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Supplemental information

Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy

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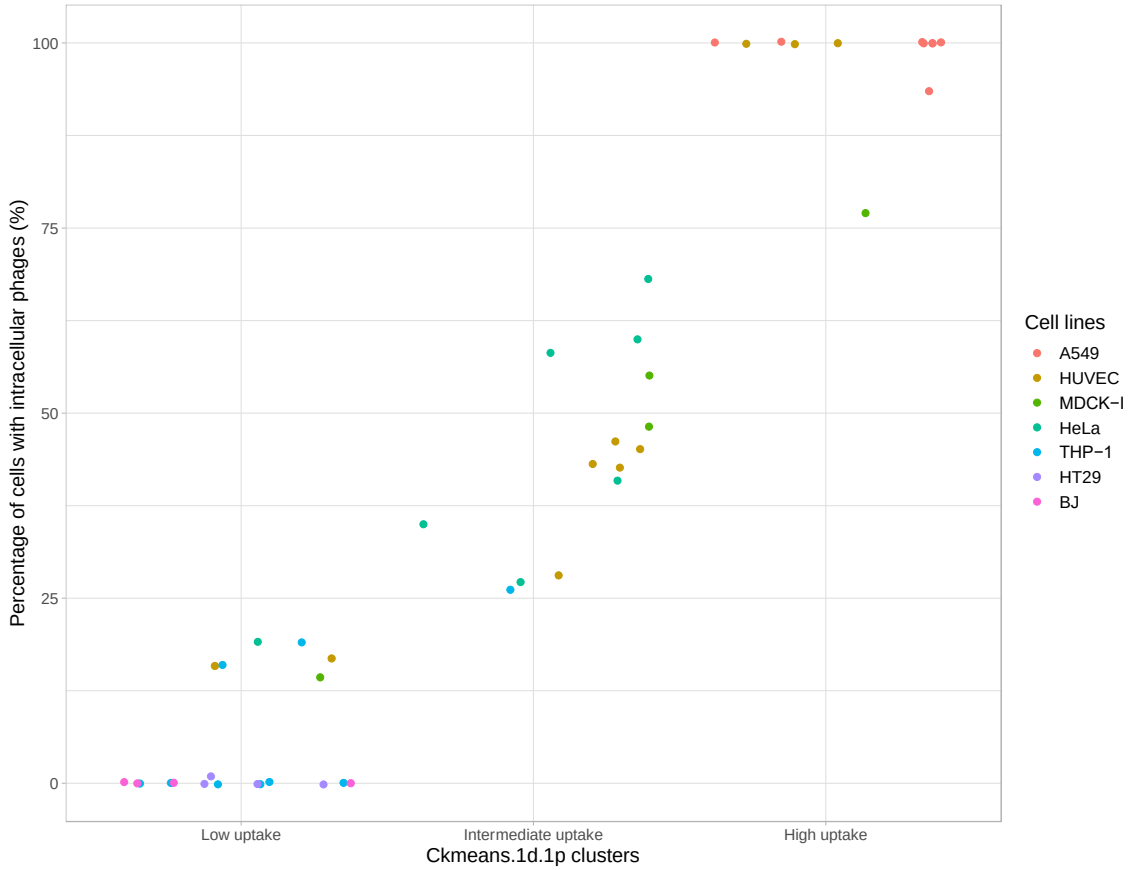

Figure S1. Clustering of cell lines, related to Figure 1. Clustering was performed using the dynamic programming algorithm in the R package Ckmeans.1d.dp. The given number of clusters was three shown here in the X axis (1 – low uptake, 2 – intermediate uptake and 3 – high uptake) and the percentage of uptake in Y axis. Each colour represents one of the cell lines tested.

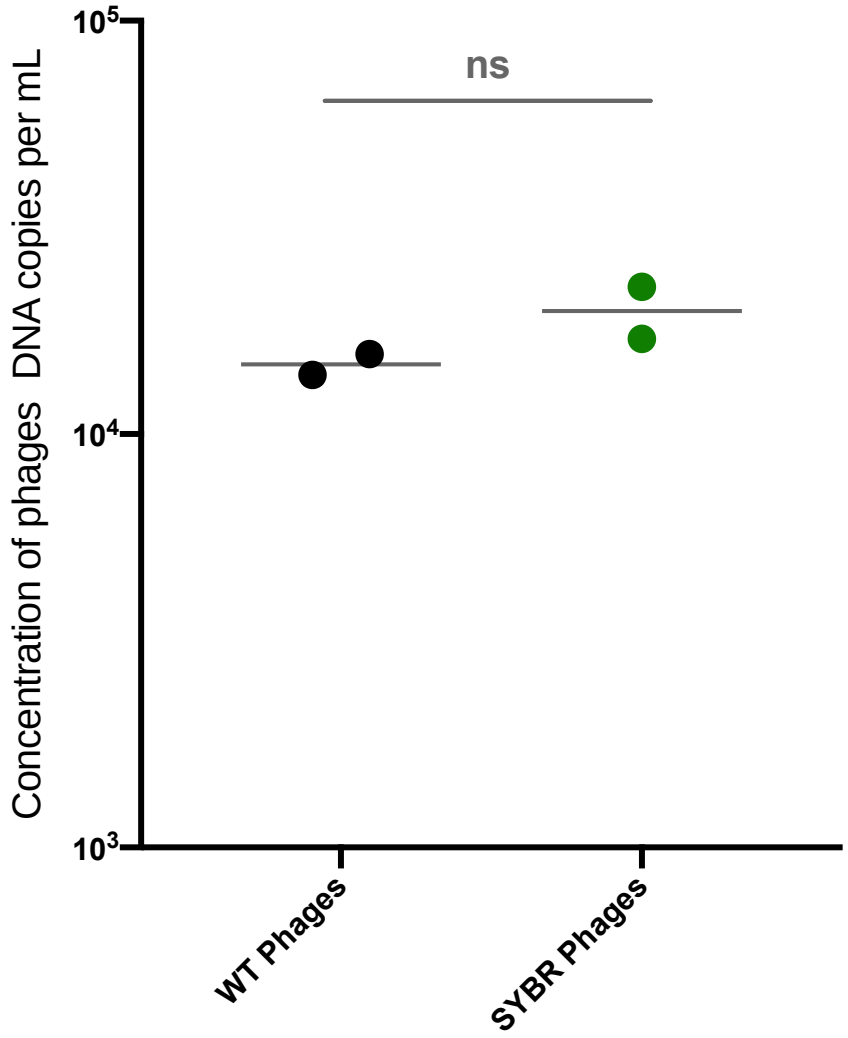

Figure S2. SYBR labelled phages rate of uptake compared to non-labelled phages, related to Figure 1. T4 phages labelled and non-labelled were applied on a confluent cell layer of A549 cells for 18 hours. The cells were then washed with DPBS and lysed for analysis using ddPCR. In the Y axis, the phages DNA copies. P-values calculated from a one-way unpaired t-test (ns: non-significant).

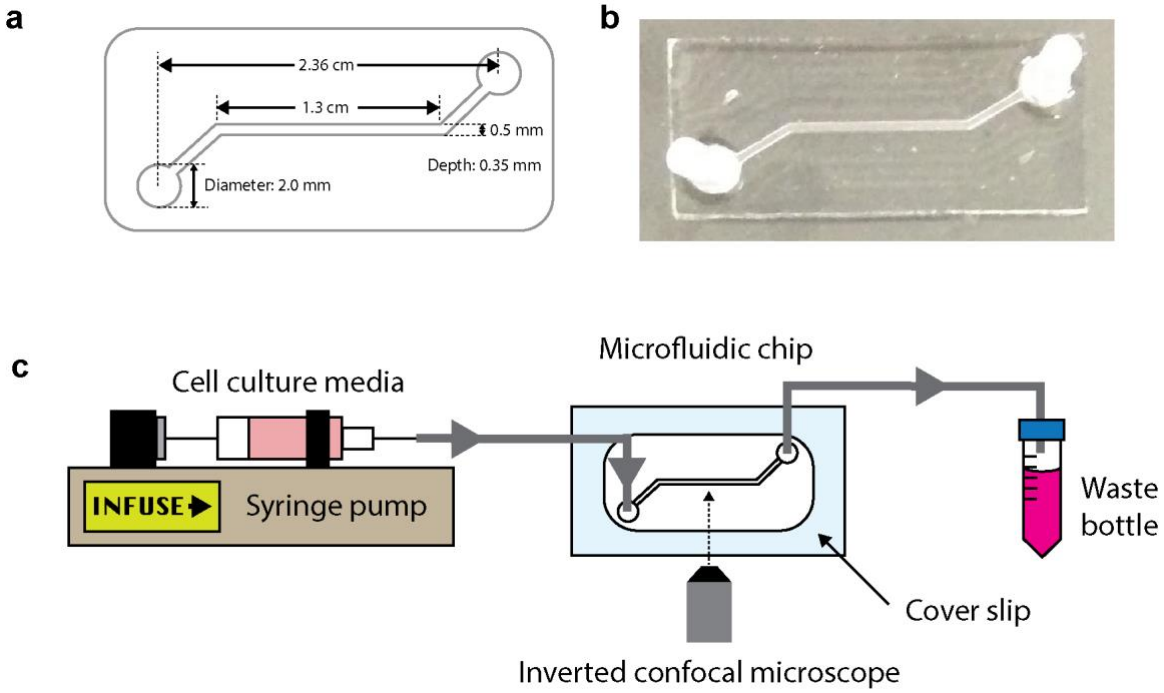

Figure S3. Microfluidic device and set-up for phage transcytosis experiment in flow conditions, related to Figure 3. **(a)** Chip channel dimensions, **(b)** the actual device picture. The chip was fabricated using PDMS and irreversibly bonded onto a glass cover slip using plasma. **(c)** Experimental set-up schematic for transcytosis experiment on flow. After inoculating the device with phages, the cell layer within the microfluidic chip was maintained under flow with egressing fluid collected as waste. Phage and cell layer behaviour were monitored and recorded via an inverted confocal microscope.

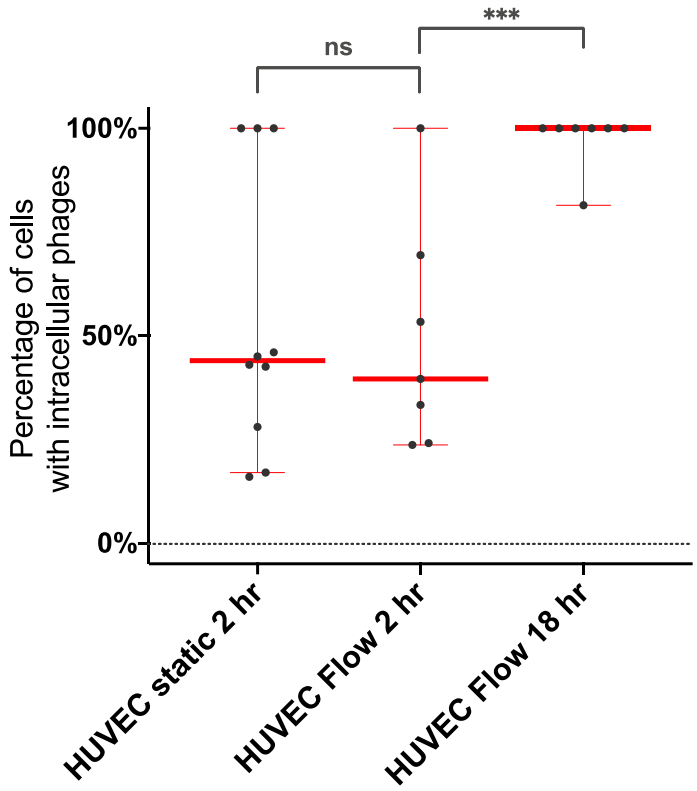

Figure S4. Intracellular phages under flow conditions, related to Figure 3. Percentage of HUVEC cells containing intracellular phages at the two-hour time point under static conditions and the two time points under flow condition of 0.1 dyne/cm^2, two hours and 18 hours. Scatter plots show medians of percentage of cells with intracellular phages; error bars represent 95% confidence intervals; each dot represents one Field of View (FOV). (static, n = 10; Flow 2 hr, n = 7; Flow 18 hours, n = 7). P-values calculated from a one-way unpaired t-test (P < 0.001: ***; ns: non-significant).

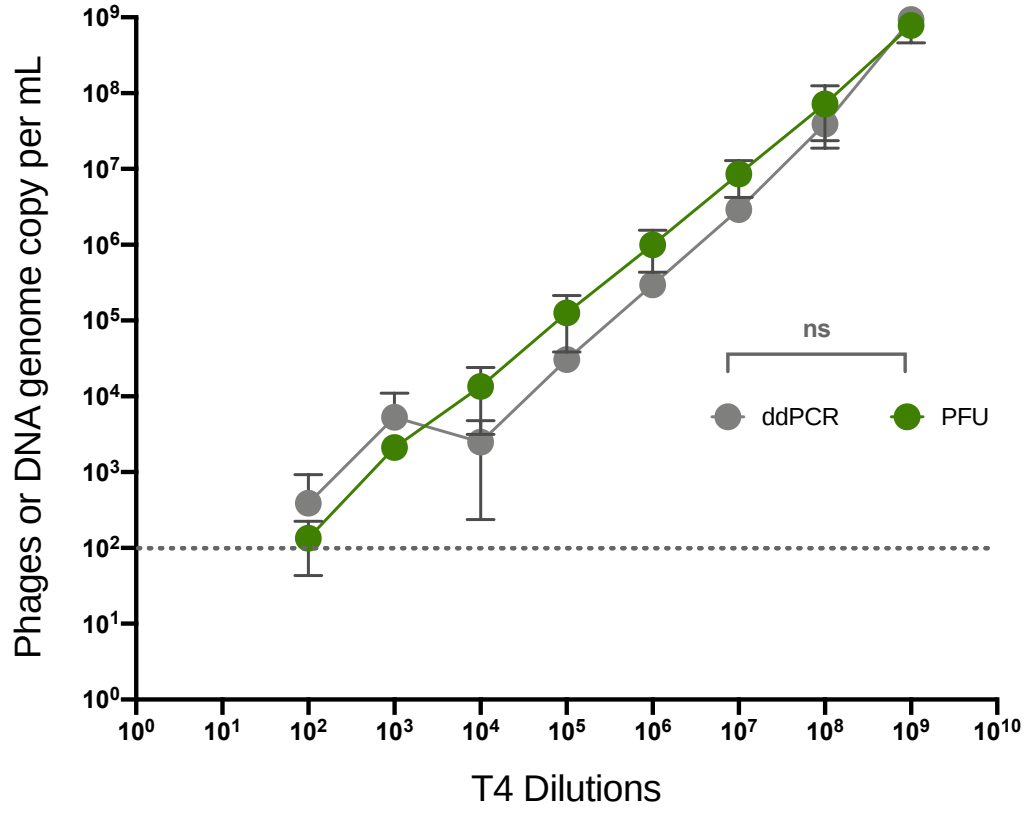

Figure S5. Dilution series of T4 phage stock using PFU and ddPCR techniques, related to Figure 5. X axis representing the dilution of the phage stock, in the Y axis the concentrations obtained with PFU or ddPCR in Phages or DNA genome copy per ml respectively. Shown in green line the PFU results and in grey the ddPCR results. Limit of detection is represented by the grey dotted line. P values calculated from paired T-test (ns: non-significant).

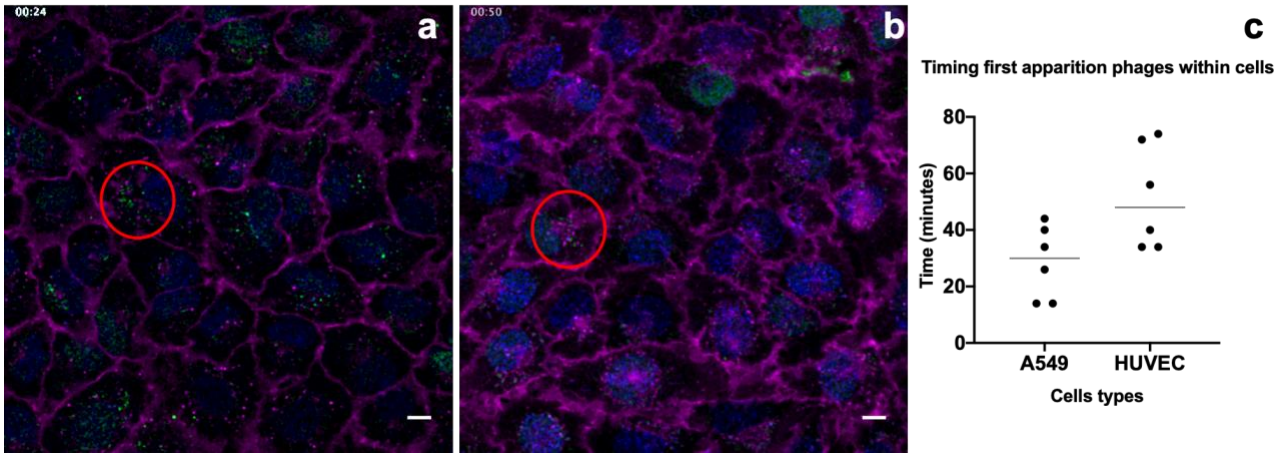

Figure S6. Time of first phage detection inside of cells, related to Figure 5. T4 phages were applied to **(a)** A549 cell layer or **(b)** HUVEC cell layer and incubated for two hours on a µ-Slide 8 well glass bottom slide on a microscope stage with temperature and CO_2 control. One image was acquired every two minutes on an inverted Leica SP8 confocal microscope with HC PL APO 63x/1.40 CS2 oil immersion objective. A hybrid detector (HyD) was used in sequential mode to visualise phage DNA. Cells were stained with nucleus stain, Hoescht 33342 (blue), plasma membrane stain, CellMask (magenta) and T4 phages labelled with DNA-complexing stain, SYBR-Gold (green). Scale bar: 10 µm; Timing: hours:minutes. The red circle represents the first detection of phages DNA within cells at 24 minutes for A549 cells and 50 minutes for HUVEC cells. **(c)** Graph representing the first time when phages appear within the cells in A549 and HUVEC cells in X axis with timing in minutes on the Y axis. Aligned dot plot with the median represented (n = 5 for A549 cells; n = 6 for HUVEC cells).

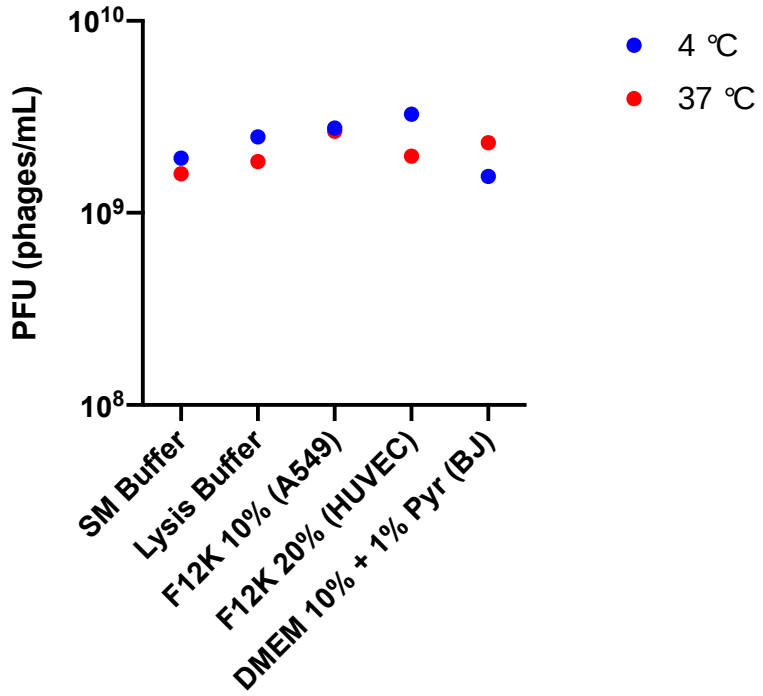

Figure S7. Concentrations of T4 phage stock using PFU techniques, related to Figure 5. X axis representing the different media of incubation: stocking media (SM Buffer), the lysis buffer used to lyse the cells and collect the intracellular phages, and the three spent cell culture media used to grow A549, HUVEC or BJ cells. The spent cell culture media was collected after two days of cells growth before being used in this control. In the Y axis the concentrations obtained with PFU in phages/ml. Shown in blue dots the results at 4 °C and in red dots the results at 37 °C. Non-significant P values calculated from one-way ANOVA.

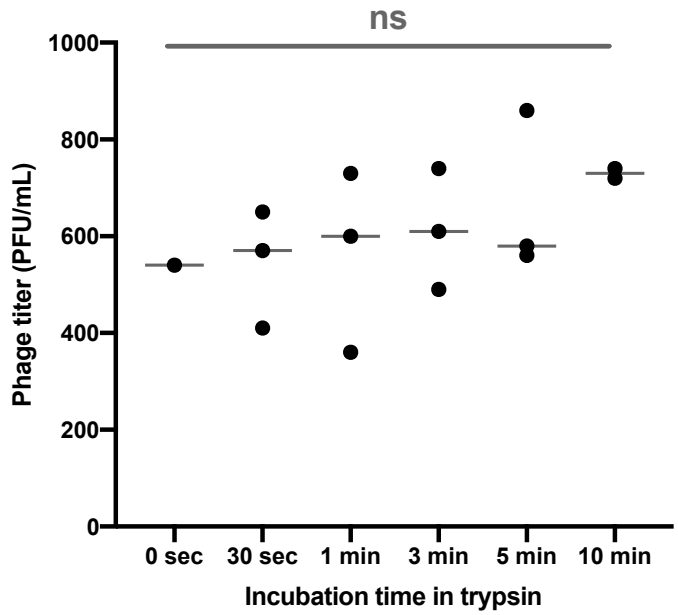

Figure S8. Concentrations of T4 phage incubated with 0.5% Trypsin at different incubation times and at 37 °C, related to Figure 5. X axis representing the different time of incubation zero to ten minutes. In the Y axis the concentrations obtained with PFU in phages/ml. Aligned dot plot with the median represented (n = 1 for 0 minutes; n = 3 for 30 sec, 1 min, 3 min and 5 min; n = 2 for 10 min). Non-significant P values calculated from one-way ANOVA.

Table S1. Shear stress calculation, related to Figure 3.

	Constant	μ: Viscosity of media at 37 °C (mPa·s)	Q: flow rate (m^3/s)	W: channel width (m)	H: channel height (m)	T: sheer stress ($dyne/cm^2$)
Values	6	0.78	1.33×10^{-10}	5×10^{-4}	3.5×10^{-4}	$T = \frac{6\mu Q}{WH^2}$

Table S2. Primers and probe DNA sequences for gp23 ddPCR assay, related to Figure 5.

Primers/probes	Sequences	Vendor
Primer gp23 Forward	5'-CTGCAGGTCAGACTTCTG-3'	Micromon
Primer gp23 Reverse	5'-CATCGGCTGAACACCAC-3'	Micromon
Probe gp23	5'-**56-FAM**/ACTCAGATT/**ZEN**/GGCCCAGCTGTT/**3IABkFQ**/-3'	Integrated DNA Technology (IDT)

Table S3. PCR mix for gp23 ddPCR assay, related to Figure 5.

	For one reaction (μl)	For 8 reactions (μl)
Super Mix for Probes	10	80
Forward gp23 Primer 10 μM	1.8	14.4
Reverse gp23 Primer 10 μM	1.8	14.4
Gp23 probe 20X	1	8
Water	5.4	43.2
Sample	2	-
Total	22	20 mix + 2 μl sample

Table S4. Thermal cycling conditions for gp23 ddPCR assay, related to Figure 5.

Cycling steps	Time (hr:min:sec)	Temperatures (°C)	Number of cycles
Enzyme activation	00:10:00	95	1
Denaturation	00:00:30	94	40
Annealing	00:01:30	55	40
Extension	00:00:30	72	40
Enzyme deactivation	00:10:00	98	1
Hold	∞	4	1

Tables S5. Calculation for math model and constants used, related to Figure 6.

Calculation of the first order constant:

$$\frac{d[ddPCR]}{dt} = k[ddPCR]$$

$$k = \frac{\ln[ddPCR_o] - \ln[ddPCR]}{t}$$

$[ddPCR]$	$phage\ conc.\ in\ central\ compartment$
$[ddPCR_o]$	$initial\ phage\ conc.\ administered$
t	$elapsed\ time$
k	$rate\ constant$
$[ddPCR_o]$ *at t:0 (DNA genome copies/mL)*	*1000000000*
$[ddPCR]$ *at t:30 seconds (DNA genome copies/mL)* *	*3392*
$[ddPCR]$ *at t:18 hours (DNA genome copies/mL)* *	*363073*

*Geometric mean of ddPCR data combined between each replicates and cells lines

83 Constants used in the model:
84

Data from	Lin et al. 2020
Central volume of distribution (mL/rat)	111
Maximum Elimination rate (PFU/h/rat)	43900000000
Peripheral volume of distribution 1 (mL/rat)	128
Peripheral volume of distribution 2 (mL/rat)	180
Intercompartmental clearance 1 (mL/h/rat)	30.4
Intercompartmental clearance 2 (mL/h/rat)	538
50% of the maximal elimination rate (PFU/mL/rat)	16400000
Dosing time	1
Number of rats per simulation	1
Simulation time (h)	50
Integration step	0.001
Phage initial dose (PFU/mL)	1000000000
First-order inactivation constant t:0 (1/h/rat)	0
First-order inactivation constant t:30 seconds (1/h/rat)	1511
First-order inactivation constant t:18 hours (1/h/rat)	0.44
Formula used to plot the graph in Fig. 6B	DV = log10(CENT/VC + 0.0001)

85

Transparent methods

Bacterial stocks and phage stocks. The bacterial strains used in this study, *Escherichia coli B* strain HER 1024 and *E. coli B* strain W3350, were cultured in lysogeny broth (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 litre of distilled water [dH_2O]) at 37 °C shaking overnight and used to propagate and titre phages T4, T3 and lambda supplemented with 10 mM $CaCl_2$ and $MgSO_4$. Phages T4, T3 and lambda were cleaned and purified using the Phage on Tap protocol (PoT) (Bonilla *et al*., 2016) and titred up to a concentration of approximately 10^{10} phages/ml. After purification, phages were stored in a final solution of SM Buffer (2.0 g $MgSO_4{\cdot}7H_2O$, 5.8 g NaCl, 50 ml of 1M Tris-HCl pH 7.4, dissolve in 1 litre of dH_2O) at 4 °C.

Endotoxin removal. For each of the phage samples, endotoxin removal protocol was followed from the Phage on Tap (PoT) protocol (Bonilla *et al*., 2016). Phages lysates were cleaned four times with octanol to remove endotoxins from the lysate and went from 5734 EU/ml, to 167 EU/ml in a phages stock solution at 10^{11} phages/ml. Only 10^9 phages/ml were used in the experiment bringing the endotoxin levels to ~1 EU/ml not including the further dilutions within the cell media. The levels of endotoxins applied to the cells were non-significant in regards to phage therapy use.

Cell line stocks. Seven cell lines were used in this study, all grown at 37 °C and 5% CO_2 and supplemented with 1% penicillin-streptomycin (Life Technologies Australia Pty. Ltd) A549 cells were grown in Ham's F-12K (Kaighn's) (also called F12-K)) (Life Technologies Australia Pty. Ltd) media with 10% Fetal Bovine Serum (FBS) (Life Technologies Australia Pty. Ltd), HUVECs were grown in F12-K media with 20% FBS, HeLa and HT-29 cells were both grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Australia Pty. Ltd) supplemented with 10% FBS, MDCK-I cells were grown in Modified Eagle Medium (MEM) (Life Technologies Australia Pty. Ltd) with 10% FBS, BJ cells were grown in DMEM media with 10% FBS and 1% sodium pyruvate (Sigma-Aldrich, Australia) and finally the suspension of THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 media (Life Technologies Australia Pty. Ltd) with 10% FBS. For differentiation, phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Australia) was added to a final concentration of 25 mM and incubated for 48 hours. After incubation PMA supplemented media was removed and cells were further grown in PMA free media for 24 hours to obtain differentiated macrophages. These differentiated cells were stable for up to one week.

Confocal microscopy. For confocal microscopy experiment, cells were seeded in an IBIDI µ-Slide 8-well glass-bottom slide (DKSH Australia Pty. Ltd) and grown to 80-90% confluency for acquisition. Cells were treated for 20 min with the respective culture media for each cell line with 5% Hoechst 33342 stain, excitation/emission 361/497 nm (Life Technologies Australia Pty. Ltd) and 1% CellMask deep red plasma membrane stain, excitation/emission 649/666 nm (Life Technologies Australia Pty. Ltd). After incubation cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) 1× and then left in Hank's Balanced Salt Solution (HBSS) with 1% FBS until acquisition. Purified phages were labelled with 1% SYBR-Gold, excitation/emission 495/537 nm (Life Technologies Australia Pty. Ltd) for one hour in the dark at 4 °C, followed by three washes with HBSS in Amicon-Ultra4 centrifugal unit 100-kDa membrane (Merck Pty. Ltd) to remove excess of stain. The washed phages were resuspended in a final volume of 1 ml in HBSS media. From a 10^9 phage per ml solution, we added 200 µl in each well to the cells under the microscope right before the start of the acquisition. The effect of SYBR-Gold on phage activity was tested using ddPCR by comparing labelled and un-labelled phages, with no significant effect detected (Fig. S2). Cells were imaged with HC PL APO 63x/1.40 Oil CS2 oil immersion objective by Leica SP8 confocal microscope on inverted stand with a hybrid detector (HyD) in real time. Excitation used for Hoechst 33342 (blue), SYBR-Gold and CellMask deep red was 405, 488 and 638 nm; corresponding emission was recorder at 412-462, 508-545 and 648-694 nm detection ranges respectively. HyD detector was used in sequential mode to detect the phages, it increases the sensitivity of detection by acquiring the same image multiple times and accumulating the fluorescence signal. All live cell imaging experiments were completed in triplicate (three fields of view in each session). One image was acquired every 2 minutes for 2 hours. Each field of view was hand-picked depending on the cell confluency and success of staining. Videos were created through post-processing using the FIJI software version 2.0.0-rc-68/1.52f (Schindelin *et al*., 2012). First, the three channels acquired were merged and processed with a Gaussian Blur filter of 0.8. Second, each channel brightness and contrast were enhanced for printing quality. Finally, the time and scale were added to the final movie saved in 12 fps.

Quantification of phages in live cell imaging. For each live cell experiment, we quantified cells that contained intracellular green fluorescence as indicative of SYBR-Gold labelled phages. Live cell images were acquired every ten minutes were quantified by manual counting the total number of cells in the field of view and the number of cells with intracellular phages to calculate the percentage of cells containing intracellular phages. Results were plotted using the GraphPad Prism version 8.4.2 for macOS GraphPad Software, San Diego, California USA, www.graphpad.com, to show uptake of phages over time.

Clustering analysis. Univariate clustering was performed using the dynamic programming algorithm in the R package Ckmeans.1d.dp (Wang and Song, 2011).

Flow conditions in microfluidic chip. A chip mould with 500 µm wide, 350 µm high and 1.3 cm long channels was designed using SolidWorks® 2017. The moulds were 3D-printed using Object Eden360V (Stratasys, USA) with a manufacturer-provided polymer FC720 and surface-salinised in a vacuum desiccator overnight with 20 µl trichloro(1*H*,1*H*,2*H*,2*H*-perfluorooctyl)silane (Sigma-Aldrich, USA). The microfluidic chips were manufactured via soft-lithography, by casting a 10:1 mixture of Sylgard 184 PDMS and its curing agent (Dowsil, USA)

respectively, onto the moulds and were cured at 90 °C until completely solidified (~2 hours). The chips were then cut with a surgical knife, gently peeled off, trimmed and their inlet and outlet were punched with 2 mm biopsy punchers (ProSciTech, Australia). Subsequently, the chips were washed in pentane and acetone to remove residual uncured PDMS. Atmospheric plasma generated at 0.65 Torr with high radio frequency was used to bound the PDMS chip to a glass cover slip No. 1.5H (0.170 mm ± 0.005 mm thickness) optimised for confocal microscopy (Marienfeld), for 20 seconds. The microchannel of the assembled microfluidic device were washed with ethanol (80% v/v)-sterilised, UV-sterilised and pre-treated with 1:50 MaxGel™ ECM (Sigma-Aldrich) in cold F12-K media for 2 hours at 37 °C and 5% CO_2. The microchannel was then washed with F12-K media to remove residual ECM. Schematic and picture of the microdevice is included in Fig. S3. The channel was seeded with 10 µl of HUVECs at a concentration of 5×10^5 cells/ml, which were carefully pipetted through the in port. The seeded chip was incubated statically for 12 hours to allow cell attachment at 37 °C and 5% CO_2. This was followed by perfusing the attached cells with complete media for 24 hours at 0.66 µl/min flow rate to establish a confluent cell layer. The cells were then perfused with cell culture media supplemented with 20% of FBS for another 24 hours at 8 µl/min to acclimate the cells to the shear stress. Perfusion was mediated by a single-channel syringe pump (New Era Pump Systems, USA) using a 10 ml 21 gauge needled-syringe fitted to Teflon tubes of 1/16" inner diameter and 1/32" outer diameter (Cole-Palmer, USA) that were previously sterilised using ethanol (80% v/v)-sterilised, DPBS and UVs. HUVECs were then stained with nucleus stain, Hoechst 33342 (blue), plasma membrane stain, CellMask (magenta) under static conditions for 20 minutes. T4 phages labelled with DNA-complexing stain, SYBR-Gold (green) were then added to the chip under 8 µl/min flow rate for either 2 or 18 hours. After incubation under flow with the phages, the in and out port of the chips were sealed using sterilized-binder paper clips and the chip placed under the microscope. The images were acquired with HC PL APO 63x/1.40 Oil CS2 oil immersion objective on an inverted Leica SP8 confocal microscope. A hybrid detector (HyD) was used to visualise phage DNA (excitation/emission 495/537 nm), other channels were acquired with conventional PMT detectors for CellMask (excitation/emission 649/666 nm) and for Hoechst 33342 (excitation/emission 361/497 nm).

Image analysis with CellProfiler. To quantify the fluorescence intensity of SYBR-Gold labelled phages (495 nm wavelength), we used a pipeline created in CellProfiler (McQuin *et al.*, 2018) (see the pipeline used in data SD1), allowing us to measure the pixel grey values as a proxy for fluorescence intensity across the image. First, we segmented regions covered by nuclei by applying the IdentifyPrimaryObjects module to the Hoechst channel image. Second, we defined expanded regions around the nuclei for cytoplasmic measurements using the IdentifySecondaryModule with the parameter Distance-N set to 200. Third, we masked out nuclei regions in the of the nuclei SYBR (phages) channel. This is to exclude fluorescence coming from the cell nuclei due to the leaking of the SYBR dye from the phage capsid to the cell nuclei, which would lead to false positive quantification. Finally, the grey values image intensity in the masked SYBR channel and additional parameters of the secondary objects were measured (Data SD1). Only a single time point at 2 hours at each field of view was used for the analysis. The number of images analysed for each condition varied, as manual quality control was applied to exclude out of focus and non-analysable fields of view.

Intracellular phages. For the intracellular phages experiment, cells were plated in T25 cm^2 flasks until they reached confluency. For the 18 hours experiment, phages were applied in volumes of 3 ml of media with 10^9 phages/ml per flask and incubated overnight at 37 °C and 5% CO_2. The control flasks were incubated with 3 ml of phage-free media. After the 18 hours incubation, control flasks were incubated with the same phage dilution for 30 seconds. The initial dilution for each flask was collected for quantification. Cells were washed three times with 5 ml of 1 × DPBS to remove non-adherent phages. Next, one ml of 0.5% trypsin was added to the flask and incubated at 37 °C and 5% CO_2 for a few minutes. Trypsin was tested to confirm that it did not have any effect on phage activity (Fig. S8). Once cells detached, the cells were resuspended in 5 ml of 1 × DPBS and spun at 1500 rpm for three minutes and washed three times with 5 ml 1 × DPBS to remove any non-adherent phages. After the washes, cells were resuspended in 1 ml of lysis buffer (0.5 M EDTA and 1 M Tris at pH 7.5, complete with dH_2O and adjust pH to 8) and left at room temperature for 20 min. After incubation the cells are passed through a 30 G syringe three times to ensure complete cell lysis. The lysis was confirmed by looking at the sample under a microscope.

ddPCR setup. Digital Droplet Polymerase Chain Reaction (ddPCR) was performed following manufacturer's instructions (Bio-Rad, Australia). A 20 µl reaction was assembled with primers, probe, ddPCR Supermix for probe (Bio-Rad, Australia) and sample. The primer and probe sequence and PCR parameters are shown in Table S2 - S4. ddPCR reaction mix was then loaded into eight channel disposable droplet generator cartridge (Bio-Rad, Australia). 70 µl of droplet generation oil was added to each channel and placed in the Bio-Rad QX200 droplet generator. The droplets were transferred into the deep well 96 well plate (Bio-Rad, Australia), using a multichannel pipette. The plate was then sealed using Bio- Rad plate sealer and then placed in a conventional thermocycler and the PCR product was amplified (Table S4). After amplification, the plate was loaded into the droplet reader (Bio-Rad, Australia) to quantify the fluorescent droplets. Analysis of the data was performed using the Poisson distribution with QuantaLife software (Bio-Rad, Australia).

PFU quantification. The Plaque Forming Unit (PFU) assay was performed using LB agar plates where a thin layer of soft LB agar was mixed with one ml of host bacterial culture and the desired dilution of phages was poured on the agar plate. The plate was incubated over-night at 37 °C before counting the number of plaques

formed on the bacterial lawn. The results were calculated in PFU. To obtain the number of inactive phages we subtracted PFU numbers (active phages) from the ddPCR numbers (total number of phages).

Pharmacokinetics model. A previously developed PK model in healthy rats was utilized to evaluate the impact of phage inactivation on *in vivo* phage disposition (Lin *et al.*, 2020). An additional compartment was incorporated to describe the inactivation and reactivation of phages by the epi- and endothelial cells. The rates of inactivation and reactivation was described by first-order rate constant, KD, and was assumed to be constant over time. The differential equations for phage disposition and inactivation were represented by:

$$\frac{dA_1(t)}{dt} = -(CL_T + Q_1 + Q_2)\frac{A_1}{V_1}(t) + Q_1\frac{A_3}{V_3}(t) - KD_1A_1(t) + KD_2A_4(t)$$

$$\frac{dA_2(t)}{dt} = Q_1\frac{A_1}{V_1}(t) - Q_1\frac{A_2}{V_2}(t)$$

$$\frac{dA_3(t)}{dt} = Q_2\frac{A_1}{V_1}(t) - Q_2\frac{A_3}{V_3}(t)$$

$$\frac{dA_4(t)}{dt} = KD_1A_1(t) - KD_2A_4(t)$$

$$CL_T = \frac{V_{max}}{K_m + \frac{A_1}{V_1}}$$

where

Q_1 = inter-compartmental clearance 1 (ml/h/rat).
Q_2 = inter-compartmental clearance 2 (ml/h/rat).
V_1 = Volume of distribution of the central compartment (ml/rat).
V_2 = Volume of distribution of the peripheral compartment 1 (ml/rat).
V_3 = Volume of distribution of the peripheral compartment 2 (ml/rat).
K_m = Phage titre that produces 50% of the maximal elimination rate of the system (PFU/ml/rat).
V_{max} = Maximum elimination rate (PFU/h/rat).
KD_1 = Inactivation rate constant (1/h).
KD_2 = Reactivation rate constant (1/h).

Deterministic was performed using model-predicted median PK parameters in rats without inter-individual variability and random unexplained variability (Code available in SD2 and SD3 and Table S5). Inactivation rate constant was determined using the ddPCR results as described in table S5. First order for the 30 seconds graph is of 1415 1/h/rat and for the 18 hours the first order is of 0.358 1/h/rat calculated from the ddPCR data. Reactivation rate constant was fixed to 0. Deterministic simulations were performed in R using mrgsolve (version 0.10.4) (Bunn and Korpela, 2019; Lin *et al.*, 2020).

244 **References**
245 Schindelin, J. *et al*. (2012) 'Fiji: an open-source platform for biological-image analysis', *Nature Methods*, 9(7).
246