Supporting Information for

Designer protein pseudo-capsids targeting intracellular bacteria

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Materials and Methods

Antibacterial agents. Polymyxin B was purchased from Fisher Scientific. Peptides (amhelin and cytolytic control peptide) were synthesized as described elsewhere.[10] D-Ψ-capsids were assembled from a lactoferrin pharmacophore re-engineered into a triskelion – rrwtwe-βA-k(rrwtwe)-k(rrwtwe)-am (Figure S1). The triskelion was assembled on a Liberty Blue microwave peptide synthesizer (CEM) using standard Fmoc/^{t}Bu solid-phase protocols with DIC/Oxyma Pure as coupling reagents on a Tentagel R RAM (TGR RAM) resin. Fmoc-D-Lys(Mtt)-OH was used to enable orthogonal conjugation on resin via a tri-functional dendritic hub: *H*-βA-k(Mtt)-k(Mtt)-TGR RAM. After post-synthesis cleavage and deprotection (94% TFA, 3% TIS, 3% water) the peptides were purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Semi-preparative and analytical RP-HPLC was performed on a Thermo Scientific UHPLC system (Dionex 3000), using 5 μm Vydac semi-preparative and analytical columns (C18 or C8), respectively, with detection at 280 and 214 nm. Semi-preparative runs were performed at 4.5 mL/min: a 0-40 % B gradient over 40 min for C18, and a 10-40 % B gradient over 60 min for C8 (buffer A, 5% and buffer B, 95% aqueous CH_3CN, 0.1% TFA). Analytical runs were performed at 1 mL/min using a 0-100% B gradient (over either 20 min or 50 min) for C18. LCMS was performed on a Thermo Scientific Q-Exactive system (equipped with a HESI probe), using 4 μm Phenomenex Synergi C12 (00F-4337-E0) analytical columns. LCMS runs used a 0-100% B gradient over 20 min at 0.8 mL/min with detection at 280 and 214 nm (buffer A, 5% and buffer B, 95% aqueous CH_3CN, 0.1% formic acid). LCMS, m/z (ESI^+), data is reported as m/z observed (assignment = m/z expected):

D-triskelion: 1030.1831 Da ($1/3[M+3H]^+$ = 1030.2100 Da), 772.8896 Da ($1/4[M+4H]^+$ = 772.9095 Da), 618.5134 Da ($1/5[M+5H]^+$ = 618.5291 Da), 515.5965 Da ($1/6[M+6H]^+$ = 515.6089 Da), 442.0834 Da ($1/7[M+7H]^+$ = 442.0945 Da), 386.9484 Da ($1/8[M+8H]^+$ = 386.9586 Da).

TAMRA-D-triskelion: 1191.2338 Da (1/3$[M+3H]^+$ = 1191.2698 Da), 893.6768 Da (1/4$[M+4H]^+$ = 893.7043 Da), 715.1432 Da (1/5$[M+5H]^+$ = 715.1650 Da), 596.1213 Da (1/6$[M+6H]^+$ = 596.1388 Da), 511.1052 Da (1/7$[M+7H]^+$ = 511.1201 Da), 447.3423 Da (1/8$[M+8H]^+$ = 447.3423 Da).

Amhelin: 1223.8179 Da (1/2$[M+2H]^+$ = 1223.8443 Da), 816.2148 Da (1/3$[M+3H]^+$ = 816.2321 Da), 612.4130 Da (1/4$[M+4H]^+$ = 612.4261 Da), 490.1321 Da (1/5$[M+5H]^+$ = 490.1424 Da), 408.6115 Da (1/6$[M+6H]^+$ = 408.6200 Da), 350.3819 Da (1/7$[M+7H]^+$ = 350.3897 Da), 306.7099 Da (1/8$[M+8H]^+$ = 306.7169 Da).

Cytolytic control peptide: 1159.8503 Da (1/2$[M+2H]^+$ = 1159.8510 Da), 773.5694 Da (1/3$[M+3H]^+$ = 773.5702 Da), 580.4288 Da (1/4$[M+4H]^+$ = 580.4296 Da), 464.5450 Da (1/5$[M+5H]^+$ = 464.5452 Da), 387.2884 Da (1/6$[M+6H]^+$ = 387.2890 Da), 332.1051 Da (1/7$[M+7H]^+$ = 332.1060 Da), 290.7178 Da (1/8$[M+8H]^+$ = 290.7187 Da).

D-Ψ-capsid assembly. D-triskelion was assembled overnight at 100-400 μM in filtered (0.22 μm), 10 mM MOPS, phosphate or PBS buffers, pH 7.4, at room temperature. For the experiments using confocal microscopy D-triskelion was co-assembled with TAMRA-D-triskelion at 10:1 molar ratio.

Macrophage cell culture. NR8383 semi-adherent macrophage cells (ATCC CRL-2192, Manassas, Virginia, USA) were maintained in Ham F-12 Kaighn's modification media (Thermo Fisher, UK) supplemented with 15% FCS (Thermo Fisher, UK) at 37°C, 5% CO_2 and 95% humidity. The cells above 80% confluency were harvested using a cell scraper. The detached cells were span down by centrifugation, and the excess solvent was replaced by cell growth media. Prior to seeding for analysis, the viability of the cells were tested by trypan blue exclusion haemocytometer counting (>95 % viability) The harvested cells were seeded into a 96-well chambers (25×10^3 cells per well) for the toxicity assay, in IBIDI glass bottom 8-well chambers (50×10^3 cells per well) for fluorescence microscopy experiments, in 48-well plates (5×10^4 cells per well) for flow cytometry and into a 25 cm^2 flask (4×10^6 cells) for electron microscopy experiments. The cells were incubated overnight prior to the experiments.

Infection model. Macrophages were infected with *E. coli* (K12) suspension (OD_{600} = 0.8) at the multiplicity of infection (MOI) of 10 and left at 37°C, 5% CO_2 and 95% humidity for 120 min, after which the culture was treated with antibacterial agents without performing an antibiotic protection assay to avoid a potential bias of lysing internalised bacteria.

Cell viability assays. Each antibacterial agent was added into the cells in Opti-MEM media (Invitrogen, UK). Cells were incubated for 24 h prior to PrestoBlue assays. PrestoBlue® reagent (Life Technologies, UK) was added to each sample according to the manufacturer's protocol: the reagent

supplied as a 10× solution was added to each well by diluting (1×) in culture medium. The cells were incubated for 120 min at 37 °C in 200 µL of the reagent. The fluorescence of each well was measured with a microplate reader (BMG Labtech, Germany), with 544 nm excitation and 590 nm emission filters. Standard calibration curves were generated by plotting measured fluorescence values versus cell numbers. All samples were tested in triplicate.

Cellular uptake assays. Before uptake experiments, the cells were washed (x3) with OptiMEM media. TAMRA-labelled D-Ψ-capsids were transferred to the wells containing macrophages and incubated up to 120 min marking different time points. Cell uptake was visualised using confocal fluorescence microscopy. Uptake efficacy is expressed as percentage of fluorescent cells of the total number of cells.

Flow cytometry assay. Quantification of TAMRA-labelled D-Ψ-capsids uptake was performed by flow cytometry. After 30-, 60- and 90-min incubations with complexes, cells were trypsinized and re-suspended in 150 µL Dulbecco's Modified Eagle Medium (DMEM). TAMRA fluorescence was measured on a CytoFLEX S flow cytometer (Beckman Coulter) and analysed using CytExpert software. 10^{4-5} events were gated from each $>10^5$ subset measured for each sample by forward scatter and side scatter on the 585 nm laser to extract and analyse $>10^4$ single viable cells. From untreated control cells, incubated with 150 µL Opti-MEM containing no antimicrobial agents, a TAMRA negative population was selected and taken as 0% negative allowing for false positive samples. This gate was then used on all other samples to measure the relative population of TAMRA positive cells, which was expressed in percentage in Figure 2C.

Confocal, fluorescence and optical microscopy. High resolution confocal images were acquired using a confocal laser scanning microscope equipped with 408 nm LD class 2 laser with 5-100x objective lenses giving a total magnification of 120-14400x. Images were processed using the proprietary software. Fluorescence was monitored using an Olympus IX81 confocal microscope at 543 nm using x20 and x60 objectives with appropriate filters. Cell imaging was performed under controlled environmental conditions (37 °C, 5% CO_2). Images were processed using Imaris v5.1 and Image J software. The staining was performed using SYTO 9 (ThermoFisher), propidium iodide (ThermoFisher) and 7-amino-actinomycin D (Abcam) as per manufacturers' protocols.

Microtome cell analysis by electron microscopy. Infected macrophages were fixed at different time points with 1% (w/w) glutaraldehyde (Agar Scientific) diluted in PBS and pre-warmed at 37°C. In 5 mins, the fixed cells were scrapped, transferred into a falcon tube and centrifuged at 150 g (5 mins) at 20°C. After centrifugation, the fixative was replaced by a 1 ml of fresh glutaraldehyde fixative solution

(1%, v/v, in PBS) at room temperature and the cells were incubated overnight at 4°C. The cells were then embedded in low melting point agarose (Sigma Aldrich) before electron microscopy processing, and the samples were post-fixed with aq. 2% (v/v) osmium tetroxide (Agar Scientific) solution in aq. 0.1 M cacodylate buffer containing aq. 1.5% (w/v) potassium ferricyanide over 1 hour on ice. Then, the samples were stained with aq. 2% (w/v) uranyl acetate for 1h at room temperature. The samples were dehydrated using an ethanol series at increasing concentrations (50%, 70% 80%, 90%, 100% (2x), 5 minutes each) and followed by washing with anhydrous acetone (twice) for 10 min at room temperature. The samples were gradually infiltrated with Durcupan resin (Sigma Aldrich) and polymerized over 48 hrs at 60°C. The prepared samples were sectioned at 70 nm (Leica Ultracut EM UCT, Leica Microsystems) and collected on Formvar coated 200-mesh copper grids and were analysed by a transmission electron microscope (JEM1400-Plus, 120kV, LaB6) equipped with a Gatan OneView 4K camera. For bacteria counting, dividing bacteria were counted as two cells. The antibacterial agents were added at x2 their minimal inhibitory concentrations[6,10] to account for the action against extracellular bacteria. After incubation with the agents (120 min), the infected cells were fixed for analysis.

Statistical Analysis. Statistical analysis was performed by OriginPro 8.5 using ANOVA and χ^2 tests for at least three independent experiments each done in triplicate, with multi-means comparisons post-test (e.g., Bonferroni), with p values <0.05 considered significant. The results are expressed as means ± SEM or means ± SD (flow cytometry). TEM analyses were performed for an average of 50 individual microtomed macrophages for each time point in each case as indicated in Figure captions (Figs 1, 3, 4, S2, S3, S4). Fluorescence microscopy analyses were performed for at least 10^2 cells in each case. Flow cytometry analyses were carried out for 10^{4-5} cells (after gating) in each case.

Figures

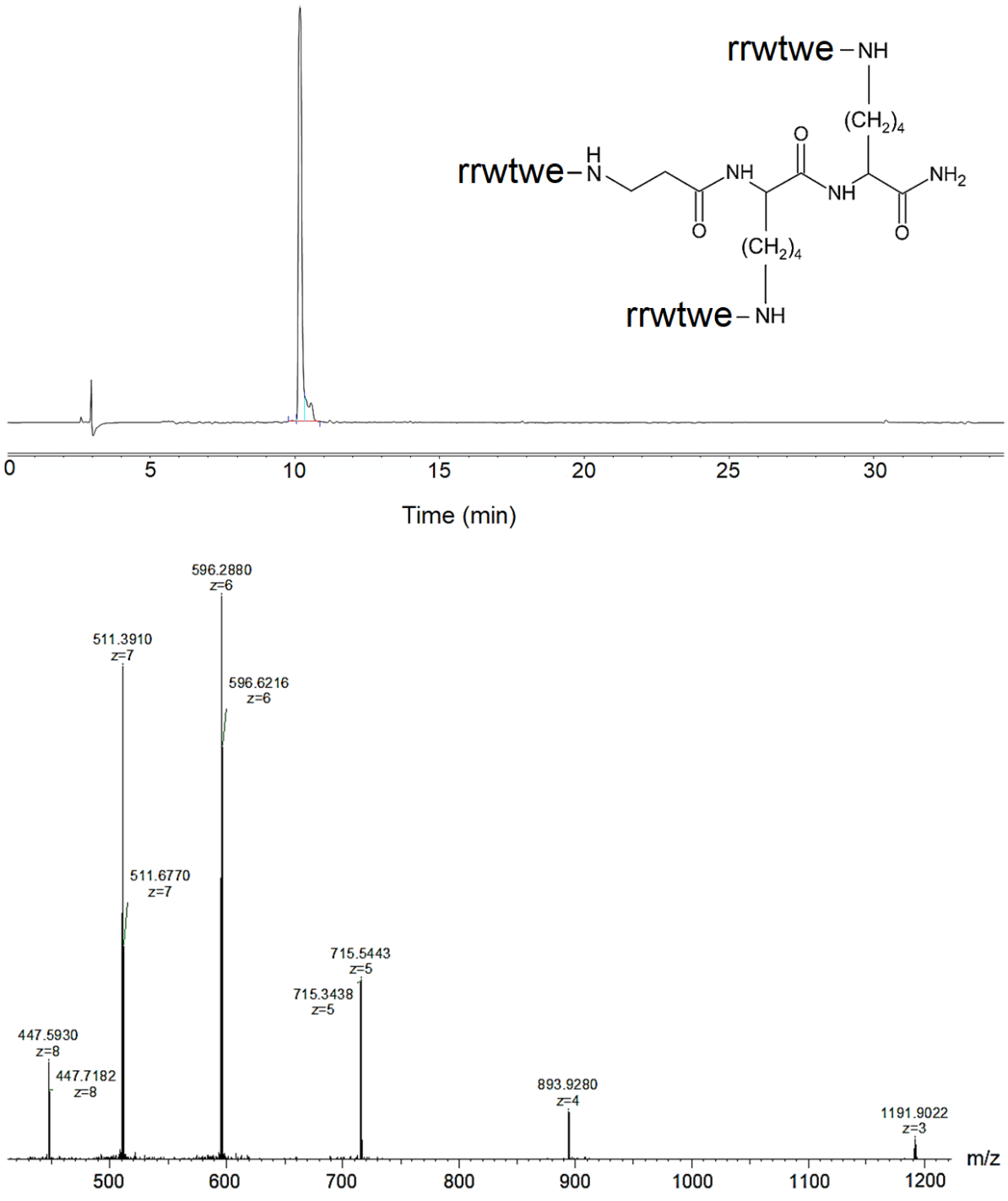

Figure S1. Representative analytical HPLC profile (upper) and mass-spectrometry spectrum (lower) for D-triskelion, with its chemical structure shown in the inset (upper right).

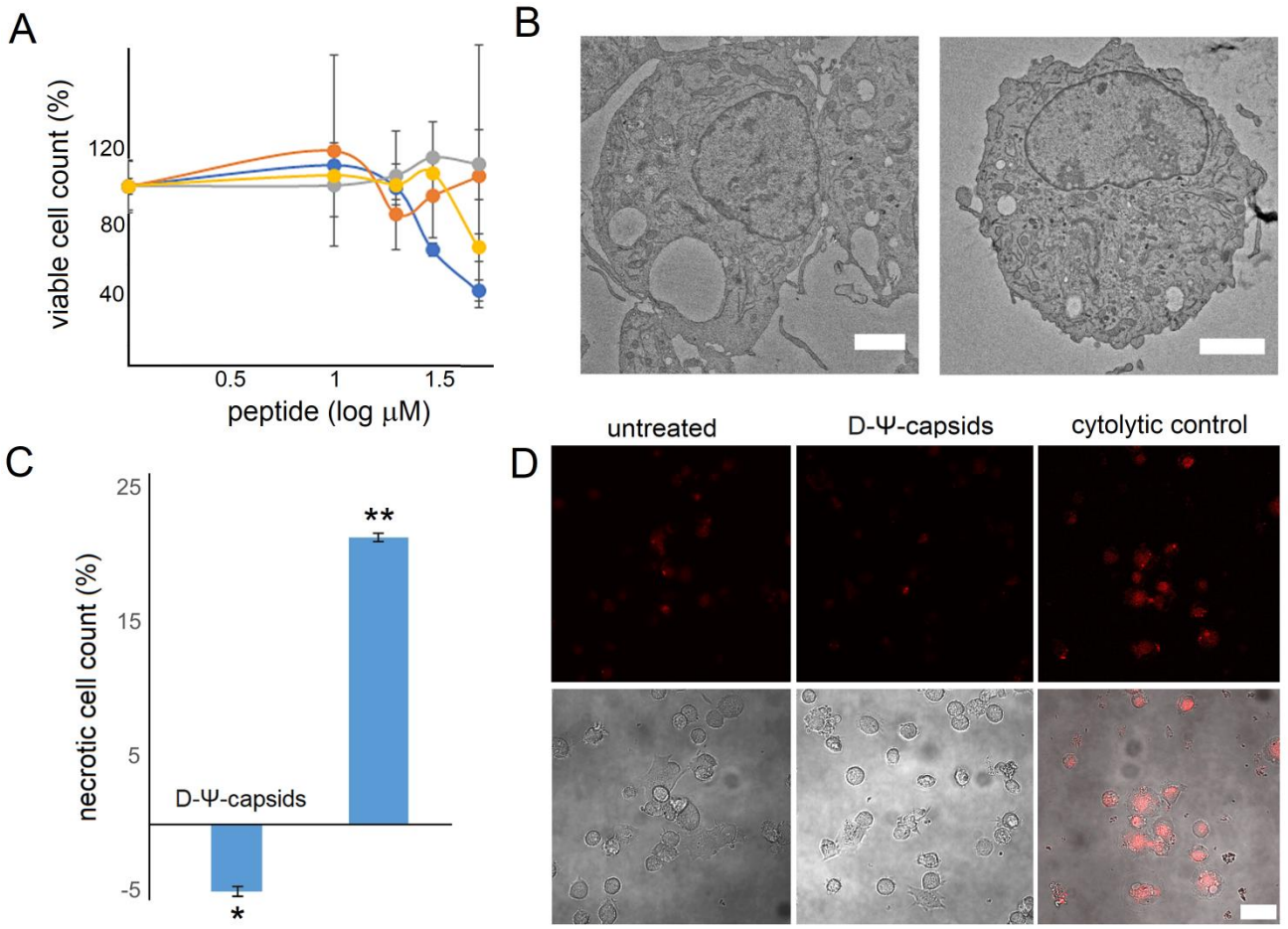

Figure S2. Intact macrophages. (A) Dose response curves showing total viable cell count over 24 hours determined by PrestoBlue cell viability assays for amhelin (grey), polymyxin B (yellow), D-Ψ-capsids (orange) and the cytolytic control peptide (blue). Total number of cells wihtout peptide treatment (blank) is taken as 100%. The data represents mean value ± standard deviation. Each experimental point is done in triplicate. (B) Representative electron micrographs of microtomed cells without peptide or bacterial treatment. Scale bars are 2 μm. On average 50 single microtomed macrophages were analyses (40-60 cells). (C) Percentage of necrotic macrophages in the total number of peptide-treated cells after subtracting background necrosis (cells without peptide treatment). On average 50 single microtomed macrophages (40-60 cells) were analysed in each case. The data shows the mean ± SEM for three independent experiments performed for each case. According to a χ^2 test samples treated with the cytolytic control peptide had higher numbers of necrotic macrophages than untreated samples. Significant differences are represented with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. (D) Fluorescence micrographs (upper panel) of macrophages incubated over 120 min with D-Ψ-capsids and stained with 7-amino-actinomycin D (red, 647 nm emission maximum) as a marker for necrotic cells. Corresponding DIC micrographs of the microphages (lower panel). For the cytolytic control peptide the DIC image is shown merged with the fluorescence image. Scale bars are 30 μm. Over 10^2 cells were analysed by fluorescence microscopy in each experiment for each time point.

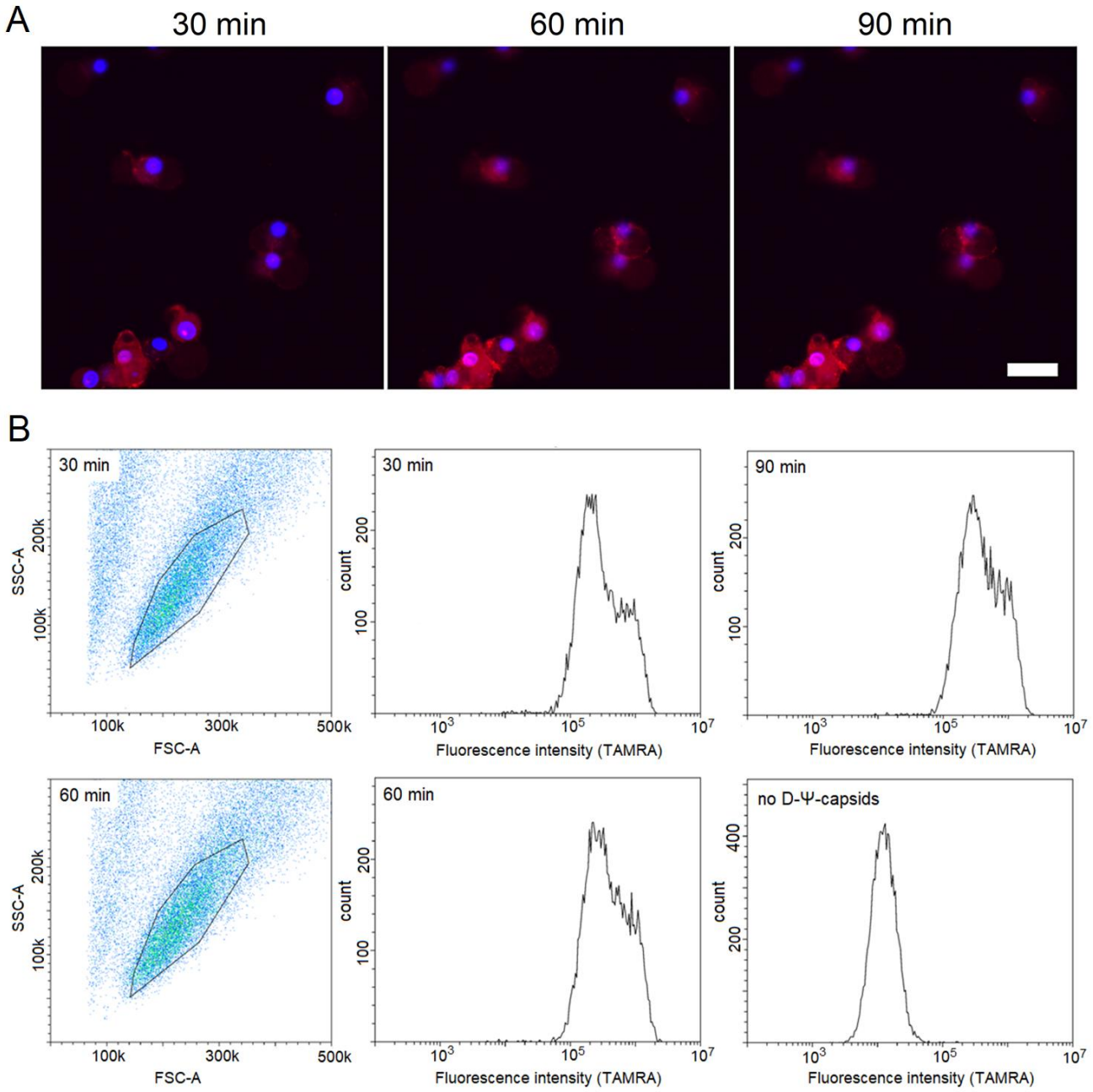

Figure S3. Cell uptake of D-Ψ-capsids. (A) Fluorescence micrograph of macrophages incubated with D-Ψ-capsids labelled with 5-carboxytetramethylrhodamine (TAMRA) (red) over 30, 60, and 90 min. Cell nuclei are counterstained with Hoechst (blue). Scale bar is 30 μm. (B) Flow cytometry charts. $10^{4\text{-}5}$ events were gated (circled in the charts) from each $>10^5$ subset measured for each sample by forward scatter and side scatter (X and Y axis, respectively) on the 585 nm laser to extract and analyse $>10^4$ singlet viable cells at different time points (30, 60, 90 minutes). From the untreated control cells, incubated without antimicrobial reagents, a TAMRA negative population was taken as 0% negative allowing for false positive samples. The gate was used on all other samples to express the count of TAMRA-positive cells in percentage in Figure 2C.

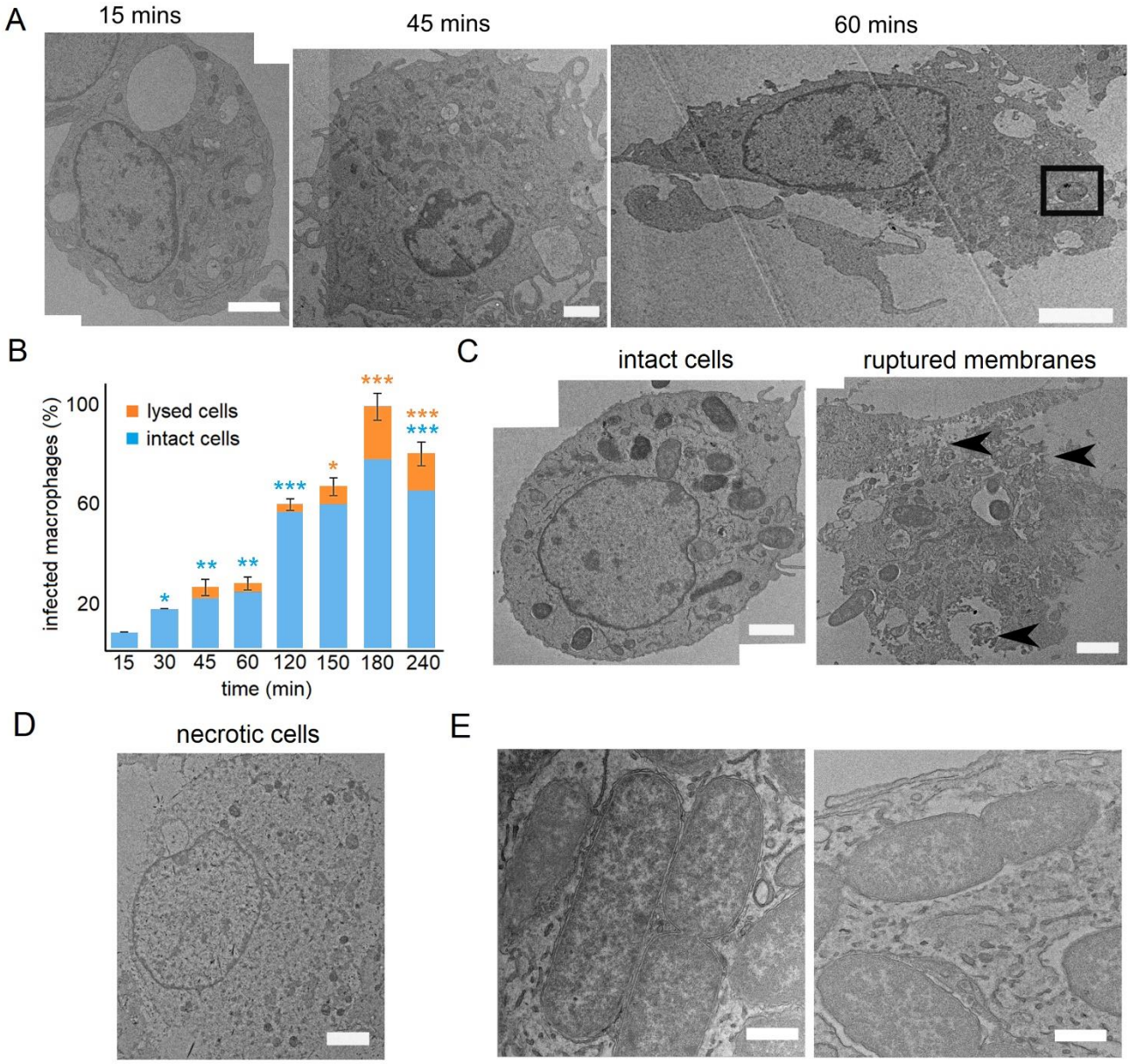

Figure S4. Infected macrophages. (A) Representative electron micrographs of infected macrophages microtomed at different time points during the first hour of incubation with *E. coli*, with a bacterium highlighted in a black frame at 60 min. (B) Percentage of infected macrophages, intact and lysed cells, in the total counts of macrophages at different time points. Macrophages containing at least one bacterial cell per 70 nm microtomed section are counted as infected. The data shows the mean ± SEM for three independent experiments performed for each time point. According to the χ^2 test, later time points had significantly higher numbers of macrophages than at earlier time points (15 min). Significant differences are represented with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$. On average 50 single microtomed macrophages (40-60 cells) were processed in each case. (C) Representative electron micrographs of infected macrophages microtomed at 240 min of incubation, showing intact and ruptured (black arrows) membranes. (D) Representative electron micrograph of infected macrophages microtomed at 240 min showing a necrotic macrophage. (E) Representative electron micrographs of microtomed macrophages infected with *E. coli* at t = 120 min wtihout antibacterial treatment. Scale bars are 2 µm for A, C and D, and 500 nm for E.

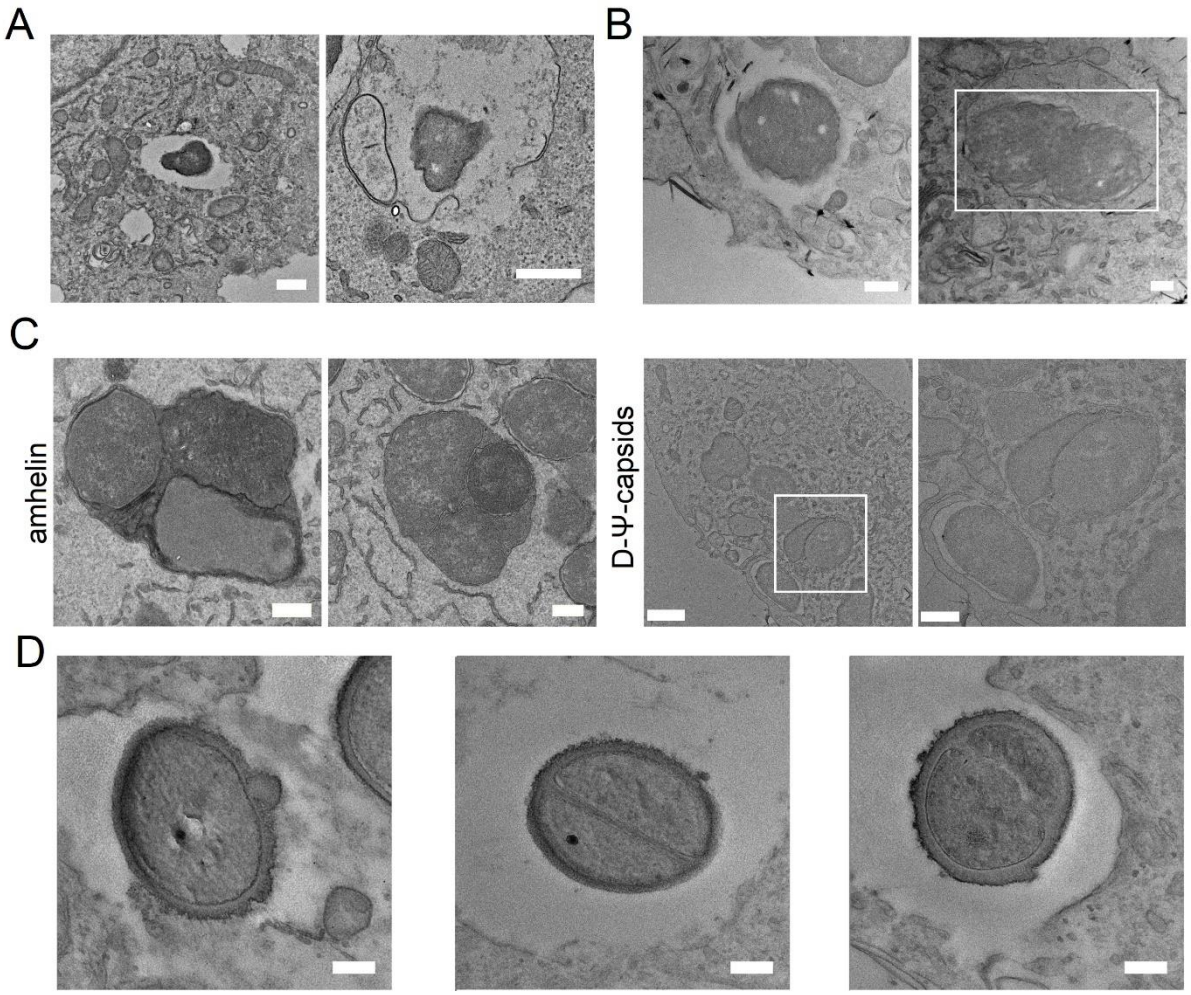

Figure S5. Targeting intracellular *E. coli* in macrophages. Representative electron micrographs of microtomed macrophages infected with *E. coli* at t = 120 min after treatment with polymyxin B (A), amhelin (B and C), and D-Ψ-capsids (C and D). White square frames in (B) and (C) highlight clumping bacteria. (C) Clumping effects of bacteria, with two-three bacteria appeared to fuse together. (D) "Raisin"-like morphologies observed for D-Ψ-capsids. Scale bars are 500 nm for (A); 200 nm for (B, D); 300 nm for amhelin (C) and 1 µm (left) and 500 nm (right) for D-Ψ-capsids (C). On average 50 single microtomed macrophages (30-80) were analysed in each case.

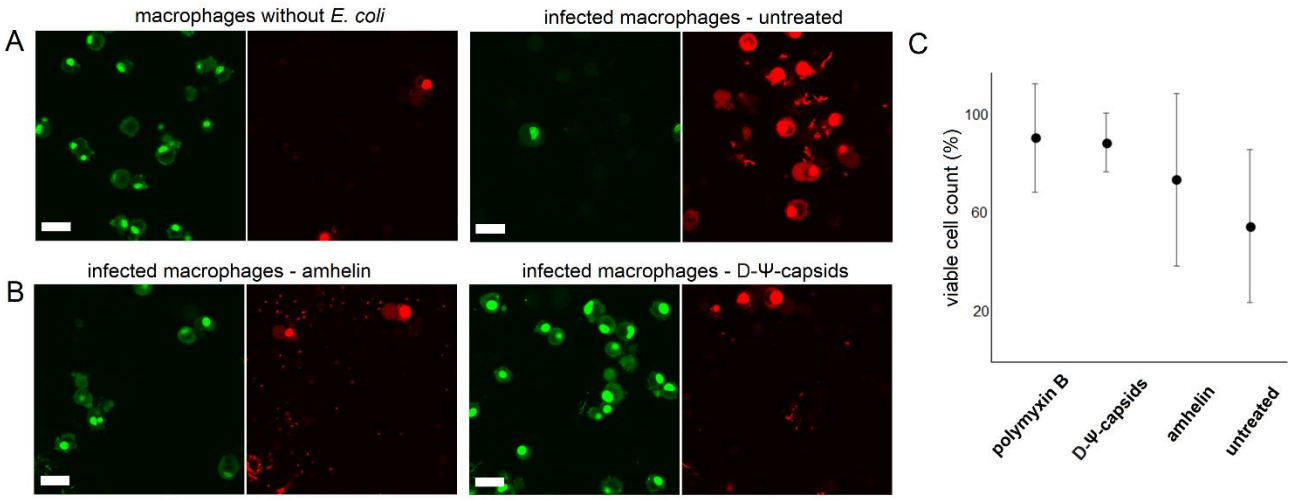

Figure S6. Impact of antibacterial treatment on infected macrophages using live-dead assays. Representative fluorescence micrographs at t = 240 min for (A) untreated macrophages without and with *E. coli* infection, and (B) infected macrophages. The cells were co-stained with SYTO 9 (green), which penettrates both dead and live cells, and propidium iodide – a membrane impermeant dye, which penetrates dead cells with damaged membranes. (C) Recovery rates of infected macrophages after antibacterial treatments. The recovery rate of macrophages without *E. coli* is taken as 100%. The rates are expressed as the percentage of viable cells in the total number of infected macrophages in each case. The data shows the mean ± SEM for three independent experiments performed in triplicate for each case.