SUPPLEMENTARY MATERIAL

Material and Methods

Zebrafish husbandry

Embryos were collected by natural spawning, staged according to Kimmel *et al*. (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995) and raised at 28°C in fish water (Instant Ocean, 0,1% Methylene Blue) in Petri dishes, according to established techniques. After 24 hpf, to prevent pigmentation 0,003% 1-phenyl-2-thiourea (PTU, Sigma-Aldrich, Saint Louis, Missouri, USA) was added to the fish water. Embryos were washed, dechorionated and anaesthetized with 0.016% tricaine (Ethyl 3-aminobenzoate methanesulfonate salt; Sigma-Aldrich), before observations, microinjection and picture acquisitions.

Bacterial strain preparation

P. aeruginosa PAO1 strain (Stover et al., 2000) cultures were grown with shaking at 37°C to OD_{600}= 0.5 (corresponding to about 5×10^8 cfu/ml) in LD broth (Ghisotti et al., 1992) added with carbenicillin (300 μg/ml). Then, culture was pelleted and resuspended in the same volume of physiological solution. Dilutions were used for microinjecting about 30 cells of PAO1 in *myd88*-MO embryos.

Phage cocktail (CKϕ) preparation.

The four virulent phages able to infect *P. aeruginosa* were isolated and characterized previously (Forti et al., 2018a). The phages belong to *Caudovirales* order and in particular two are *Podoviridae*, PYO2 (GenBank accession numbers vB_PaeP_PYO2, MF490236) and DEV (vB_PaeP_DEV, MF490238), and two *Myoviridae*, E215 (vB_PaeM_E215, MF490241), and E217 (vB_PaeM_E217, MF490240). Details of phages are reported in Table S1. The phage preparations were grown and purified as described (Forti et al., 2018a). Briefly, high-titer phage lysates of PAO1 cultures were filtrated with 1.2 μm diameter filters and incubated with DNase (1 μg/ml) and RNase

(1 µg/ml); then, treated lysates were PEG-precipitated, purified by cesium chloride ultracentrifugation and dialyzed against TN buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7). Finally, phage preparations were passed through endotoxin removal columns (EndoTrap HD; Hyglos, Germany). The levels of residual endotoxins in the phage preparations were below the limit value recommended for intravenous administration (5.0 international units/kg body mass/hour; http://www.who.int/medicines/publications/pharmacopoeia/Bacterial-endotoxins_QAS11-452_FINAL_July12.pdf) as assessed by measuring the endotoxin level with the LAL Chromogenic Endotoxin Quantitation assay (Pierce). The phage cocktail was assembled immediately before each experiment by mixing equivalent volumes of the four phage preparations at the same titer (phage cocktail titer, 5×10^8 pfu/ml).

Generation of zebrafish *cftr* and *myd88* morpholino knockdown

Injection of oligo-antisense morpholino were carried out on 1- to 2-cell stage embryos. Morpholinos were diluted in 1x Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, 5.0 mM HEPES (pH 7.6)) and the dye tracer rhodamine dextran was co-injected when necessary to allow visualization. *cftr* mRNA translation repression was achieved by co-injecting 0.25 pmole/embryo of each *cftr*-ATG-MO and *cftr*-splice-MO (Gene Tools LLC, Philomath, OR), as previously described (Phennicie, Sullivan, Singer, Yoder, & Kim, 2010). The block of *myd88* mRNA translation was achieved through the co-injection of 0.5 pmole/embryo of each *myd88*-ATG-MO and *myd88*-splice-MO, previously used and characterized (Llamas MA, 2014; Stockhammer, Zakrzewska, Hegedûs, Spaink, & Meijer, 2009; van der Vaart, van Soest, Spaink, & Meijer, 2013). A standard control morpholino oligonucleotide with no target in zebrafish (Gene Tools LLC) was injected as control.

Microinjection of zebrafish embryos with phage cocktail or PAO1.

Phage cocktail or PAO1 were microinjected into the duct of Cuvier to obtain a systemic delivery. For immune response experiments, 2 nl of TN buffer (TN) or phage preparation (phage cocktail, CKϕ, or single phage preparation) containing approximately 500-1000 pfu/embryo ($5x10^8$ pfu/ml) were microinjected into circulation of zebrafish embryos at 48 hpf. To titre the injected phages, drops of 2 nl of phage suspension were diluted in TN buffer and measured by agar overlay method (Gratia, 1932) to determine the pfu number. The titre of the injected phages/embryo was extrapolated from the average of five independent measures.

For infection experiments, 2 nl of PAO1 suspension containing approximatively 30 cells/embryo was microinjected into the duct of Cuvier, as previously described (Clatworthy et al., 2009). To titre the injected bacteria (cfu/embryo), drops of 2 nl of PAO1 suspension were diluted in physiological solution and plated. The evaluation of bacterial infection was performed following the guidelines of Takaki and colleagues (Takaki, Davis, Winglee, & Ramakrishnan, 2013) 20-30 embryos were injected for each single treatment, and each experiment was repeated at least three times.

Determination of PAO1 bacterial burden.

To measure bacterial burden related to PAO1 infection, embryos injected at 48 hpf were incubated at 28°C and were thoroughly washed in sterile PBS at 20 hpi and analyzed. Three groups of 15 embryos for each treatment were mechanically homogenized in 1% Triton X-100 in PBS by means of an insulin syringe (with a 27-gauge needle). The resulting homogenates were serially diluted and plated on LD agar. Ampicillin (100 μg/ml) was added to LD medium to select for the amp-resistant PAO1 strain, by limiting the growth of other bacterial strains (present in embryos and/or in embryo medium). Plates were incubated at 37°C for 16-20 hours. Then, colonies were counted and corresponding bacterial titers were calculated as the mean of the titers obtained for the three groups of homogenized embryos. The average cfu *per* embryo was extrapolated by dividing the obtained bacterial titer by the number of embryos in one group.

Determination of endogenous bacterial burden.

At least 15 embryos injected with TN buffer or CKϕ were thoroughly washed in sterile PBS at 20 hpi, homogenized 20 h post-injection and plated onto LD agar to allow the growth of colonies formed by the endogenous bacteria. Six morphologically different colonies were selected, inoculated in LD in different tubes and incubated 16 h at 37°C. The CKϕ was plated by agar overlay method using the above independent cultures as the source of bacterial indicator.

Phage inactivation treatments.

For phage inactivation experiments, UV-treated CKϕ (CKϕ UV) was produced as previously described (Hudson, Billington, Premaratne, & On, 2016), with some modifications. Briefly, 150 μl of phage suspension was placed in a petri dish and exposed to a germicidal UV lamp (254 nm) at room temperature; the distance from the UV source was 20 cm and the UV dose applied was 150 mJ/cm^{-2} for three cycle of exposition. To produce heat-treated CKϕ (CKϕ heat), phage suspension was placed in a centrifuge tube and heated to 100°C for 30 minutes in water bath. CKϕ was titred before and after each of the two physical inactivation treatments by means of agar overlay method. CKϕ UV and CKϕ heat were kept in ice until use.

Phage DNA isolation.

Genomic DNA was extracted from purified high titer phage preparations. 1 ml of the lysate was treated for 30 min at RT with $MgCl_2$ (12,5 mM final concentration), DNaseI (0,8U/ml) and RNase A (0,1 mg/ml). EDTA 20 mM, proteinase K 0,05 mg/ml and SDS 0,5% were added and the sample incubated at 55° for 60 min, by vortexing every 20 min. The DNA preparation was purified with phenol-chloroform and precipitated with ethanol.

Determination of the expression level of inflammation mediator genes.

Reverse transcription-PCR and real-time quantitative-PCR (RT-qPCR) assays were carried out to detect the mRNA expression levels of inflammation mediator genes, including cytokines, *IL-1β*, *TNF-α*, *IL-6* and *IFN-γ*; chemokines, *IL-8* and *CXCL-12a*; and neutrophil marker *mpx*. Total RNA was extracted from zebrafish embryos using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the producer's instructions. Concentration and purity of RNA were measured using the Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, US). To avoid possible genomic contamination, RNA was treated with DNase I RNase-free (Roche Diagnostics, Basel, Swiss). 1 µg of DNase-treated RNA was reverse-transcribed by means of the "ImProm-II™ Reverse Transcription System" (Promega, Madison, Wisconsin USA), using a mixture of random primers and oligo(dT), following the manufacturer's protocol. qPCRs were performed in a total volume of 20 µl containing 1X iQ SYBR Green Super Mix (Promega), using proper amount of synthesized cDNA. qPCRs were performed using the QuantStudio 5 (Thermo Fisher Scientific) following the manufacturer's guidelines. Thermocycling conditions were: 95°C for 10 min, 95°C for 10 s, and 55°C for 30 s. All reactions were performed at least in triplicate for 40 cycles. Primers used for mRNA expression analysis are listed in Table S2. The relative expression level of each gene was calculated according to the 2−ΔΔCt method (Livak & Schmittgen, 2001). For normalization purposes, *rpl8* was used as internal reference gene.

Live imaging of neutrophil migration assay.

For this assay zebrafish *Tg*BAC(*mpx*:EGFP)i114 line was used. To induce an acute inflammatory response, a portion of 3 dpf embryo tailfins was transected using a scalpel blade (5 mm depth), by slicing immediately posterior to the circulatory loop (avoiding to damage the circulatory loop), as previously described (Isles et al., 2019; Renshaw et al., 2006). Amputated embryos were locally microinjected repetitively for three times with 2 nl (each time) of TN buffer or CKϕ (5 x 10^8 pfu/ml) at wound site, spreading injection content in adjacent tissues. Injected embryos were incubated in fresh PTU, and at 6 hours post-amputation (hpa) single slice images were acquired using a fluorescence stereomicroscope (M205FA, Leica, Wetzlar, Germany) equipped with fluorescent lamp and a digital camera, and mounting GFP-filter (excitation of 488 nm). Bright-field and fluorescence images were sequentially acquired. Images were processed using the Adobe software and when necessary, different focal images planes were merged in a single image. Neutrophils count at the wound site were measured in *Tg*BAC(*mpx*:EGFP)i114 embryos using ImageJ software (Developer: Wayne Rasband) with "Cell Counter" plugin.

Neutrophil migration assay through Sudan black staining.

Embryos were processed as described above. At 6 hpa, embryos were fixed overnight in 4% paraformaldehyde (Sigma-Aldrich) PBS at 4°C, rinsed in PBS, then incubated in Sudan Black (Sigma-Aldrich, Saint-Quentin Fallavier, France) for 20 minutes and washed as described by Le Guyader *et al.* (Le Guyader et al., 2008). Single slice images of stained embryos were acquired using a microscope equipped with a digital camera with LAS Leica imaging software (Leica, Wetzlar, Germany) and processed as described above. Neutrophils count were measured as described above, using ImageJ software.

SUPPLEMENTARY FIGURE

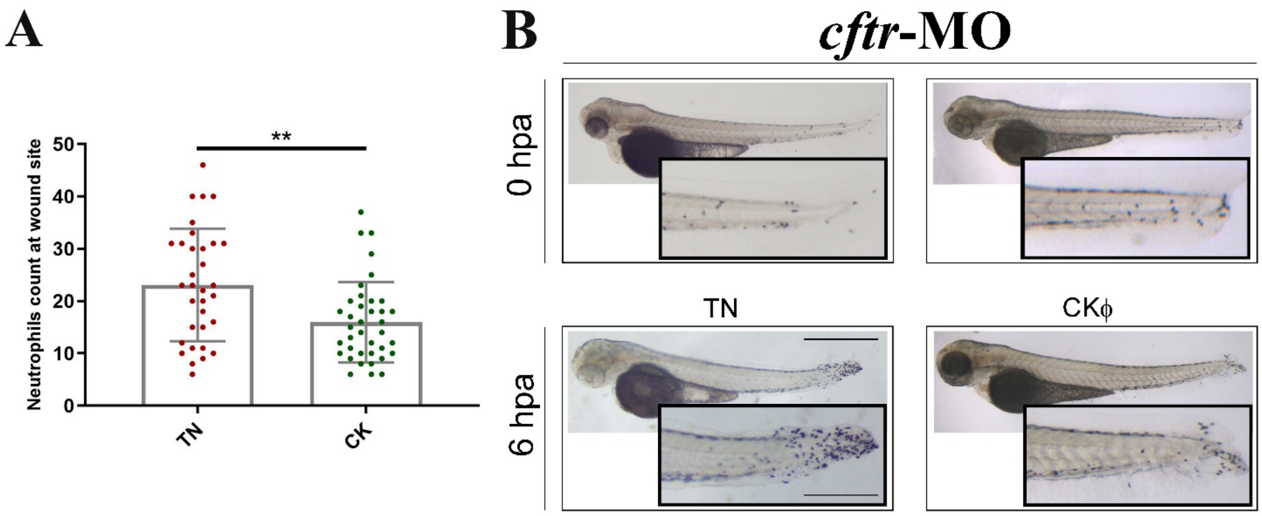

Figure S1. Local administration of phage cocktail (CKϕ) limits neutrophils migration toward the site of inflammation in CF embryos. (A) Neutrophils count at wound site at 6 hpa visualized by Sudan Black staining of 3 dpf CF embryos treated with TN or CKϕ. Each single dot represented a single embryo. Mean and SD of the two groups were given. Groups were assessed for statistical significance by unpaired Student's *t* test. (B) Representative images of Sudan Black-stained injured or uninjured CF embryos at 0 and 6 hpa, locally treated with TN or CKϕ. Images represented lateral views of whole embryo and a magnification of the corresponding caudal section. The upper panels showed uninjured and injured embryos at 0 hpa; the lower panels showed injured embryos treated with TN or CKϕ at 6 hpa. Scale bar, 50 μm and 100 μm. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns = not significant.

SUPPLEMENTARY TABLE

Table S1 genome sequence of phages. The genome sequences of the phages are deposited in GenBank. Growth parameters have been described in Forti et al. (Forti et al., 2018b)**.**

Table S2 primer list.

PHAGE	**Gene Bank Accession Number**	**Taxonomy**	**Genome lenght (bp)**	
vB_PaeP_PYO2	MF490236	Caudovirales; Podoviridae; Lit1virus	72697	
vB_PaeP_DEV	MF490238	Caudovirales; Podoviridae; Lit1virus	72697	
vB_PaeM_E215	MF490241	Caudovirales; Myoviridae; P1virus; unclassified Punalikevirus	66789	
vB_PaeM_E217	MF490240	Caudovirales; Myoviridae; P1virus; unclassified Punalikevirus	66291	

Gene	*Primer ff (5’- 3’)*	*Primer rev (5’- 3’)*
rpl8	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG
β-actin		
IL-1β	TGGACTTCGCAGCACAAAATG	CGTTCACTTCACGCTCTTGGATG
TNF-α	CTTCACGCTCCATAAGACCC	GCCTTGGAAGTGAAATTGCC
IL-6	TCAGAGACGAGCAGTTTGAG	GAGAGGAGTGCTGATCCTGA
IL-8	CGACGCATTGGAAAACACAT	TGTCATCAAGGTGGCAATGA
CXCL12a	CGTTCCACAGTCAACACAGT	GGCAATGACTTGGAAGGGG
IFN-γ	TGAATCTTGAGGAAAGTGAGCA	TCATCCACGCTGTCATTCTG
mpx	GCTGCTTACAAGTATTCTCG	ACGGCCTCCCGTGTCTTTCG

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