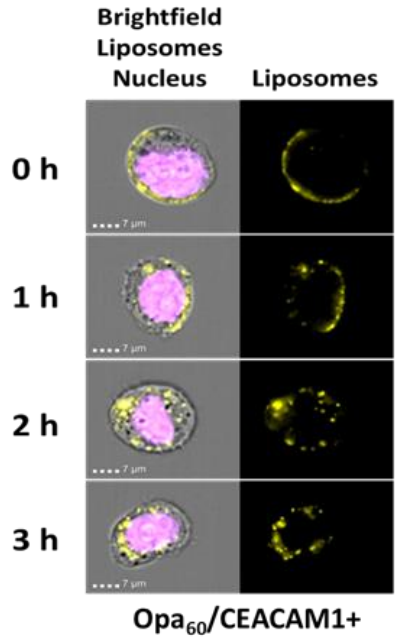


Figure S7. Representative images of Opa60 proteoliposomes with CEACAM1 HeLa cells for three hours following liposome exposure. CEACAM1 cells were briefly exposed to Opa_{60} proteoliposomes and then washed, after which bound Opa_{60} proteoliposomes were allowed to internalize into cells over the course of three hours. Opa_{60} proteoliposomes (yellow) appear primarily at the cell surface immediately after cell exposure (0 h) as seen in Brightfield images that are representative of the full cell population. As Opa_{60} proteoliposomes internalize into cells (1, 2, 3 h), fluorescence decreases from the cell surface and increases in the cytosol nuclei are stained with DAPI (blue).

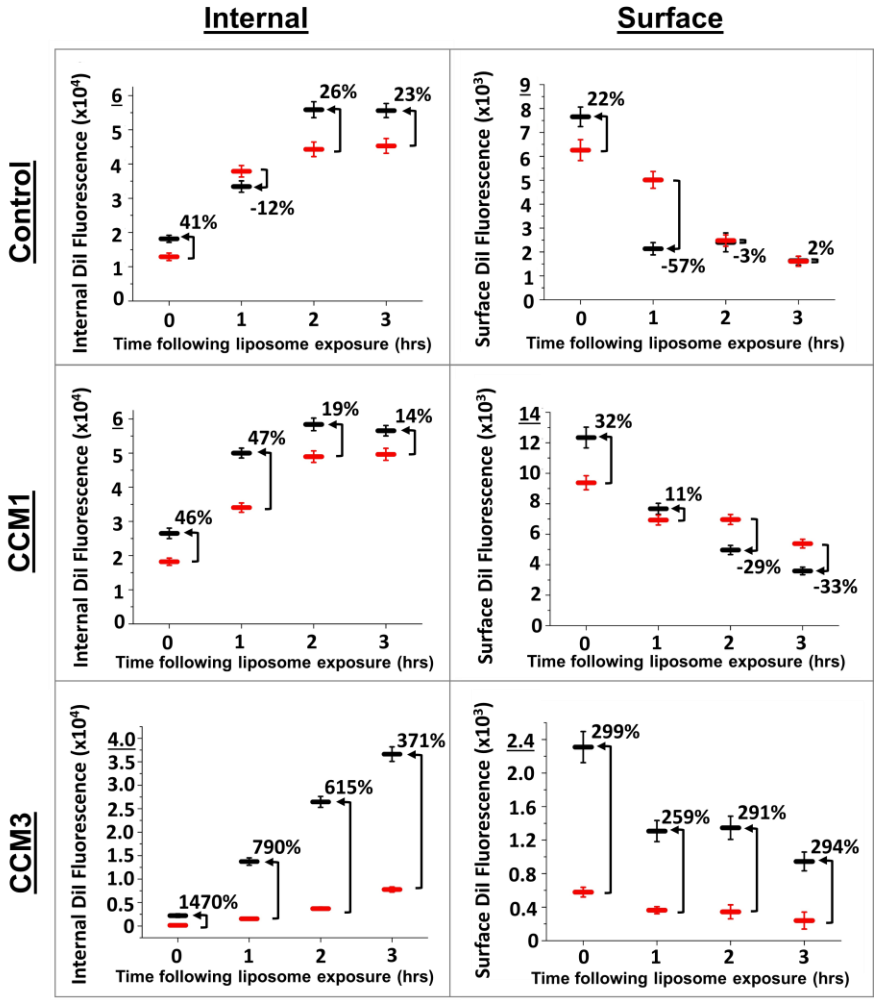


Figure S8. Opa$_{60}$ promotes proteoliposome uptake into HeLa cells compared to Opa(HV-) control liposomes. HeLa cells were pulsed briefly with proteoliposomes before incubating further (0, 1, 2, or 3 hrs). Internal and surface liposome fluorescence was determined for control (A), CEACAM1 (B), and CEACAM3 (C) HeLa cells following exposure to Opa$_{60}$ (black) and Opa(HV-) (red) proteoliposomes. The increase or decrease in Opa$_{60}$ proteoliposome fluorescence is given as a percent change of Opa(HV-) fluorescence. Error bars represent 95% C.I.

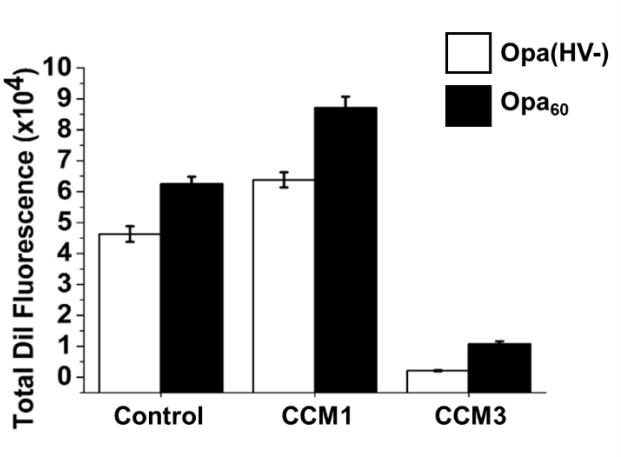


Figure S9. Total cellular fluorescence of liposomes after cell exposure. Opa(HV-) (white) and Opa_{60} (black) proteoliposomes were exposed to cells for 15 minutes. Cells were then washed, lifted and fixed. Total fluorescence measures all liposomes, both bound and internalized, associated with cells at that time. Opa_{60} was found to promote liposome association of proteoliposomes with cells, while the highest total cellular fluorescence was seen in CEACAM1 HeLa cells exposed to Opa_{60} proteoliposomes. Error bars represent 95% C.I.

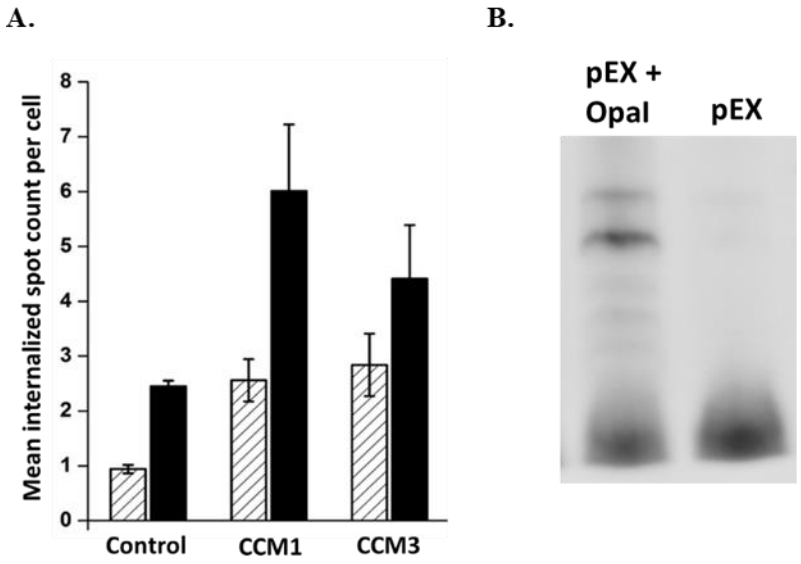


Figure S10. OpaI promotes E. coli internalization into CEACAM1 and CEACAM3 HeLa cells. (A) *E. coli* expressing OpaI (black) were found to internalize into cells at higher levels than plasmid control *E. coli* (grey stripes) as determined by a spot-count algorithm. *E. coli* internalization into CEACAM1 and CEACAM3 cells was higher than control HeLa cells one hour after exposure, suggesting that OpaI promotes *E. coli* internalization through CEACAM. Error bars represent 99% C.I. (B) *E. coli* transformed with pEX plasmid with OpaI insert express Opa as shown by Western blot staining. A 1:1000 αOpa primary antibody and 1:5000 Alexa-555 goat anti-mouse secondary antibody show bands around 29 kDa in *E. coli* transformed with OpaI+ pEX plasmid.

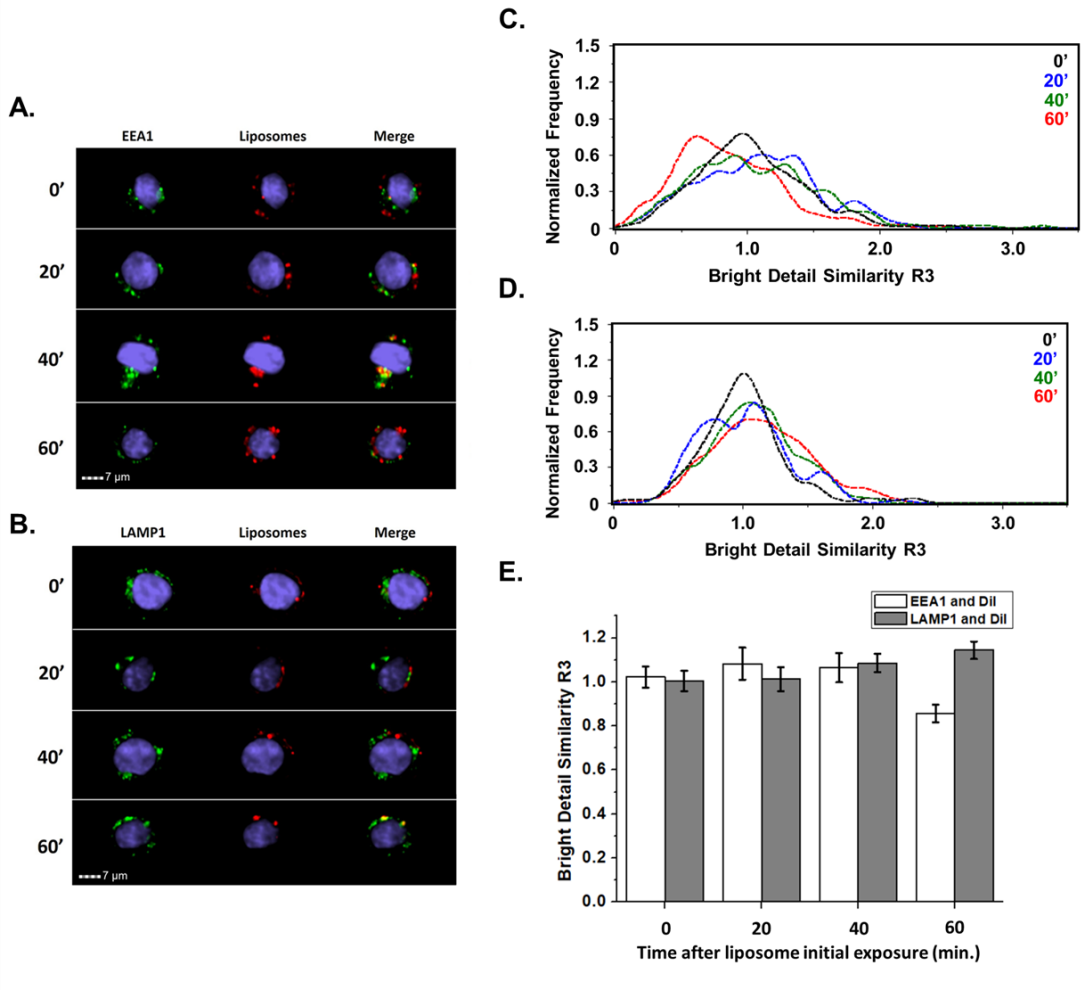


Figure S11. Colocalization occurs between liposomes and markers for early endosomes and lysosomes following Opa_{60} proteoliposome exposure. Representative images of cells with labelled early-endosomes (A, green) or lysosomes (B, green), and DiI-labelled Opa_{60} proteoliposomes (red) at different timepoints after initial liposome exposure. Red or green channel fluorescence is shown with DAPI nuclear staining (blue), along with a merged image in which colocalization between green EEA1 or LAMP1 antibody fluorescence and red liposome fluorescence appears yellow. Plots for bright detail similarity (BDS) score between liposomal DiI and markers for early endosomes (C) or lysosomes (D) show population-level BDS data not indicative of significant liposomal colocalization with either marker. Only cells that were positive for both liposomal fluorescence (DiI) and early endosomal or lysosomal fluorescence (Alexa488) within a 75% Adaptive Erode brightfield mask are shown here. (E) Average BDS values for DiI with EEA1 or LAMP1 at each of the four different time points following initial liposome exposure. Error bars represent 95% C.I.

Additional Methods and Materials:

E. coli growth conditions and labelling

A BL21 (DE3) *E. coli* cell strain was transformed with either a pEX vector subcloned with the *opaI* gene or an empty control vector. Cells were grown for 17 hours in ampicillin-supplemented Lysogeny Broth (LB) media containing 15 µg per mL of 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate (DiI) (Thermo Fisher, D282) until they reached an OD_{600} of 1.0 (approximately 8.0 x 10^8 cfu/mL). *E. coli* were centrifuged at 4000 g for fifteen minutes and red cell pellets were observed. The pellets were washed twice in LB media before being suspended in LB to a final concentration of approximately 2.5 x 10^9 cfu/mL.

Incubation of Fluorescent E. coli with HeLa cells

HeLa cells stably-transfected to express CEACAM1, CEACAM3, or a vector control line were seeded onto plastic plates approximately 20 hrs prior to the experiment. The day of the experiment HeLa cells were washed with fresh media without FBS and cell counts for each line were determined. *E. coli* cells were diluted in DMEM and exposed to cells at a MOI of 50 cfu/cell for one hour at 37°C. Following a one hour exposure to bacteria, the HeLa cells were washed with fresh DMEM and allowed to incubate at 37°C for another four hours. At the end of the experiment, the cells were washed with DMEM before being lifted by 2mM EDTA in PBS (pH 7.4). Cells were spun at 300 x g for 10 minutes and then fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature. Cells were centrifuged at 400 x g for 10 minutes, washed with PBS, and then stained with 1:1000 DAPI in PBS for 1 hour. Cells were washed again with PBS and then stored at 4°C prior to imaging.

Determining Macropinocytic Uptake of HeLa Cells

Macropinocytosis was quantified according to a published protocol[1]. Briefly, 2.0x10^6 cells were plated one day prior to the experiment. The day of the experiment, HeLa cells were washed with serum-free DMEM and incubated with 1 mg ml^{-1} lysine-fixable 70kDa Tetramethylrhodamine (TMR)-dextran (Invitrogen, D1818) at 37°C for 30 min. After, the cells were washed twice with serum-free DMEM then lifted with 2 mM EDTA in PBS and centrifuged at 300 x g for 10 minutes. Cells were fixed with 4% PFA in PBS for 15 minutes at room temperature, centrifuged at 400 x g, and stained with 1:1000 DAPI in PBS for one hour. Cells were finally centrifuged again and washed in PBS before being stored at 4°C prior to imaging. To inhibit macropinocytosis, cells were pre-exposed to 100 µM 5-(N-Ethyl-N-isopropyl)amiloride (EIPA) (Sigma) for 30 minutes in serum-free DMEM just prior to liposome exposure.

Colocalization of Opa_{60} Proteoliposomes with EEA1 or LAMP1

0.2 mM [phosopholipid] of Opa_{60} proteoliposomes were exposed to $2.0x10^6$ CEACAM1 HeLa cells for 20 minutes in DMEM. Following liposome exposure, cells were washed and allowed to incubate further for 0, 20, 40, or 60 minutes. After incubation, cells were washed again with media, lifted with 2mM EDTA in PBS, and fixed with 4% PFA in PBS as described in Materials and Methods. Cells were centrifuged and resuspended in 10% normal-goat serum in PBS with 0.2% saponin to permeabilize the membranes. Antibodies to EEA1 (BD Biosciences, 610456) or LAMP1 (DSHB, H4A3) were incubated with cells 1:100 concentration along with 1:1000 DAPI, following which cells were centrifuged and washed with PBS. A goat anti-mouse Alexa488 secondary antibody was incubated with cells 1:1000 for 1 hour before cells were centrifuged and washed again in PBS for imaging. After imaging, for data analysis, only single cells positive for both DiI and Alexa488 were analysed. Bright detail similarity (BDS) was calculated from the log transformed Pearson correlation coefficient for each cell. An intensity threshold was set for DiI fluorescence between 100-4095 greyscale value to remove background fluorescence in Ch 03, and a similar threshold was set for Alexa488 in Ch 02. Additionally, a 75% internal mask was applied to ensure that only internalized DiI fluorescence was used to calculate BDS as including surface DiI fluorescence would prove a confounding factor for EEA1 and LAMP1 colocalization.

Calculation of the Number of Lipids, Number of Liposomes, and Impact on Fluorescence Intensity

The liposome diameters presented in Table 1 were used to calculate the number of lipids per liposome (N_{lipids}) according to Equation 1 below, where d is the liposome diameter, h is the bilayer thickness of approximately 5 nm, and a is the lipid headgroup area approximated close to a PC headgroup of around 0.71 square nm[2,3].

Eqn. 1 $$N_{lipids} = \frac{\left[4\pi\left(\frac{d}{2}\right)^2 + 4\pi[\left(\frac{d}{2}\right)-h]^2\right]}{a}$$

Liposomes extruded through a membrane with a 0.03 µm pore size (resulting in a 47.8 nm diameter, d) are calculated to contain approximately 1.64×10^4 lipids per liposome. In contrast, the largest liposomes analysed in this experiment were extruded through 0.4 µm pores (calculated d=298.4 nm) and contain approximately 7.69×10^5 lipids per liposome, nearly 47 times more lipids. Because DiI is at the same molar concentration in all liposome preps, 400 nm liposomes could be assumed to show ~47 times higher fluorescence per liposome than 30 nm liposomes due to their higher fluorophore per liposome number.

Assuming each liposome is a sphere, the volume of a single 298.4 nm liposome is around 247 times the volume of a 47.8 nm liposome; therefore, while approximately 47 liposomes extruded through a 30 nm pore are required to equal the fluorescence intensity of a single 400 nm-extruded liposome, the small liposomes reach this equivalent fluorescence intensity in approximately 20% the volume. The reduced volume requirement for smaller liposomes to equal the fluorescence intensity of larger liposomes may explain the significantly higher

fluorescence seen with smaller Opaless liposomes internalizing into HeLa cells since smaller liposomes may have access to a larger set of internalization pathways than LUVs, which may be sterically excluded from certain uptake compartments that smaller liposomes can access.

Supporting References

1 Commisso, C., Flinn, R. J. & Bar-Sagi, D. Determining the macropinocytic index of cancer cells through a quantitative image-based assay. *Nat Protoc* **9**, 182-192, doi:10.1038/nprot.2014.004 (2014).

2 Kučerka, N., Tristram-Nagle, S. & Nagle, J. F. Structure of fully hydrated fluid phase lipid bilayers with monounsaturated chains. *J Membrane Biol* **208**, 193-202 (2006).

3 Heberle, F. A. *et al.* Model-based approaches for the determination of lipid bilayer structure from small-angle neutron and X-ray scattering data. *Eur Biophys J* **41**, 875-890 (2012).