## TITLE

# Phages as immunomodulators and their promising use as anti-inflammatory agents in Cystic Fibrosis

## AUTHORS

Marco Cafora[1], Alessia Brix[1], Francesca Forti[2], Nicoletta Loberto[1], Massimo Aureli[1], Federica Briani[2], Anna Pistocchi[1*]

[1]Dipartimento di Biotecnologie Mediche e Medicina Traslazionale – Università degli Studi di Milano – LITA – via Fratelli Cervi 93 – 20090 Segrate (MI) - Italy

[2]Dipartimento di Bioscienze - Università degli Studi di Milano - Via Celoria 26 - 20133 Milano – Italy

*Correspondence to: anna.pistocchi@unimi.it

## DECLARATION OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. The funder has no role in data interpretations.

19 **ABSTRACT**

20 Cystic Fibrosis (CF), one of the most frequent hereditary disease due to mutations in the *CFTR*
21 gene, causes mortality in humans mainly due to infection in the respiratory system. However,
22 besides the massive inflammatory response triggered by chronic bacterial infections, a
23 constitutive pro-inflammatory state associated with the most common *CFTR* mutations has
24 been reported in paediatric cases before the onset of bacterial colonization. In a previous works
25 we isolated and characterized a mix of virulent bacteriophages (phage cocktail) able to
26 efficiently counteract *Pseudomonas aeruginosa* infection in a zebrafish model of CF, but also
27 showing anti-inflammatory effects in CF zebrafish embryos not infected by bacteria.
28 On these premises, in this work we demonstrated the anti-inflammatory role of the phage
29 cocktail both in the wild-type (WT) and hyper-inflamed CF zebrafish embryos in terms of
30 reduction of pro-inflammatory markers. We also dissect that only the virion proteinaceous
31 components but not the phage DNA, are responsible for the immune-modulatory effect and
32 that this action is elicited through the activation of the Toll-like Receptor (TLR) pathway. In
33 the CF context, we demonstrated that phages injection significantly reduces neutrophil
34 migration following acute inflammatory induction. The elucidation of the molecular interaction
35 between phages and the cells of vertebrate immune system might open new possibility in their
36 manipulation for therapeutic benefits especially in diseases such as cystic fibrosis,
37 characterized by chronic infection and inflammation.

38

## INTRODUCTION

Cystic Fibrosis (CF) is a complex disease in which bacterial infections and inflammation are challenging for successful therapies[1]. Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene lead to an imbalanced electrolyte composition of cellular secretions that accumulate in the organs, providing an ideal environment for bacterial colonization[2]. Chronic lung infections of pathogens like *Pseudomonas aeruginosa* are the primary cause of mortality in patients with CF[3]. Pro-inflammatory cytokines and chemokines recruit neutrophils and macrophages in the infected tissues which, in turn, further sustain their production, creating a positive feedback loop that strengthens the inflammatory response[4]. Inappropriate retention of neutrophils, which release their granule contents, worsens inflammation causing progressive tissue damages and fibrosis[1,5].

Besides the strong inflammatory response triggered by chronic bacterial infections, a constitutive inflammatory state associated with the most common *CFTR* mutations has been reported in paediatric cases before the onset of bacterial colonization[5,6]. Surprisingly, elevated levels of pro-inflammatory cytokines have been found even in the lungs of a 24-weeks old CF fetus[7]. So far, it is not clear how the constitutive inflammation is initiated, but some studies reported that accumulation of the mutant misfolded CFTR protein in the endoplasmic reticulum (ER) causes ER stress and initiates autophagy, triggering inflammatory stimuli[5,6]. Currently, both steroidal and non-steroidal drugs are used for long-term anti-inflammatory therapies in patients with CF[8]. However, the multiple adverse effects of anti-inflammatory therapies demand for new therapeutic treatments.

In the last years, the use of bacteriophages to treat bacterial infections (phage therapy) has regained interest as an alternative to classical antibiotics. In addition to their proven antibacterial action[9], bacteriophages can act as immunomodulators exerting anti-inflammatory activity. Evidence of this capability have been observed *in vitro* and in *in vivo* in several animal

64 models[10]. So far, little is known about the molecular mechanisms involved in such
65 immunomodulatory action but some studies reported an interaction between the host immune
66 system and phages[11–13]. The interplay between phages and innate immunity seems to be
67 dependent on the TLRs-Myd88-NFκB axis[14].
68 Zebrafish represents an ideal model for studies on innate immunity that is highly conserved
69 between fish and mammals with respect to both immune cell populations and inflammatory
70 pathways[15]. Moreover, some typical aspects of patients with CF, such as the lower capability
71 of counteracting bacterial infections and the altered neutrophils migration, have been
72 successfully recapitulated in *cftr*-loss-of-function zebrafish embryos[16]. In our previous work,
73 we reported that a four-phage cocktail (CKϕ) efficiently counteracted *P. aeruginosa* infections
74 but also mitigated the constitutive inflammatory state of the CF zebrafish embryos in the
75 absence of bacterial infections[17].
76 In this work, we investigated the immunomodulatory action of phages, focusing on pro-
77 inflammatory markers and on neutrophils-mediated inflammation, confirming the ability of
78 CKϕ to down-regulate the expression levels of pro-inflammatory genes in both the wild-type
79 (WT) and in the constitutive inflamed CF embryos.
80
81

82 **Material and Methods**

83 **Zebrafish husbandry**

84 Zebrafish (*Danio rerio*) were maintained at the University of Milan, Via Celoria 26 – 20133
85 Milan, Italy (Aut. Prot. n. 295/2012-A – December 20, 2012). Zebrafish strains AB, and
86 *Tg*BAC(*mpx*:EGFP)i114 strains, known as *Tg(mpx*:EGFP)[18], were maintained according to
87 international (EU Directive 2010/63/EU) and national guidelines (Italian decree No 26 of the
88 4th of March 2014). Embryos were staged and used as described in Supplementary Material.
89 For Sudan black staining analyses, embryos were fixed overnight in 4% paraformaldehyde
90 (PFA; Sigma-Aldrich) in Phosphate Buffer Saline (PBS; Sigma-Aldrich) at 4°C, then rinsed in
91 PBS.
92

93 **Bacterial strain preparation**

94 *P. aeruginosa* PAO1 strain[19] has been prepared as described in Supplementary Material.
95

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

97 Injection of oligo-antisense morpholino were carried as described in Supplementary Material.
98

99 **Microinjection of zebrafish embryos with phage cocktail or PAO1.**

100 Phage cocktail (CKϕ) or PAO1 were microinjected into the duct of Cuvier. For immune
101 response experiments, 2 nl of TN buffer (TN) or phage preparation (CKϕ or single phage
102 preparation) containing approximately 500-1000 pfu/embryo ($5x10^8$ pfu/ml) were
103 microinjected as described in Supplementary Material.
104 For infection experiments, 2 nl of PAO1 suspension containing approximatively 30
105 cells/embryo was microinjected into the duct of Cuvier, as described in[20] and in the
106 Supplementary Material.

107

108 **Determination of PAO1 and endogenous bacterial burden.**

109 To measure the bacterial burden related to PAO1 infection, embryos injected with PAO1 were
110 incubated at 28°C and were thoroughly washed in sterile PBS at 20 hpi and analyzed as
111 described in Supplementary Material. To measure the endogenous bacterial burden, embryos
112 injected with TN buffer or CKϕ were washed in sterile PBS, homogenized and plated onto LD
113 agar to allow the growth of colonies formed by the endogenous bacteria

114

115 **Phage inactivation treatments.**

116 For phage inactivation experiments, UV-treated CKϕ (CKϕ UV) was produced as previously
117 described in[21] and Supplementary Material.

118

119 **Phage DNA isolation.**

120 Genomic DNA was extracted from purified high titer phage preparations as described in
121 Supplementary Material.

122

123 **Determination of the expression level of inflammation mediator genes.**

124 Reverse transcription and quantitative-PCR (RT-qPCR) assays were carried out to detect the
125 mRNA expression levels of inflammation mediator genes, including cytokines, *IL-1β*, *TNF-α*,
126 *IL-6* and *IFN-γ*; chemokines, *IL-8* and *CXCL-12a*; and neutrophil marker *mpx* as described in
127 Supplementary Material.

128

129 **Neutrophil migration assays.**

130 To induce an acute inflammatory response, a portion of embryo tailfins was transected as
131 described in Supplementary Material. Amputated embryos were locally microinjected with 6
132 nl (of TN buffer or CKϕ (5 x $10^8$ pfu/ml) at wound site and analyzed at 6 hours post-amputation
133 (hpa). For live imaging of neutrophil migration analyses, we used the
134 *Tg*BAC(*mpx*:EGFP)i114[18] line and neutrophils were analyzed under a fluorescence
135 stereomicroscope (M205FA, Leica, Wetzlar, Germany) as described in Supplementary
136 Material. We also performed the staining of neutrophils with Sudan black as described in
137 Supplementary Material.

138

139 **Statistical analyses.**

140 Statistical analyses were performed as described in Supplementary Material. P-value $< 0.05$
141 was considered to indicate statistically significant differences. Data represent results of at least
142 three independent experiments, and mean ± SD or mean with min to max values were reported
143 in graphs.

144

145 **RESULTS**

146 **The phage cocktail (CKϕ) induces *per se* anti-inflammatory effects.**

147 In a previous work we demonstrated that a phage cocktail (CKϕ) capable of counteracting *P.*
148 *aeruginosa* infections[22] reduced the level of the pro-inflammatory cytokine *IL-1β* in infected
149 zebrafish embryos. Surprisingly, this happened also in the absence of bacterial infection[23]. To
150 better elucidate if the CKϕ is able to induce *per se* anti-inflammatory effects, we analyzed the
151 expression levels of a panel of pro-inflammatory markers. We microinjected the CKϕ into the
152 duct of Cuvier of 48 hpf embryos to generate a systemic delivery. For each treatment, 25-30
153 embryos were injected with approximately 500-1000 CKϕ pfu/embryo and the expression
154 levels of pro-inflammatory markers were measured by RT-qPCR at 20 hours post injection
155 (hpi). According to our previous evidence[23], the expression levels of *IL-1β*, *IL-6*, *IL-8* and
156 *CXCL12a,* but not TNF-α, were decreased in embryos treated with CKϕ in comparison to
157 embryos treated with TN buffer (**Fig. 1A**).

158 Although the effect of the CKϕ is strictly specific for *P. aeruginosa*, to exclude that the
159 decrease expression of pro-inflammatory markers was correlated with the reduction of
160 endogenous bacteria colonizing zebrafish larvae from early developmental stages[24], we
161 compared the basal bacterial titer of embryos treated with CKϕ or TN and we did not observe
162 significant differences (**Fig.1B**). Furthermore, we homogenized untreated embryos and plated
163 them on agar to allow the growth of endogenous bacteria. We than randomly selected six
164 different types of bacterial colonies and verified that the CKϕ was not able to infect and lyse
165 them (data not shown), demonstrating that the modulation of the inflammatory response is not
166 dependent to the CKϕ-mediated modulation of endogenous bacteria.

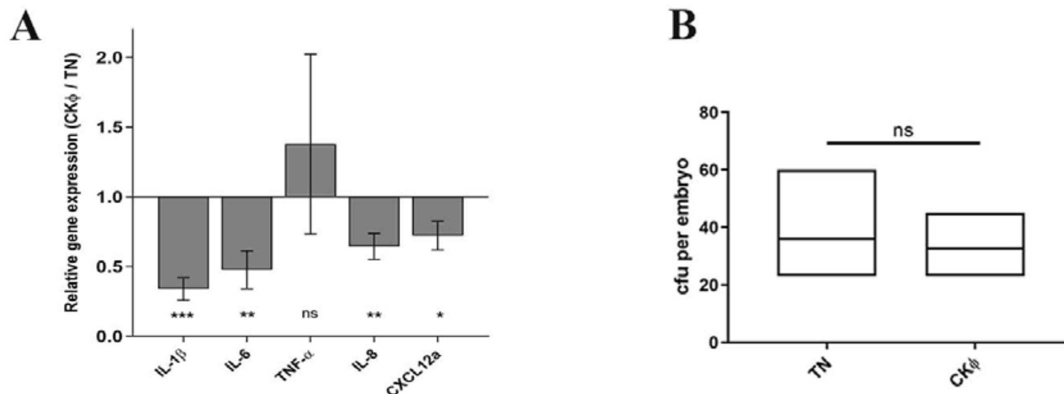


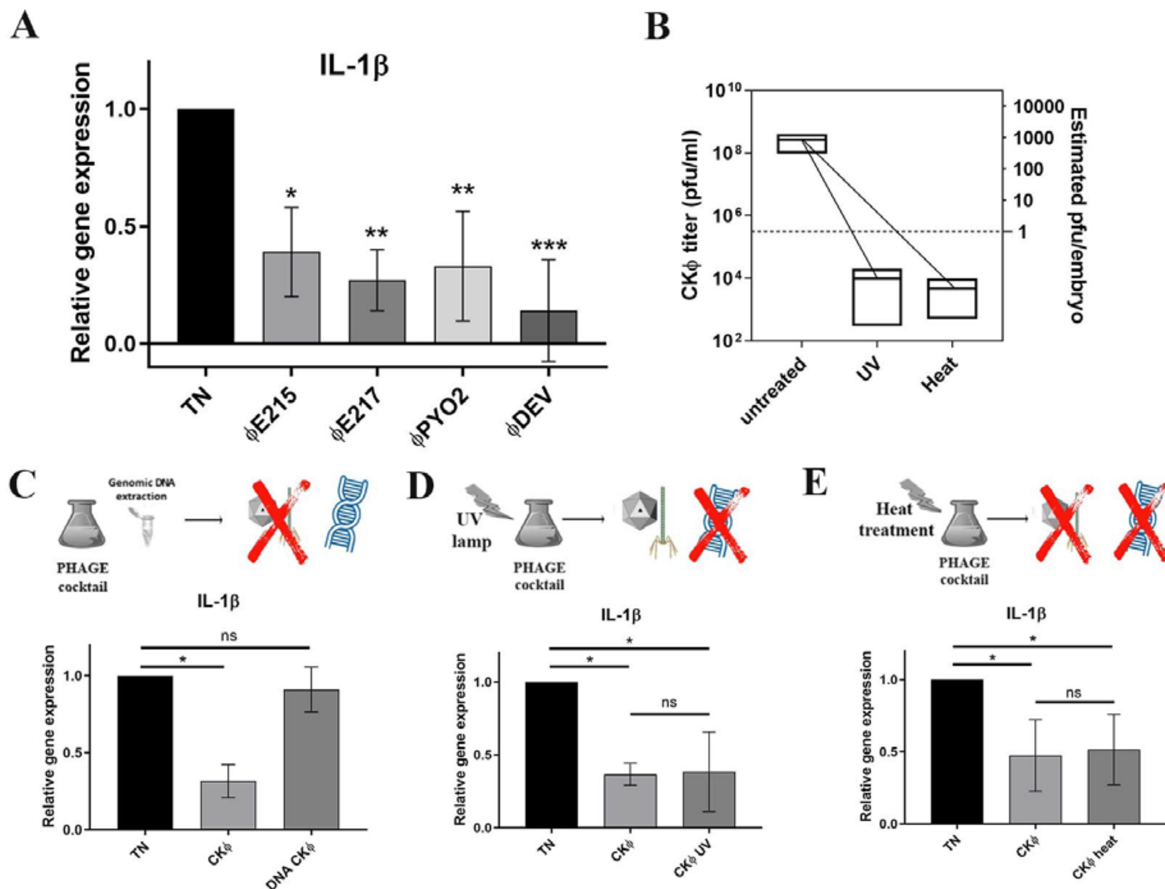

**Figure 1. Phage cocktail (CKϕ) modulates the immune response in zebrafish WT embryos without affecting the endogenous bacterial burden.** (A) Relative expression levels of inflammation mediator genes measured by RT-qPCR at 20 hpi in WT embryos treated with TN or CKϕ at 48 hpf. Data were normalized on expression levels of embryos treated with TN. The mean and SD of at least three independent experiments were reported. For each gene statistical significance was assessed by unpaired Student's *t* test, comparing TN and CKϕ expression levels. (B) Endogenous bacterial burden at 20hpi in WT embryos treated with TN or CKϕ at 48 hpf. Total cfu/embryo counts were given. In each experiment 30 or 45 embryos/treatment were included. The mean and the min to max values of three independent experiments were reported on floating bars chart. *p<0.05, **p<0.01, ***p<0.001, ns = not significant.

## The CKϕ and single phages composing it show similar immunomodulatory effects

The CKϕ is composed by four different *Caudovirales* lytic phages, two *Myoviridae* and two *Podoviridae*, as described in[22]. To elucidate the contribution to the immunomodulatory effect of each phage in the CKϕ, we microinjected zebrafish embryos at 48 hpf with single phage preparations at the same titer as CKϕ (approximately 500-1000 pfu/embryo), and measured by RT-qPCR the expression level of *IL-1β* at 20 hpi. Both *Myoviridae* (E215 and E217) and *Podoviridae* (PYO2 and DEV) treatments showed similar decrease in *IL-1β* expression levels in comparison to TN-injected controls (**Fig.2A**) suggesting that the immunomodulatory effect is not dependent to the phage type in the CKϕ.

190 **Phage proteins, and not phage DNA, have immunomodulatory effects.**

191 All the CKϕ phages have a dsDNA genome contained in the protein capsid[22]. To dissect the
192 contribution in immunomodulation of DNA *vs.* protein phage component, we assessed the
193 effect of: i) phage DNA (DNA CKϕ) isolated from the DEV phage; ii) CKϕ exposed to UV
194 irradiation to damage the phage DNA (CKϕ UV)[21] and iii) CKϕ heated at 100°C, to denature
195 both DNA and proteins (CKϕ heat)[25]. To verify the effect of UV and heat exposition on CKϕ,
196 we measured the CKϕ titre before and after the treatments and we found that CKϕ was
197 inactivated by the treatments as we observed a 4-5 $\log_{10}$ titer drop (**Fig.2B**). We assessed the
198 immunomodulatory effects of both phage DNA and inactivated CKϕ in comparison to
199 untreated CKϕ and TN buffer as negative control by microinjecting in the duct of Cuvier of 48
200 hpf embryos. We observed a significant reduction in the expression of *IL-1β* in embryos treated
201 with either UV- and heat-inactivated CKϕ as well as with active CKϕ, whereas the purified
202 phage DNA was not able to elicit such effect (**Fig.2 C,D,E**).

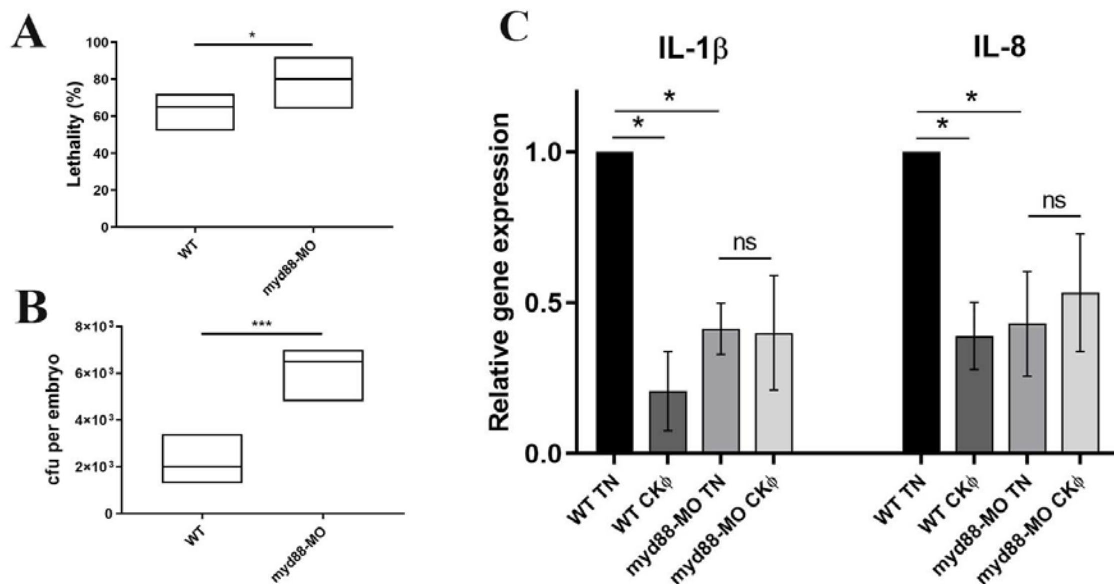



203

204 **Figure 2. Immunomodulatory effects of different components of phage cocktail (CKϕ) in**
205 **WT embryos.** (A) Relative expression levels of *IL-1β* gene measured by RT-qPCR at 20 hpi
206 in WT embryos treated with TN or with single phages of the CKϕ at 48 hpf. Data were
207 normalized on expression levels of TN-treated embryos. The mean and SD of three
208 independent experiments were reported. Statistical significance was assessed by One-way
209 ANOVA test followed by Tukey's post hoc correction. (B) Phage titre of treated or untreated
210 CKϕ. CKϕ was treated with an UV dose of 150 mJ/cm$^{-2}$ (CKϕ UV) or heated at 100°C for 30
211 minutes (CKϕ heat) before titration. Pfu/ml of UV-treated (UV), heat-treated (Heat) or
212 untreated CKϕ was reported on floating bars chart. On the chart was indicated also the
213 corresponding number of viable phage particles injected *per* embryo: the dotted line represents
214 the threshold below which less than one phage particle is injected. The mean and the min to
215 max values of three independent experiments were reported. (C, D, E). Relative expression
216 levels of *IL-1β* gene measured by RT-qPCR at 20 hpi in WT embryos treated with TN, CKϕ,
217 and CKϕ DNA (C), CKϕ UV (D) or CKϕ heat (E) at 48 hpf. Data were normalized on
218 expression levels of TN-treated embryos. The mean and SD of at least three independent
219 experiments were reported. Statistical significance was assessed by One-way ANOVA test
220 followed by Tukey's post hoc correction. *p<0.05, **p<0.01, ***p<0.001, ns = not significant.
221

222 **Immunomodulatory effects of phages are elicited through the Toll-like receptor pathway.**

223 Since studies have speculated that phages could elicit immunomodulatory effects in
224 mammalian systems through TLRs pathway[14], we aimed to evaluate if the immunomodulatory
225 effect of CKϕ was related to an interaction with the myeloid differentiation factor 88 (MyD88)-
226 dependent TLRs pathway highly conserved between zebrafish and mammals[26]. We generated
227 zebrafish *myd88*-loss-of-function embryos through the injection of a couple of *myd88*
228 morpholinos previously used and characterized[27]. We verified that embryos injected at 48 hpf
229 with a mix of 0.5 pmole/embryo of each *myd88*-MO and infected with approximately 30
230 cfu/embryo of *P. aeruginosa,* showed higher bacterial burden and, consistently, higher lethality
231 in comparison to controls at 20 hpi, as reported by other authors[27] (**Fig. 3A,B**). Moreover, in
232 *myd88*-MO embryos treated with TN buffer we observed a reduced basal level of
233 inflammation, (*IL-1β* and *IL-8* expression) in comparison to TN-injected WT embryos (**Fig.**
234 **3C**). On the contrary, the injection of the CKϕ in *myd88*-MO embryos did not elicit alteration
235 in inflammation, supporting the hypothesis that phages may induce an anti-inflammatory effect
236 in zebrafish embryos by modulating the MyD88-dependent TLRs pathway (**Fig. 3C**).
237




238

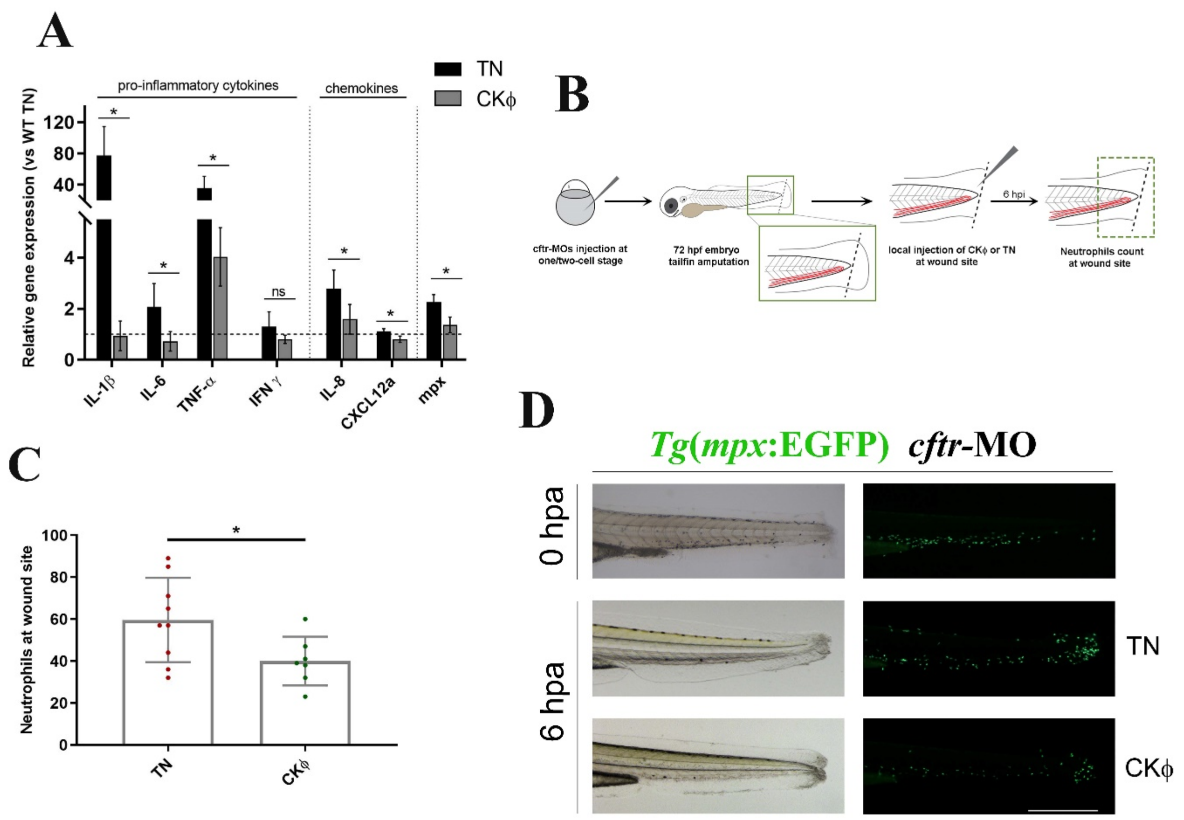
239 **Figure 3. Immunomodulatory effects of phages is dependent on Myd88 expression.** (A, B)
240 Validation of *myd88*-MO. WT and *myd88*-MO embryos were infected with approximately 30

241 cfu/embryo of PAO1 at 48 hpf: (A) embryos lethality at 20 hpi; (B) bacterial burden at 20hpi;
242 total cfu/embryo counts were given; the mean and the min to max values of three independent
243 experiments, each with 30-45 embryos/treatment, were reported on floating bars charts;
244 statistical significance was assessed by unpaired Student's *t* test. (C) Relative expression levels
245 of *IL-1β* and *IL-8* genes measured by RT-qPCR at 20 hpi in WT and *myd88*-MO embryos
246 treated with TN (WT TN and *myd88*-MO TN) or CKϕ (WT CKϕ and *myd88*-MO CKϕ) at 48
247 hpf. Data were normalized on the expression levels of WT TN. Mean and SD of three
248 independent experiments were reported. Statistical significance was assessed by One-way
249 ANOVA test followed by Tukey's post hoc correction. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns =
250 not significant.
251

252 **The CKϕ induces anti-inflammatory effects in CF zebrafish embryos.**

253 The reduced activity of Cftr determines *per se* a constitutive inflammatory state in CF zebrafish
254 embryos[23] as well as in human patients[5–7]. Therefore, we aimed to better elucidate the anti-
255 inflammatory effect of CKϕ in a CF background. We generated CF zebrafish embryos through
256 the injection of a couple of *cftr* morpholinos as previously described[23]. CF embryos were
257 microinjected at 48 hpf into the duct of Cuvier with CKϕ (approximately 500–1000
258 pfu/embryo) or TN buffer as a control and the expression of inflammatory markers was
259 evaluated. Interestingly, the expression of *IL-1β*, *TNF-α*, *IL-6*, *IL-8* and the neutrophil marker
260 *mpx* was significantly higher in CF embryos in comparison to WT at 20 hpi, in agreement with
261 our previous evidence[23], whereas *CXCL12a* and *IFN*-γ expression was not altered (**Fig. 4A**).
262 Previous studies have shown that phages directly interact with immune system cells or
263 influence their functions[28] with a possible interplay between phages and neutrophils[29,30]. Since
264 the inappropriate retention of neutrophils at inflammation sites in lungs represents a crucial
265 problem in CF patients and leads to tissue damage[6], we investigated if the CKϕ interacts with
266 neutrophils in CF zebrafish embryos. To study neutrophils migration, we used the transgenic
267 reporter line *Tg*BAC(*mpx*:EGFP)i114 (hereafter *Tg*(*mpx*:EGFP)), with GFP positive
268 neutrophils[18]. We induced an acute inflammatory response in 3 dpf *Tg*(*mpx*:EGFP) embryos,
269 previously injected with *cftr*-MOs, by means of amputation of a small portion of embryo tailfin

270 without damaging the circulatory loop. Immediately after the amputation, CF embryos were
271 locally microinjected with TN buffer or CKϕ at the wound site and incubated at 28°C for 6
272 hours post-tail fin amputation (6 hpa), to reach the peak of neutrophils recruitment at the wound
273 site[18,31] (**Fig. 4B**). We observed that GFP+ neutrophils were recruited at the wound site but the
274 migration was significantly reduced in CKϕ-injected than in TN-injected CF embryos (**Fig.**
275 **4C,D**). Indeed, while TN-injected CF embryos presented a mean of 59.6 GFP+ neutrophils at
276 wound site *per* embryo, in CKϕ-injected CF embryos the number decreased to 40 *per* embryo
277 (**Fig. 4C**). Similar result was obtained through Sudan black staining, considering
278 myeloperoxidase positive neutrophils (**Suppl. Fig.1**).




279

280 **Figure 4. Phage cocktail (CKϕ) down-regulates inflammatory mediators and limits**
281 **neutrophils migration toward the site of inflammation in CF embryos.** (A) Relative
282 expression levels of inflammation mediator genes measured by RT-qPCR at 20 hpi in CF
283 embryos treated with TN or CKϕ at 48 hpf. Data were normalized on expression levels of WT
284 embryos treated with TN (horizontal dotted line represent relative expression level of genes in
285 WT embryos treated with TN). Mean and SD of at least three independent experiments were
286 reported. For each gene statistical significance was assessed by unpaired Student's *t* test,

287 comparing expression levels in CF embryos treated with TN and CKϕ. (B, C, D) CKϕ local
288 administration limits neutrophils migration toward amputation site in 3 dpf CF embryos. (B)
289 Experimental schematic of neutrophils migration assay in 3dpf *Tg*(*mpx:*EGFP) embryos. (C)
290 Number of neutrophils at the wound site at 6 hpa in injured 3 dpf *Tg*(*mpx:*EGFP) CF embryos
291 treated with TN or CKϕ. Each single dot represented a single embryo. Mean and SD of the two
292 groups were given. Groups were assessed for statistical significance by unpaired Student's *t*
293 test. (D) Representative images of *Tg*(*mpx:*EGFP) CF embryos at 0 and 6 hpa, locally treated
294 with TN or CKϕ. Adjacent bright field and fluorescent images represent lateral views of caudal
295 section of the same embryo. Scale bar, 100 μm. *p<0.05, **p<0.01, ***p<0.001, ns = not
296 significant.
297

298 **Conclusions**

299 Progressive lung disease is the main cause of mortality in patients with cystic fibrosis (CF) and
300 is characterized by chronic bacterial airway infections with specific flora. This situation leads
301 to persistent airway inflammation, a major factor in irreversible lung damage[1]. However, it has
302 emerged that inflammation is an early event in CF patients, even in the absence of bacterial or
303 viral infections, thus representing a constitutive state[6,7]. In our previous work, we also observed
304 that the reduced levels of Cftr determines *per se* an altered constitutive inflammatory state in
305 CF zebrafish embryos and that systemic administration of a four-phage cocktail (CKϕ) was
306 able to mitigate it[23].
307 Although phages have been effectively used to fight bacterial infections (phage therapy) in a
308 large number of different vertebrate models including zebrafish[10], their immunomodulatory
309 abilities have been poorly studied. In this work, we confirmed the anti-inflammatory effect of
310 a phage cocktail, both in WT and CF zebrafish embryos. In zebrafish WT embryos we detected
311 a slight basal level of inflammation presumably due to the endogenous bacterial colonization[24],
312 and we showed that CKϕ administration down-regulated the expression of key pro-
313 inflammatory cytokines such as *IL-1β, IL-6* and chemokines *IL-8* and *CXCL12a*. Since we did
314 not detect alterations in the total bacterial burden of the embryos after the administration of the
315 phages, we assumed that the immunomodulatory effect of CKϕ is independent to a possible
316 antimicrobial activity of phages against the endogenous bacteria.

317 Our CKϕ is made up of four different virulent *Caudovirales* phages, belonging to two different
318 families, *Podoviridae* and *Myoviridae*. Considering the expression level of pro-inflammatory
319 cytokine *IL-1β*, which was the most affected inflammatory marker following CKϕ treatment,
320 we demonstrated that the contribution of each single phage to immunomodulation was
321 comparable, independently to the phage family. Similar results were obtained by administrating
322 four different *P. aeruginosa* phages belonging to three different *Caudovirales* families to
323 mononuclear human blood cells[32].
324 By investigating which component of the phage particle provokes immunomodulation, we
325 excluded phage DNA as it did not elicit immunomodulatory effects when systemically injected
326 in zebrafish embryos. The finding that phage nucleic acid, which is considered a potentially
327 dangerous component for therapeutic purposes, is dispensable for achieving phage-dependent
328 immunomodulation, could enhances the translational application of phage proteins as anti-
329 inflammatory agents in chronic deleterious inflammation as those present in patients with CF.
330 We also verified that heated-CKϕ retained anti-inflammatory properties and that that the
331 immunomodulation capabilities of CKϕ were not related to the geometry of the phage particle.
332 Therefore, we hypothesize that individual proteins of the virion, or even small peptides derived
333 from them, could exert immunomodulatory effects. This hypothesis is not new and phages were
334 demonstrated to bind to human lymphocytes and induce immunosuppressive effects through
335 mechanisms unrelated to their classic antibacterial action[12].
336 In a mouse model, it was demonstrated that the immunomodulatory effects of phages were
337 dependent on the TLR pathway[14]. To study a potential relationship between TLR pathways and
338 CKϕ, we generated a zebrafish model with impaired TLR functionality by downregulating
339 Myd88, the universal adapter for the signaling of almost all TLRs. Zebrafish has a functional
340 orthologues of Myd88[26] and its deficiency generated by means of morpholino injection
341 (*myd88*-MO embryos), led to an increased sensitivity to *P. aeruginosa* infection than the WT

342 siblings, due to the loss of immune system activation[27]. The administration of CKϕ in *myd88*-
343 MO embryos did not elicit immunomodulatory effects as in the WT siblings, confirming that
344 the TLR pathway is necessary for the CKϕ-mediated immune response.
345 As patients with CF, zebrafish embryos also show constitutive hyper-inflammation[23]. We
346 demonstrated that CKϕ administration mitigates basal hyper-inflammation in CF embryos by
347 reducing pro-inflammatory markers to levels comparable to WT embryos. In human patients
348 with CF, airway inflammation is characterized by inappropriate influx and retention of
349 neutrophils at inflammation sites, mainly drowned by host and bacteria produced chemo-
350 attractants, that leads to tissue damage. In particular, the increased levels of chemokine *IL-8*
351 amplify the neutrophil response through a positive feedback loop[4]. Therefore, phage-mediated
352 inhibition of neutrophilic chemotactic stimuli could lead to protective effects, as already
353 speculated in studies analyzing the interplay between phages and neutrophils[29,30]. In zebrafish
354 we demonstrated that, following acute inflammatory induction by tailfin amputation,
355 neutrophil migration was significantly reduced when the CKϕ was injected in the amputation
356 site. The reduced migration of neutrophils towards the inflammation site might be a
357 consequence of the CKϕ-mediated down-regulation of chemokines expression or of different
358 mechanisms such as early neutrophil desensitization or retrograde chemotaxis.
359 In summary, our results demonstrate that phages mitigate inflammation in zebrafish by
360 reducing the expression levels of pro-inflammatory cytokines and the neutrophilic recruitment
361 to the infection site. We speculate that the immunomodulatory action of phages occurs through
362 an interaction between TLRs and phage proteins (or peptides). Future work is necessary to
363 further elucidate the molecular mechanism of the interaction and how phages can be
364 manipulated for therapeutic benefits especially in diseases such as cystic fibrosis, characterized
365 by chronic infection and inflammation.
366

367 **References**


368 (1) Cantin, A. M.; Hartl, D.; Konstan, M. W.; Chmiel, J. F. Inflammation in Cystic
369 Fibrosis Lung Disease: Pathogenesis and Therapy. *Journal of Cystic Fibrosis*. 2015.
370 https://doi.org/10.1016/j.jcf.2015.03.003.
371 (2) Vankeerberghen, A.; Cuppens, H.; Cassiman, J. J. The Cystic Fibrosis Transmembrane
372 Conductance Regulator: An Intriguing Protein with Pleiotropic Functions. *J. Cyst.*
373 *Fibros.* **2002**. https://doi.org/10.1016/S1569-1993(01)00003-0.
374 (3) Stoltz, D. a; Meyerholz, D. K.; Welsh, M. J. Origins of Cystic Fibrosis Lung Disease.
375 *N. Engl. J. Med.* **2015**, *372* (4), 351–362. https://doi.org/10.1056/NEJMra1300109.
376 (4) De Rose, V. Mechanisms and Markers of Airway Inflammation in Cystic Fibrosis.
377 *Eur. Respir. J.* **2002**. https://doi.org/10.1183/09031936.02.00229202.
378 (5) Nichols, D. P.; Chmiel, J. F. Inflammation and Its Genesis in Cystic Fibrosis. *Pediatric*
379 *Pulmonology*. 2015, pp S39–S56. https://doi.org/10.1002/ppul.23242.
380 (6) Schultz, A.; Stick, S. Early Pulmonary Inflammation and Lung Damage in Children
381 with Cystic Fibrosis. *Respirology* **2015**, *20* (4), 569–578.
382 https://doi.org/10.1111/resp.12521.
383 (7) Verhaeghe, C.; Delbecque, K.; de Leval, L.; Oury, C.; Bours, V. Early Inflammation in
384 the Airways of a Cystic Fibrosis Foetus. *J. Cyst. Fibros.* **2007**.
385 https://doi.org/10.1016/j.jcf.2006.12.001.
386 (8) De Boeck, K.; Amaral, M. D. Progress in Therapies for Cystic Fibrosis. *The Lancet*
387 *Respiratory Medicine*. 2016. https://doi.org/10.1016/S2213-2600(16)00023-0.
388 (9) Kutter, E.; De Vos, D.; Gvasalia, G.; Alavidze, Z.; Gogokhia, L.; Kuhl, S.; Abedon, S.
389 Phage Therapy in Clinical Practice: Treatment of Human Infections. *Curr. Pharm.*
390 *Biotechnol.* **2010**. https://doi.org/10.2174/138920110790725401.
391 (10) Brix, A.; Cafora, M.; Aureli, M.; Pistocchi, A. Animal Models to Translate Phage

392 Therapy to Human Medicine. *Int. J. Mol. Sci.* **2020**, *21* (10), 3715.
393 https://doi.org/10.3390/ijms21103715.
394 (11) Barr, J. J. A Bacteriophages Journey through the Human Body. *Immunological*
395 *Reviews*. 2017. https://doi.org/10.1111/imr.12565.
396 (12) Górski, A.; Dąbrowska, K.; Międzybrodzki, R.; Weber-Dąbrowska, B.; Łusiak-
397 Szelachowska, M.; Jończyk-Matysiak, E.; Borysowski, J. Phages and
398 Immunomodulation. *Future Microbiol.* **2017**, *12* (10), 905–914.
399 https://doi.org/10.2217/fmb-2017-0049.
400 (13) An, T. W.; Kim, S. J.; Lee, Y. D.; Park, J. H.; Chang, H. I. The Immune-Enhancing
401 Effect of the Cronobacter Sakazakii ES2 Phage Results in the Activation of Nuclear
402 Factor-KB and Dendritic Cell Maturation via the Activation of IL-12p40 in the Mouse
403 Bone Marrow. *Immunol. Lett.* **2014**, *157* (1–2), 1–8.
404 https://doi.org/10.1016/j.imlet.2013.10.007.
405 (14) Gogokhia, L.; Buhrke, K.; Bell, R.; Hoffman, B.; Brown, D. G.; Hanke-Gogokhia, C.;
406 Ajami, N. J.; Wong, M. C.; Ghazaryan, A.; Valentine, J. F.; Porter, N.; Martens, E.;
407 O'Connell, R.; Jacob, V.; Scherl, E.; Crawford, C.; Stephens, W. Z.; Casjens, S. R.;
408 Longman, R. S.; Round, J. L. Expansion of Bacteriophages Is Linked to Aggravated
409 Intestinal Inflammation and Colitis. *Cell Host Microbe* **2019**, *25* (2), 285-299.e8.
410 https://doi.org/10.1016/j.chom.2019.01.008.
411 (15) Forn-Cuní, G.; Varela, M.; Pereiro, P.; Novoa, B.; Figueras, A. Conserved Gene
412 Regulation during Acute Inflammation between Zebrafish and Mammals. *Sci. Rep.*
413 **2017**. https://doi.org/10.1038/srep41905.
414 (16) Phennicie, R. T.; Sullivan, M. J.; Singer, J. T.; Yoder, J. A.; Kim, C. H. Specific
415 Resistance to Pseudomonas Aeruginosa Infection in Zebrafish Is Mediated by the
416 Cystic Fibrosis Transmembrane Conductance Regulator. *Infect. Immun.* **2010**, *78* (11),

417 4542–4550. https://doi.org/10.1128/IAI.00302-10.
418 (17) Cafora, M.; Deflorian, G.; Forti, F.; Ferrari, L.; Binelli, G.; Briani, F.; Ghisotti, D.;
419 Pistocchi, A. Phage Therapy against Pseudomonas Aeruginosa Infections in a Cystic
420 Fibrosis Zebrafish Model. *Sci. Rep.* **2019**. https://doi.org/10.1038/s41598-018-37636-
421 x.
422 (18) Renshaw, S. A.; Loynes, C. A.; Trushell, D. M. I.; Elworthy, S.; Ingham, P. W.;
423 Whyte, M. K. B. Atransgenic Zebrafish Model of Neutrophilic Inflammation. *Blood*
424 **2006**. https://doi.org/10.1182/blood-2006-05-024075.
425 (19) Stover, C. K.; Pham, X. Q.; Erwin, A. L.; Mizoguchi, S. D.; Warrener, P.; Hickey, M.
426 J.; Brinkman, F. S. L.; Hufnagle, W. O.; Kowallk, D. J.; Lagrou, M.; Garber, R. L.;
427 Goltry, L.; Tolentino, E.; Westbrock-Wadman, S.; Yuan, Y.; Brody, L. L.; Coulter, S.
428 N.; Folger, K. R.; Kas, A.; Larbig, K.; Lim, R.; Smith, K.; Spencer, D.; Wong, G. K.
429 S.; Wu, Z.; Paulsen, I. T.; Relzer, J.; Saler, M. H.; Hancock, R. E. W.; Lory, S.; Olson,
430 M. V. Complete Genome Sequence of Pseudomonas Aeruginosa PAO1, an
431 Opportunistic Pathogen. *Nature* **2000**. https://doi.org/10.1038/35023079.
432 (20) Clatworthy, A. E.; Lee, J. S. W.; Leibman, M.; Kostun, Z.; Davidson, A. J.; Hung, D.
433 T. Pseudomonas Aeruginosa Infection of Zebrafish Involves Both Host and Pathogen
434 Determinants. *Infect. Immun.* **2009**, *77* (4), 1293–1303.
435 https://doi.org/10.1128/IAI.01181-08.
436 (21) Hudson, J. A.; Billington, C.; Premaratne, A.; On, S. L. W. Inactivation of Escherichia
437 Coli O157:H7 Using Ultraviolet Light-Treated Bacteriophages. *Food Sci. Technol. Int.*
438 **2016**. https://doi.org/10.1177/1082013214560445.
439 (22) Forti, F.; Roach, D. R.; Cafora, M.; Pasini, M. E.; Horner, D. S.; Fiscarelli, E. V.;
440 Rossitto, M.; Cariani, L.; Briani, F.; Debarbieux, L.; Ghisotti, D. Design of a Broad-
441 Range Bacteriophage Cocktail That Reduces *Pseudomonas Aeruginosa* Biofilms and

442 Treats Acute Infections in Two Animal Models. *Antimicrob. Agents Chemother.* **2018**,
443 AAC.02573-17. https://doi.org/10.1128/AAC.02573-17.
444 (23) Cafora, M.; Deflorian, G.; Forti, F.; Ferrari, L.; Binelli, G.; Briani, F.; Ghisotti, D.;
445 Pistocchi, A. Phage Therapy against Pseudomonas Aeruginosa Infections in a Cystic
446 Fibrosis Zebrafish Model. *Sci. Rep.* **2019**, *9*, 1527.
447 https://doi.org/doi.org/10.1038/s41598-018-37636.
448 (24) Galindo-Villegas, J.; Garciá-Moreno, D.; De Oliveira, S.; Meseguer, J.; Mulero, V.
449 Regulation of Immunity and Disease Resistance by Commensal Microbes and
450 Chromatin Modifications during Zebrafish Development. *Proc. Natl. Acad. Sci. U. S.*
451 *A.* **2012**. https://doi.org/10.1073/pnas.1209920109.
452 (25) Duda, R. L.; Ross, P. D.; Cheng, N.; Firek, B. A.; Hendrix, R. W.; Conway, J. F.;
453 Steven, A. C. Structure and Energetics of Encapsidated DNA in Bacteriophage HK97
454 Studied by Scanning Calorimetry and Cryo-Electron Microscopy. *J. Mol. Biol.* **2009**,
455 *391* (2), 471–483. https://doi.org/10.1016/j.jmb.2009.06.035.
456 (26) Jault, C.; Pichon, L.; Chluba, J. Toll-like Receptor Gene Family and TIR-Domain
457 Adapters in Danio Rerio. *Mol. Immunol.* **2004**.
458 https://doi.org/10.1016/j.molimm.2003.10.001.
459 (27) van der Vaart, M.; van Soest, J. J.; Spaink, H. P.; Meijer, A. H. Functional Analysis of
460 a Zebrafish Myd88 Mutant Identifies Key Transcriptional Components of the Innate
461 Immune System. *Dis. Model. Mech.* **2013**, *6* (3), 841–854.
462 https://doi.org/10.1242/dmm.010843.
463 (28) Jault, P.; Leclerc, T.; Jennes, S.; Pirnay, J. P.; Que, Y. A.; Resch, G.; Rousseau, A. F.;
464 Ravat, F.; Carsin, H.; Le Floch, R.; Schaal, J. V.; Soler, C.; Fevre, C.; Arnaud, I.;
465 Bretaudeau, L.; Gabard, J. Efficacy and Tolerability of a Cocktail of Bacteriophages to
466 Treat Burn Wounds Infected by Pseudomonas Aeruginosa (PhagoBurn): A

467 Randomised, Controlled, Double-Blind Phase 1/2 Trial. *Lancet Infect. Dis.* **2019**.
468 https://doi.org/10.1016/S1473-3099(18)30482-1.
469 (29) Roach, D. R.; Leung, C. Y.; Henry, M.; Morello, E.; Singh, D.; Di Santo, J. P.; Weitz,
470 J. S.; Debarbieux, L. Synergy between the Host Immune System and Bacteriophage Is
471 Essential for Successful Phage Therapy against an Acute Respiratory Pathogen. *Cell*
472 *Host Microbe* **2017**, *22* (1), 38-47.e4. https://doi.org/10.1016/j.chom.2017.06.018.
473 (30) Pabary, R.; Singh, C.; Morales, S.; Bush, A.; Alshafi, K.; Bilton, D.; Alton, E. W. F.
474 W.; Smithyman, A.; Davies, J. C. Antipseudomonal Bacteriophage Reduces Infective
475 Burden and Inflammatory Response in Murine Lung. *Antimicrob. Agents Chemother.*
476 **2016**. https://doi.org/10.1128/AAC.01426-15.
477 (31) Isles, H. M.; Herman, K. D.; Robertson, A. L.; Loynes, C. A.; Prince, L. R.; Elks, P.
478 M.; Renshaw, S. A. The CXCL12/CXCR4 Signaling Axis Retains Neutrophils at
479 Inflammatory Sites in Zebrafish. *Front. Immunol.* **2019**, *10*.
480 https://doi.org/10.3389/fimmu.2019.01784.
481 (32) Van Belleghem, J. D.; Clement, F.; Merabishvili, M.; Lavigne, R.; Vaneechoutte, M.
482 Pro- and Anti-Inflammatory Responses of Peripheral Blood Mononuclear Cells
483 Induced by Staphylococcus Aureus and Pseudomonas Aeruginosa Phages. *Sci. Rep.*
484 **2017**. https://doi.org/10.1038/s41598-017-08336-9.
485
486

487 **Authors contribution:**

488 AP, MA, FB Conceptualization; AP, FB, MA Data curation; AP, FB, FF, NL, AB, MC, MA

489 Formal analysis; AP, MA Funding acquisition; FF, NL, AB, MC Investigation; FF, NL, AB,

490 MC Methodology; AP, FB, MA Project administration; AP, FB, MA Resources; AP Software;

491 AP Supervision; FF, NL, AB, MC Validation; AP, FB, FF, NL, AB, MC Visualization; AP,

492 AB, MC Roles/Writing - original draft.

493