The small RNA ErsA plays a role in the regulatory network of *Pseudomonas aeruginosa* pathogenicity in airways infection

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Abstract

Bacterial small RNAs play a remarkable role in the regulation of functions involved in host-pathogen interaction. ErsA is a small RNA of *Pseudomonas aeruginosa* that contributes to the regulation of bacterial virulence traits such as biofilm formation and motility. Shown to take part in a regulatory circuit under the control of the envelope stress response sigma factor σ^{22}, ErsA targets post-transcriptionally the key virulence-associated gene *algC*. Moreover, ErsA contributes to biofilm development and motility through the post-transcriptional modulation of the transcription factor AmrZ. Intending to evaluate the regulatory relevance of ErsA in the pathogenesis of respiratory infections, we analyzed the impact of ErsA-mediated regulation on the virulence potential of *P. aeruginosa* and the stimulation of the inflammatory response during the infection of bronchial epithelial cells and a murine model. Furthermore, we assessed ErsA expression in a collection of *P. aeruginosa* clinical pulmonary isolates and investigated the link of ErsA with acquired antibiotic resistance by generating an *ersA* gene deletion mutant in a multidrug-resistant *P. aeruginosa* strain which has long been adapted in the airways of a cystic fibrosis (CF) patient. Our results show that the ErsA-mediated regulation is relevant for the *P. aeruginosa* pathogenicity during acute infection and contributes to the stimulation of the host inflammatory response. Besides, ErsA could be subjected to selective pressure for *P. aeruginosa* patho-adaptation and acquirement of resistance to antibiotics commonly used in clinical practice during chronic CF infections. Our findings establish the role of ErsA as an important regulatory element in the host-pathogen interaction.

Author summary

Pseudomonas aeruginosa is one of the most critical multi-drug resistant opportunistic pathogen in humans, able to cause both lethal acute and chronic lung infections. Thorough knowledge of the regulatory mechanisms involved in the establishment and persistence of the airways infections by *P. aeruginosa* remains elusive. Emerging candidates as molecular regulators of pathogenesis in *P. aeruginosa* are small RNAs, which act post-transcriptionally as signal transducers of host cues. Known for being involved in the regulation of biofilm formation and responsive to envelope stress response, we show that the small RNA ErsA can play regulatory roles in acute infection, stimulation of host inflammatory response, mechanisms of acquirement of antibiotic resistance and adaptation during the chronic lung infections of cystic fibrosis patients. Elucidating the complexity of the networks regulating host-pathogen interaction is crucial to identify novel targets for future therapeutic applications.

54 ## Introduction

55 The bacterium *Pseudomonas aeruginosa* is a common pathogen associated with
56 respiratory tract infections in patients with diverse diseases (1-3). *P. aeruginosa* causes
57 fatal acute lung infections in critically ill individuals who are for instance hospitalized,
58 intubated in an intensive care unit, or immune-compromised (e.g. transplant recipients,
59 patients with burns, cancer, and neutropenia, or infected with HIV). In acute pneumonia,
60 *P. aeruginosa* causes necrosis of the lung epithelium and disseminates into the
61 circulation, resulting in septic shock and multiple organ failure. *P. aeruginosa* is also a
62 major cause of chronic lung infections in individuals with cystic fibrosis (CF), non-CF
63 bronchiectasis, and chronic obstructive pulmonary disease (COPD). It was shown that
64 long-term *P. aeruginosa* persistence in CF airways triggers tissue remodeling that finally
65 leads to lung function decline and ultimately results in respiratory failure.
66 Biofilm formation is a well-known essential requisite for *P. aeruginosa* during chronic
67 airways infections (4). However, the relevant role of biofilm aggregation of *P. aeruginosa*
68 on the apical surface of polarized epithelial cells at early time points of acute lung
69 infections has also been pinpointed (5-7), challenging the classical notion that acute
70 infections are associated only with the planktonic lifestyle. Indeed, *P. aeruginosa* initiates
71 most acute infections with a transition from planktonic bacteria to host cell-attached
72 aggregates (7). Initial binding of individual sentinel bacteria at the mucosal barrier through
73 two major adhesins, flagella, and retractile type IV pili (8) leads in few minutes to the
74 recruitment of free-swimming bacteria, with the resultant formation of antibiotic-resistant
75 biofilm-like bacterial aggregates of ten to hundreds of bacteria embedded in an
76 exopolysaccharide (EPS) and extracellular DNA (eDNA) matrix and localized in spots on

77 the host cell surface (5, 6). Surface-bound bacterial aggregates, and not individual
78 bacteria, trigger a dramatic remodeling of the apical membrane, namely the formation of
79 protrusions (6). Apical membrane remodeling is linked with localized nuclear translocation
80 of NF-kB underneath aggregates but not beneath single bacteria (9). This indicates the
81 activation of the innate immune response to bacterial aggregates (9). However,
82 aggregate-induced protrusion formation is necessary, but not sufficient, for activation of
83 the innate immune response (9). Indeed, NF-kB activation and the subsequent production
84 of pro-inflammatory cytokines require both pathogen-induced membrane protrusions and
85 the recognition of pathogen-associated molecular patterns (PAMPs) such as flagellin or
86 lipopolysaccharide (LPS) via the cognate Toll-like receptors (TLRs) (10). Once mucosal
87 colonization is established, *P. aeruginosa* delivers a large battery of virulence factors to
88 cause disease, for instance through the type III secretion system (T3SS) that is also
89 required for the bacterial aggregate-mediated induction of membrane protrusions (6). At
90 this stage of acute infection, all virulence factors participate, at different levels, in the
91 cytotoxicity of *P. aeruginosa* that leads to bypassing the epithelial barrier and then to
92 invasion and systemic dissemination (1). Most of the *P. aeruginosa* invasive functions
93 characteristics of acute infection are selected against in CF chronic infection leading to
94 less virulent but more persistent phenotypes (4, 10).
95 The two pathogenetic processes associated to the progression of *P. aeruginosa* airways
96 infection towards either rapid and acute systemic dissemination or chronic colonization
97 are complex and depend on the coordinate up- or down-regulation of several virulence
98 lifestyle functions that imply both short- and long-term adaptation to host environment (4,
99 11, 12). For instance, in the pathogenesis of CF chronic infections, *P. aeruginosa* adapted

100 variants can shape the innate immune response favoring their persistence and contribute
101 to the emergence of CF airway hallmarks (13). *P. aeruginosa* adaptive response leading
102 to pathogenesis relies on a wide, intricate, and "prone to remodeling" regulatory network
103 formed both by transcription factors and post-transcriptional regulators including also
104 small RNAs (sRNAs) (14-16). The dynamicity of this regulatory network is frequently
105 observed during the adaptive radiation of *P. aeruginosa* for long-term persistence in the
106 CF lung environment, where bacteria endure various attacks, encompassing oxidative
107 stresses, immune responses, and prolonged antibiotic treatments. To survive these harsh
108 conditions, initial infecting *P. aeruginosa* clones undergo substantial phenotypic changes
109 that may include slow growth, auxotrophy, virulence attenuation, loss of motility, mucoid
110 capsule, biofilm formation, hypermutability, LPS modifications and antibiotic resistance
111 (17). Analysis of several CF clinical isolates showed that adaptive mutations in about 50
112 genes are mainly responsible for the convergent molecular evolution towards the above-
113 mentioned phenotypes. Of these genes, common mutations occur in around 15 regulatory
114 genes for transcription factors and are supposed to be at the base of remodeling of the
115 infection regulatory network leading to *P. aeruginosa* adaptation to CF lung (17). These
116 regulatory patho-adaptive mutations also involve genes for alternative sigma factors such
117 as PvdS, σ^{54} (RpoN), σ^{22} (AlgT/U), and its repressor MucA (17, 18). *P. aeruginosa* σ^{22} is
118 the functional homolog of *E. coli* σ^{E} (19) that, along with several σ^{E}-regulated sRNAs (20,
119 21), orchestrates the envelope stress response, which in Gram-negative bacteria is
120 critical for maintaining envelope integrity in the host environment and thus to successfully
121 cause infection (20-22).

122 Generally, sRNAs are key components of the regulatory networks involved in the adaptive
123 response to the stressful conditions that pathogenic bacteria experience during host
124 infection (16, 20-25). Specific protein-RNA and RNA-RNA interactions in the *P.*
125 *aeruginosa* adaptive regulatory network have been identified for about 16 sRNAs (16).
126 One such *P. aeruginosa* sRNA is ErsA, a 132 nt long transcript that was described for the
127 first time in a work in which 52 novel sRNAs were identified in PAO1 (26) and PA14 (27),
128 two prototype laboratory strains in which ErsA showed to be similarly expressed under
129 laboratory conditions (28). Later, ErsA expression was shown to be strictly dependent on
130 and responsive to envelope stress by σ^{22} (29). Other infection cues such as temperature
131 shifts from environmental to body temperature and reduced oxygen conditions up-
132 regulate ErsA expression (29). Functional studies showed that ErsA contributes to the
133 regulation of virulence traits such as biofilm formation and motility (29, 30).
134 Phenotypically, the knock-out *ersA* mutant strain forms a flat and uniform biofilm and
135 shows enhanced swarming and twitching capability (30). ErsA influences the dynamics
136 of exopolysaccharide production, and the consequent biofilm formation, via negative post-
137 transcriptional regulation of *algC* mRNA (29). The *algC* gene encodes a key point enzyme
138 that coordinates the alginate biosynthetic pathway and the synthesis of several
139 *P. aeruginosa* polysaccharide exoproducts such as Psl, Pel, LPS, and rhamnolipids (31-
140 33). Like ErsA, the expression of *algC* is also dependent on σ^{22} (34, 35), which generates
141 an incoherent feed-forward loop to fine-tune the expression of the AlgC enzyme. Besides,
142 acting as a positive post-transcriptional regulator, ErsA stimulates exopolysaccharide
143 production and biofilm formation also through the post-transcriptional activation of A*mrZ*
144 (30), a transcription factor known to regulate alginate production and motility, and

145 indicated as a molecular switch that triggers biofilm maturation in *P. aeruginosa*.
146 Moreover, ErsA regulatory activity impacts considerably the *P. aeruginosa* transcriptome.
147 More than 160 genes are differentially expressed in RNA-seq experiments comparing the
148 knock-out *ersA* mutant with the PAO1 wild-type. Among these are genes for biofilm
149 formation and motility regulation that also belong to the AmrZ regulon. Furthermore, other
150 differentially expressed genes in the Δ*ersA* mutant are involved in several aspects of *P*.
151 *aeruginosa*-host interaction, such as denitrification and nitrate metabolism, nitrate
152 transport, type VI and III secretion systems effectors, energy and carbon metabolism,
153 heat-shock proteins and pyocyanin production (30).
154 Overall, the ErsA ability to respond to host cues and influence the expression of several
155 virulence-associated genes was thought to play a relevant role during host infection. Also,
156 ErsA was implicated in other aspects of *P*. *aeruginosa* lifestyle linked to infection
157 processes, such as niche establishment/protection in mixed populations, and antibiotic
158 resistance. Indeed, ErsA was suggested to coordinate biofilm maturation dynamics also
159 during mixed-species biofilm growth (36). In the presence of *Staphylococcus aureus*,
160 ErsA is part of that 0.3% of *the P. aeruginosa* genome which becomes differentially
161 expressed. The increase of its transcription levels suggests a role not only in
162 counteracting agents produced by *S. aureus* but also in modulating the state of the
163 exopolymeric matrix for typical biofilm maturation (36). ErsA was also shown to negatively
164 regulate *oprD* mRNA (37), coding for the OprD porin that is the major channel for entry of
165 the carbapenem antibiotics into the periplasm of *P. aeruginosa*. Coherently with these
166 results, strains lacking ErsA were more susceptible to meropenem than the PAO1 wild-
167 type strain (37).

168 This study aimed to assess the role of ErsA in the regulatory network of *P. aeruginosa*
169 pathogenicity in the infection of the airways. Here we provide evidence that the ErsA-
170 mediated regulation is relevant during acute infection and contributes to the stimulation
171 of the host inflammatory response. Besides, ErsA could also play a regulatory role during
172 chronic infection, in mechanisms of adaptation and acquirement of antibiotic resistance
173 leading to the typical resilient phenotype of *P. aeruginosa* in the CF airways.
174

175 ## Results

176 **ErsA contributes to the regulation of bacterial functions involved in cytotoxicity**
177 **and stimulation of the pro-inflammatory response**

178 We started to evaluate the role of ErsA in virulence and pathogenicity of *P. aeruginosa*
179 by using an *in vitro* infection system based on pulmonary cell lines. Infections were
180 performed with two prototype laboratory strains, PA14 and PAO1, which are hyper-
181 virulent and moderately virulent, respectively, along with their knock-out counterparts
182 (Δ*ersA*), or with *ersA*-overexpressing PAO1 strains in a wild-type genetic background.
183 The overexpression of ErsA by the vector pGM-*ersA* mimics the increase in ErsA levels
184 induced by the σ^{22}-mediated envelope stress response, producing a fivefold-increase of
185 the sRNA levels (29).
186 We evaluated the influence of ErsA on the cytotoxicity elicited by *P. aeruginosa* during
187 the infection of proliferating CF bronchial epithelial cells IB3-1 through the 3-(4,5-
188 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
189 (MTS) colorimetric assay. We exposed pulmonary IB3-1 cells to PA14 or PAO1 wild-type
190 strains and their corresponding Δ*ersA* mutants and followed cell viability by MTS assay

191 during the subsequent 3 hrs. Non-exposed cells were used as control of unaffected
192 viability in the experiment time window (Figs 1A and B). Infection of IB3-1 cells with the
193 PA14 wild-type strain (Fig 1A) caused significant cytotoxic effects, 30% of cell death (loss
194 of viable cells), compared to the non-infected control at the first time point post-infection
195 (60 min). At later time points of 120 and 180 min, the percentage of dead cells was
196 supposed to mirror the pattern of the ratio between death and proliferation rates. In the
197 same conditions, PA14 Δ*ersA* strain showed an interestingly different trend of infection-
198 induced cytotoxicity. Following the killing effects detected at 60 min post-infection, the
199 percentage of dead IB3-1 cells significantly and progressively decreased relatively to
200 PA14 wild-type infection at time points of 120 and 180 min, indicating a cytotoxicity
201 attenuation of PA14 Δ*ersA* strain (Figs 1A and B).
202 The infection-induced cytotoxicity was assessed for PAO1 wild-type and PAO1 Δ*ersA* in
203 the same experiments described above. Fig 1B reports the results in terms of percentage
204 of cytotoxicity attenuation compared to PA14 wild-type infections shown in Fig 1A, namely
205 the differences, at each time point, between the percentage of dead IB3-1 cells elicited
206 by PA14 wild-type, the most virulent strain of the panel, and those for the other strains.
207 As expected, the PAO1 wild-type strain showed an attenuated phenotype compared to
208 PA14 wild-type. At each time point, the percentages of dead IB3-1 cells were significantly
209 lower than during the infection with PA14 wild-type (i.e. a positive % of attenuation; Fig
210 1B). The same was true for the PAO1 Δ*ersA* strain but with an extremely relevant
211 difference. Indeed, the percentage of cytotoxicity attenuation resulted significantly higher
212 than PAO1 wild-type at 120 min and further increased at 180 min post-infection. These

213 results for PAO1 are consistent with those presented above for PA14 and strongly
214 suggest that the loss of ErsA affects the cytotoxic potential of *P. aeruginosa*.
215 We then evaluated the impact of ErsA on the inflammatory response of the IB3-1 cells by
216 monitoring the infection-induced secretion of the pro-inflammatory marker interleukin IL-
217 8, the major chemokine associated with neutrophil extravasation from the vasculature into
218 the lumen of the airways when respiratory epithelial cells are exposed to *P. aeruginosa*.
219 To minimize negative effects that could perturb a robust evaluation of IL-8 secretion, we
220 set out to expose the IB3-1 cells to *P. aeruginosa* strains at a multiplicity of infection (MOI)
221 10^3-fold lower than the infection experiments described above. However, PA14-based
222 strains at this MOI still caused relevant cell death, suffering, and detachment from the
223 plastic surface. Such effects were negligible following infection with PAO1-based strains
224 that thus were chosen for the subsequent analyses as follows.
225 IB3-1 cells were exposed to bacteria for 2 hrs, washed, supplemented with amikacin to
226 kill bacteria, and further incubated in the presence of fresh medium with amikacin.
227 Uninfected cells treated and incubated in the same conditions were used as a control of
228 non-stimulated IL-8 production. The amounts of IL-8 released in the supernatants by IB3-
229 1 cells at 24 hrs post-infection were measured through enzyme-linked immunosorbent
230 assay (ELISA). Our results showed that the infection of IB3-1 cells with *P. aeruginosa*
231 PAO1 Δ*ersA* causes a significant decrease in the secretion of IL-8 of about 37%
232 compared to the infection with PAO1 wild-type (Fig 2A). Consistently, IB3-1 cells infected
233 with the ErsA-overexpressing PAO1 strain showed an increase of IL-8 secretion of about
234 30% compared to those infected with the PAO1 strain harboring the empty vector
235 pGM931 (Fig 2B). Given the difference in the growth conditions of the marker-less strains

236 (PAO1 wild-type and Δ*ersA*, Fig 2A) and the vector-harboring strains (PAO1 pGM931 and
237 pGM-*ersA*, Fig 2B), the absolute amounts of secreted IL-8 are not comparable in these
238 two experimental settings. In the latter case, the presence of carbenicillin for vector
239 maintenance, and arabinose for induction of ErsA expression, may influence the bacterial
240 physiology and, as a consequence, affect the degree of the IB3-1 inflammatory response.
241 In any case, higher ErsA levels in bacterial cells result in a significantly higher secretion
242 of IL-8 in infected IB3-1 cells, as can be concluded from the analysis of both these
243 experimental sets.
244 Overall, these *in vitro* infection models indicated that ErsA regulatory role impacts *P.*
245 *aeruginosa*-induced cytotoxicity and contributes to the stimulation of the pro-inflammatory
246 response of infected epithelial cells.

247 **The deletion of ErsA impairs virulence and decreases pro-inflammatory response**
248 **in a murine model of airways infection.**

249 To further assess the ErsA involvement in the pathogenicity of *P. aeruginosa*, we
250 observed the infection outcomes in two groups of immunocompetent C57BL/6NCrlBR
251 mice whose lungs were inoculated with either PAO1 wild-type or PAO1 Δ*ersA* strain. For
252 this assessment, we followed a protocol of airways infection in which mice are inoculated
253 with bacterial cells embedded in agar beads and monitored for 13 days. We selected this
254 model of infection since it allows the simultaneous analysis of the effects of bacterial
255 mutations on both acute and chronic infection rates. Indeed, agar beads provide
256 microaerobic/anaerobic conditions that allow bacteria to experience a lung environment
257 resembling that of CF (and COPD) patients characterized by thick mucus (13, 38-40). In
258 these conditions, infecting bacteria can either colonize, spread locally and persist in lung

259 establishing a chronic infection, or undertake early systemic dissemination and eventually
260 induce death (acute infection). Alternatively, bacterial cells can be cleared by the host.
261 Depending on virulence, CF airways-adaptation, and the dose of the inoculated bacteria,
262 the fatality rate due to acute infection and the percentage of surviving mice with stable
263 bacterial loads in the lung, signs of chronic infection, can differ considerably in this model
264 of airways infection. For example, the *P. aeruginosa* CF-adapted strain RP73 elicits very
265 low mortality and a high percentage of chronic infection (about 80%) while PAO1 causes
266 significantly higher acute infection-induced mortality and lower chronicity rates (about 15-
267 20 %), thus showing more virulence and lower resilience to host-mediated clearance (38).
268 Since the PAO1 Δ*ersA* strain showed to be less pro-inflammatory than wild-type in the
269 infection experiments in *vitro* (Fig 2A), in addition to the assessment of bacterial chronic
270 colonization *vs* clearance, surviving mice were also inspected for immune system-
271 activation markers, both in lung and bronchoalveolar lavage fluid (BALF). Specifically,
272 neutrophil and macrophage titers were measured in BALFs while the levels of two pro-
273 inflammatory mediators of the response of airway epithelial cells to *P. aeruginosa*
274 infection, namely the keratinocyte chemoattractant KC (homologous to human IL-8), and
275 the monocyte secretory protein JE (homologous to human monocyte chemoattractant
276 protein-1 MCP-1), were assessed in lung homogenates.
277 As shown in Fig 3A, the PAO1 Δ*ersA* mutant strain caused significantly lower mortality
278 compared with the PAO1 wild-type counterpart with an infection fatality rate of 0% for
279 Δ*ersA* mutant *vs* 50% for wild-type. Hence, the loss of ErsA resulted in a strong decrease
280 in virulence. Conversely, the incidence of chronic colonization in surviving mice at 13 days
281 post-infection did not differ significantly between Δ*ersA* and wild-type strains (20% *vs* 14%

282 for Δ*ersA* and wild-type strains, respectively; Fig 3B). Likewise, colony-forming unit (CFU)
283 counts were similar in the lung of mice infected with Δ*ersA* mutant and with the wild-type
284 strain (median values of total CFUs: 3.63×10^2 Δ*ersA* mutant *vs* 3.14×10^2 wild-type; Fig
285 3C). The inflammatory response of mice infected by PAO1 Δ*ersA* in terms of leukocytes
286 recruitment in the bronchoalveolar lavage fluid (BALF) was only slightly lower compared
287 to PAO1 wild-type (mean values of total cells: 2.28×10^4 Δ*ersA* mutant *vs* 2.81×10^4 wild-
288 type; Fig 3D). However, when chemokines were measured in lung homogenates, we
289 found that PAO1 Δ*ersA* induced significantly lower levels of both KC and JE in
290 comparison to PAO1 wild-type (mean values of KC: 3.21×10^3 pg/ml Δ*ersA* mutant *vs*
291 4.07×10^3 pg/ml wild-type; mean values of JE: 85.7 pg/ml Δ*ersA* mutant *vs* 115.6 pg/ml
292 wild-type; Figs 4A and 4B).
293 Overall, these results indicated that the ErsA regulatory function strongly impacts the
294 cascades of events leading to acute infection and lethality and pro-inflammatory
295 response. No influence of ErsA on chronic bacterial colonization of airways is visible in
296 this experimental system.

297 **Variable ErsA expression in *P. aeruginosa* isolates recovered from human airways**
298 **chronic infections**

299 Despite the lack of influence on chronic colonization of murine airways, it could be
300 speculated that the attenuation of virulence and a lower stimulation of the immune
301 response potentially deriving from spontaneous deletion, point mutations, or even
302 downregulation of *ersA* gene might favor *P. aeruginosa* persistent lifestyle in human
303 lungs. In PAO1 and PA14 strains, we showed that ErsA expression is strictly dependent
304 on the envelope stress-responsive sigma factor σ^{22} (29). Moreover, ErsA levels can be

305 fine-tuned in response to other environmental cues by additional transcription factors (29).
306 Comparison of the *ersA* gene along with its upstream DNA region in several *P. aeruginosa*
307 isolates from clinical and environmental niches indicated high and extended sequence
308 conservation, including also the -10/-35 core promoter motifs recognized by the RNA
309 polymerase containing σ^{22} (29). This preliminary observation hinted at the possibility that
310 the *ersA* gene itself and its expression responsiveness might be conserved independently
311 of the origin of the *P. aeruginosa* isolates. Nevertheless, it may be feasible that patho-
312 adaptive mutations leading to *ersA* down-regulation contributed to the chronic
313 colonization of the human lung by *P. aeruginosa*.
314 To address this issue, the presence of *ersA* gene and its expression levels were assessed
315 in a panel of 31 *P. aeruginosa* strains isolated in respiratory samples from CF and COPD
316 patients collected during intermittent or chronic infections at different stages, and
317 compared with those in 5 *P. aeruginosa* isolates from environmental habitats (13, 41, 42)
318 using as references the PAO1 strain. Detection by PCR of the e*rsA* gene and Northern
319 blot analyses are shown in Fig 5 and summarized in S1 Table. The *ersA* gene was
320 detected in 30 out of the 31 clinical isolates and in all environmental strains. In about
321 55.6% of the analyzed strains, the expression levels of ErsA were not significantly
322 different from those detected in PAO1. This set included the 5 environmental strains and
323 15 clinical isolates. In another assembly of 13 CF strains, ErsA was significantly down-
324 regulated: in 10 strains from 2 to 7.5 folds, in 2 strains, MI2-3 and TR1, it was strongly
325 downregulated of 28 and 120 folds, respectively, and in one strain, MI1-3, no expression
326 was detected because of the loss of the *ersA* gene as mentioned above. In only three
327 clinical strains ErsA was significantly upregulated from 2 to 3 folds. Hence, these results

328 indicated that, in 13 out of 31 clinical strains analyzed (about 42%), ErsA was moderately
329 to strongly downregulated (in one case lost) relative to the environmental strains that
330 showed to express ErsA at levels comparable to PAO1. This would suggest that ErsA
331 expression is under selective pressure in the CF lung and that mutation(s) resulting in
332 ErsA down-regulation might contribute in some cases to *P. aeruginosa* patho-adaptation
333 during CF chronic infections.

334 **ErsA can contribute to *P. aeruginosa* adaptation to long-term antibiotic treatment.**

335 To further investigate the potential role of ErsA in the *P. aeruginosa* adaption to the CF
336 lung environment, we considered the emergence of antibiotic resistance that is observed
337 frequently in *P. aeruginosa* isolates from CF patients following prolonged antibiotic
338 treatment. To this end, we generated a knock-out *ersA* mutant in RP73, one member of
339 the panel of *P. aeruginosa* clinical isolates that we inspected for ErsA expression (Fig 5,
340 lanes 28 and 29). We selected RP73 because it was isolated from a CF patient 16.9 years
341 after the onset of chronic colonization and showed acquired multi-drug resistance to
342 amikacin, gentamicin, ceftazidime, imipenem, and meropenem (43).
343 The RP73 Δ*ersA* mutant was tested for the Minimum Inhibitory Concentrations (MICs) of
344 seven antibiotics, commonly used in the clinical practice, to which RP73 is resistant. As
345 shown in Table 1, RP73 Δ*ersA* showed to be sensitive to ceftazidime (MIC from 16 in
346 RP73 to 8 μg/ml) and cefepime (MIC from >= 64 in RP73 to 8 μg/ml), and intermediate to
347 meropenem (MIC from >= 16 in RP73 to 4 μg/ml). Furthermore, RP73 Δ*ersA* showed a
348 decrease of MIC from 2 to 1 μg/ml for ciprofloxacin. These results suggest that ErsA could
349 contribute to *P. aeruginosa* adaptation to long-term antibiotic treatment undergone by CF
350 patients.

351 **Table 1. Antibiotic sensitivity of RP73 and RP73 Δ*ersA* strains.**

Antibiotic	Strain	MIC[a]	Interpretation[b]
Ceftazidime	RP73	16	R
	RP73 Δ*ersA*	8	S
Cefepime	RP73	≥ 64	R
	RP73 Δ*ersA*	8	S
Imipenem	RP73	≥ 16	R
	RP73 Δ*ersA*	≥ 16	R
Meropenem	RP73	≥ 16	R
	RP73 Δ*ersA*	4	I
Amikacin	RP73	≥ 64	R
	RP73 Δ*ersA*	≥ 64	R
Gentamicin	RP73	≥ 16	R
	RP73 Δ*ersA*	≥ 16	R
Ciprofloxacin	RP73	2	R
	RP73 Δ*ersA*	1	R

352 [a]Minimum Inhibitory Concentration expressed in µg/ml.

353 [b]R: resistant, I: intermediate, or S: sensitive to the indicated antibiotic according to the
354 European Society of Clinical Microbiology and Infectious Diseases (EUCAST) guidelines.

355

356 ## Discussion

357 We investigated the regulatory role of ErsA in the pathogenicity and adaptation of *P.*
358 *aeruginosa* during the infection of the airways. Before this study, several features of ErsA
359 suggested its involvement in the interaction with the host. ErsA regulates EPS production
360 (16) and positively influences biofilm formation and maturation (17). Besides, ErsA
361 responds to cues that are related to airways infection, both at early and late stages, such
362 as a shift from room to body temperature, oxygen availability, iron concentration, and σ^{22}-
363 mediated envelope stress response (29), the latter strongly involved in pathogenicity

364 regulation in Gram-negative bacteria (21). ErsA is also involved in the resistance to
365 carbapenem antibiotics through the negative regulation of the porin OprD (37). The
366 results herein presented indicate that the regulatory function exerted by ErsA is relevant
367 in the airways for the progress of *P. aeruginosa* acute infection and might also endure
368 remodeling during the adaptive process leading to *P. aeruginosa* persistence in CF lungs.
369 The lower *in vitro* cytotoxicity induced by the *P. aeruginosa* Δ*ersA* mutants is consistent
370 with the strong decrease of the fatality rate due to acute infection observed for the PAO1
371 Δ*ersA* strain. Remarkably, we administered to mice the median lethal dose (LD_{50}) of
372 1×10^6 CFU for PAO1, which was completely ineffective in the case of PAO1 Δ*ersA*.
373 Besides, there is another consistency of results between the *in vitro* and *in vivo* infection
374 experiments: the loss of ErsA determines a lower activation of the innate immune
375 response, measured in terms of levels of the NF-kB-dependent pro-inflammatory
376 mediators IL-8 (*in vitro*), and KC and JE (*in vivo*). Hence, the regulatory role of ErsA
377 impacts both the virulence of the acute infection and the innate immune response. To
378 explain these results, we first speculated that ErsA could positively regulate in
379 response to lung environment invasive functions that can also act as PAMPs (e.g.
380 flagella, LPS, T3SS, and ExoS) (10), or the expression of non-PAMP invasive
381 functions and PAMPs products. Alternatively, the significant impairment of PAO1
382 Δ*ersA* in biofilm formation and maturation (30) could justify the simultaneous involvement
383 of ErsA in acute infection and immune response activation. Previous relevant studies (5,
384 6, 9) have evidenced that, at initial stages of *P. aeruginosa* acute infection, biofilm
385 aggregates with a canonical matrix composed of Psl, Pel, alginate, and eDNA (4)
386 assemble on airways mucosal surface and trigger both a dramatic remodeling of the

387 apical membrane (i.e. protrusions) of epithelial cells and NF-kB-dependent activation of
388 the innate immune response. Neither protrusion formation nor NF-kB activation was
389 observed upon binding of individual bacteria to epithelial cells. This strongly indicated that
390 biofilm formation is a key *P. aeruginosa* function to initiate acute infection and, through
391 the induced changes in epithelial cell polarity, a danger signal for host cells that warns of
392 an incoming threat (7). On the bases of this model, we suggest that acute virulence
393 attenuation and decreased pro-inflammatory stimulation of PAO1 Δ*ersA* could be due to
394 its defect in biofilm formation and maturation (30). This phenotype induced by the loss of
395 ErsA was attributed to the dysregulation of the expression of AlgC (29, 33) and AmrZ (44,
396 45), two proteins that play important roles in the post-transcriptional and transcriptional
397 regulation, respectively, of the production of Psl, Pel, and alginate. Furthermore,
398 transcriptomics analysis indicated that the *pelCDEFG* genes for Pel biosynthesis (46), the
399 *ppyR* gene for an activator of the Psl operon coding for the Psl biosynthetic pathway (47),
400 and the *algD* gene for alginate biosynthesis are significantly down-regulated in the PAO1
401 Δ*ersA* mutant (30).
402 Specifically, Psl and Pel (referred to as aggregative EPS) are important for initiating and
403 maintaining cell-cell interaction in biofilms, while alginate (referred to as capsular EPS) is
404 instrumental in biofilm maturation, structural stability and, protection from antibiotics (48).
405 To further assess the role of ErsA in the regulation of Psl- and Pel-linked aggregation and
406 adherence, we performed the experiments shown in S1 Fig. As a result of ErsA deletion,
407 aggregation, and adherence potentials of PAO1 are strongly reduced, while they are
408 enhanced when ErsA is overexpressed. Overall, ErsA could participate in the regulation
409 of biofilm formation at the early stages of acute infection. However, this scenario might

410 be wider since i) transcriptomics data (30) suggested that ErsA deletion can affect other
411 aspects of *P. aeruginosa* interaction with its host and ii) at the post-transcriptional level,
412 ErsA could influence the expression of virulence-associated genes other than *algC* and
413 *amrZ*.
414 The deletion of ErsA did not influence the chronic infection rate of PAO1 in the mouse
415 model. However, virulence attenuation and lower recognition by the immune system
416 showed by PAO1 Δ*ersA* are favorable traits for *P. aeruginosa* chronic infection of CF
417 airways (4, 10). Therefore, we evaluated whether ErsA expression could be
418 downregulated or even deleted in a panel of CF clinical isolates.
419 A significant proportion (about 42%) of the clinical strains analyzed showed that ErsA was
420 moderately to strongly downregulated (in one case lost) relative to both environmental
421 strains and PAO1. This suggested that the *ersA* gene could be under selective pressure
422 for lowering its expression in a CF context. This phenomenon might contribute in some
423 cases to *P. aeruginosa* patho-adaptation towards low virulence and evasion of the
424 immune system during CF chronic infections (10). We speculate that this putative
425 evolution of ErsA expression occurs in the frame of the remodeling process involving the
426 infection regulatory network, in which σ^{22} is one main component that leads to *P.*
427 *aeruginosa* adaptation to CF lung (17).
428 Finally, we found that loss of ErsA induces sensitization to ceftazidime, cefepime, and
429 meropenem in the multidrug-resistant clinical isolate RP73, which was in the group of CF
430 clinical isolates analyzed for ErsA expression. It is worth noting that RP73 was
431 demonstrated to establish long-term infection replacing an initial isolate (RP1), and
432 adapting within CF airways compared to its clonal ancestor RP45 (43). The adaptive

433 microevolution has led RP73 to differentiate significantly from RP45 in terms of virulence
434 and antibiotic resistance, being RP45 more virulent than RP73 and sensitive to amikacin,
435 ceftazidime, imipenem, and meropenem (43). The multi-antibiotic resistance that RP73
436 has acquired compared to its clonal ancestor RP45, and that is lost in the RP73 Δ*ersA*,
437 reveals an interesting link between ErsA and mechanisms of adaptation to host
438 environment during *P. aeruginosa* chronic infection of CF patients.
439 The emergence and rapid dissemination of antibiotic resistance demand the development
440 of new antibiotics and antivirulence agents (49). These latter compounds directly target
441 virulence factors or virulence regulators. The contribution to acute infection regulation and
442 the acquirement of antibiotic resistance suggest that ErsA may be a candidate target for
443 the development of novel anti-virulence and co-antibiotic drugs.
444

445 ## Materials and methods

446 ### Ethics Statement

447 The study on human *P. aeruginosa* isolates from Hannover has been approved by the
448 Ethics Commission of Hannover Medical School, Germany (41). The patients and parents
449 gave oral informed consent before the sample collection. Approval for storing the
450 biological materials was obtained by the Ethics Commission of Hannover Medical School,
451 Germany. The study on human *P. aeruginosa* isolates from the Regional CF Center of
452 Lombardia was approved by the Ethical Committees of San Raffaele Scientific Institute
453 and Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, Milan, Italy, and
454 written informed consent was obtained from patients enrolled or their parents according

455 to the Ethical Committees rules, under the laws of the Italian Ministero della Salute
456 (approval #1874/12 and 1084/14) (42).
457 Animal studies strictly followed the Italian Ministry of Health guidelines for the use and
458 care of experimental animals. This study was performed following protocols approved
459 by the Institutional Animal Care and Use Committee (IACUC, protocol #789) of the San
460 Raffaele Scientific Institute (Milan, Italy).

461 **Bacterial strains and culture conditions**

462 *P. aeruginosa* strains PAO1 (26), PA14 (27) and RP73 (41, 43), and the corresponding
463 deleted mutants PAO1 Δ*ersA*, PA14 Δ*ersA* (29) and RP73 Δ*ersA* were grown at 37 °C in
464 Luria-Bertani rich medium at 120 rpm. sRNA-overexpressing strains PAO1/pGM-ersA
465 and PA14/pGM-ersA and their empty vector-harboring control strains PAO1/pGM931 and
466 PA14/pGM931 (29) were grown with the addition of 300 µg/ml carbenicillin. For P_{BAD}
467 induction in vector plasmid pGM931, arabinose was added to a final concentration of 10
468 mM. The RP73 Δ*ersA* mutant strain was generated from the MDR-RP73 isolate using a
469 method of marker-less gene replacement (50) improved for *P. aeruginosa* using oligos
470 and molecular techniques as described previously (29), and cloning in the tetracycline-
471 resistant harboring plasmid pSEVA512S to allow selection of exconjugant on 30 µg/ml
472 tetracycline.

473 **Bacterial isolates analysis**

474 Bacterial isolates (13, 41, 42) were plated on 1.5% Brain Heart Infusion (BHI)-agar plates
475 and grown overnight at 37°C. Culture samples were taken and processed for genomic

476 DNA and total RNA extraction as described previously (28). PAO1 and PA14 strains
477 treated in the same conditions were used as controls.
478 Oligos CGAATGGCTTGAGCCCTTCGATGCT/AAAAAAAACCCCGAGCTTCGTA and
479 TGTCGTCAGCTCGTGTCGTGA/ATCCCCACCTTCCTCCGGT were used for PCR-
480 amplification of the genomic region containing the *ersA* and 16S (as positive PCR-control)
481 *loci*, respectively. Northern blot analyses were performed as described previously (28).
482 Briefly, DNA oligonucleotide probes were 5' end-labeled with [γ-32P]ATP (PerkinElmer,
483 NEG502A) and T4 polynucleotide kinase (Promega, M4103) according to the
484 manufacturer's instruction. Oligo CCCGAGCTTCGTATGGGG and
485 GGAGACCCCACACTACCATCGGCGATG were used to probe ErsA and 5S RNA,
486 respectively. Radioactive bands were acquired after exposure to phosphor screens using
487 a Typhoon™ 8600 variable mode Imager scanner (GE Healthcare BioSciences) and
488 visualized with image-Quant software (Molecular Dynamics). The intensities of the bands
489 were quantified using Li-cor Image Studio Lite. The signal of ErsA was normalized to
490 those of 5S RNA in the same lane. For each clinical isolate, the relative abundance of
491 ErsA was calculated comparing to the reference strain PAO1.
492

493 **Cytotoxicity and IL-8 secretion in human CF respiratory cells**

494 IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line
495 derived from a CF patient (ΔF508/W1282X) and obtained from LGC Promochem, were
496 grown as described previously (51). Cell viability was evaluated using the MTS-based
497 CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, G3582), which
498 determines viable cell number measuring the conversion at 490 nm of 3-(4,5-

499 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
500 (MTS) to formazan by the dehydrogenase enzyme of the intact mitochondria of living
501 cells. In a 96-well plate, triplicates of IB3-1 cells were infected with *P. aeruginosa* strains
502 at a multiplicity of infection (MOI) of 100, in a final volume of 100 µl. Immediately after
503 infection, 20 µl of the CellTiter 96® AQ_{ueous} One Solution Reagent were added directly to
504 culture and control wells. MTS was also added to non-infected cells and in wells
505 containing only the same bacterial load in absence of IB3-1 cells (blank/control).
506 According to the manufacturer's instructions, plates were incubated at 37°C with 5% CO_2,
507 read at 490 nm at different time-points, and returned to the incubator for further color
508 development. The average measurement of infected cells was subtracted from the
509 average of the corresponding blank. The relative percentages of cell death or cell viability
510 were calculated as the ratio between the average value in normalized infected cells
511 (blank-subtracted) and the uninfected cells. The stimulation of the host inflammatory
512 response was evaluated by monitoring the secretion of the pro-inflammatory interleukin
513 IL-8 as described previously (51). Briefly, after infection with *P. aeruginosa* strains at an
514 MOI of 0.1, IB3-1 cells were incubated at 37°C with 5% CO_2 for 2 hrs, washed with PBS
515 supplemented with 1 mg/ml amikacin, and incubated in presence of fresh medium
516 supplemented with 1 mg/ml amikacin. Uninfected cells treated and incubated in the same
517 conditions were used as a control of non-stimulated IL-8 production. Released IL-8 was
518 determined in supernatants collected at 24 hrs using an ELISA kit (Biosource Europe and
519 R&D Systems), according to manufacturer's instructions.

520 **Agar-beads preparation**

521 The agar-beads mouse model was used (39, 40). An aliquot of wild-type or Δ*ersA P.*
522 *aeruginosa* PAO1 strains from glycerol stocks was streaked for isolation on trypticase
523 soy agar (TSA) and incubated at 37°C overnight. One colony was picked from the plate
524 and used to inoculate 5 ml of tryptic soy broth (TSB) and placed in a shaking incubator at
525 37°C 200 rpm overnight. The overnight bacterial suspension was diluted to 0.15 OD/ml
526 in 20 ml of TSB / flask and grown for 4 hrs at 37°C at 200 rpm, to reach the log phase.
527 The bacteria were pelleted by centrifugation (2,700 g, 15 min, 4°C) and resuspended in
528 1 ml PBS (pH 7.4). A starting amount of 2×10^9 CFUs of *P. aeruginosa* was used for
529 inclusion in the agar-beads prepared according to the previously described method (39,
530 40, 52). Bacteria were added to 9 ml of 1.5% TSA (w/v), prewarmed to 50°C. This mixture
531 was pipetted forcefully into 150 ml heavy mineral oil at 50°C and stirred rapidly with a
532 magnetic stirring bar for 6 min at room temperature, followed by cooling at 4°C with
533 continuous slowly stirring for 35 min. The oil-agar mixture was centrifuged at 2,700 g for
534 15 min to sediment the beads and washed six times in PBS. The size of the beads was
535 verified microscopically and only those preparations containing beads of 100 μm to 200
536 μm in diameter were used as inoculum for animal experiments. The number of *P.*
537 *aeruginosa* CFUs in the beads was determined by plating serial dilutions of the
538 homogenized bacteria-bead suspension on TSA plates. The inoculum was prepared by
539 diluting the bead suspension with PBS to 2×10^7 CFUs/ml, to inoculate about 1×10^6
540 CFU/50μl. *P. aeruginosa* beads were prepared the day before inoculation, stored
541 overnight at 4°C for a maximum of two days. The number of *P. aeruginosa* CFUs in the

542 beads inoculated was determined by plating serial dilutions of the homogenized bacteria-
543 bead suspension on the day of the infection.

544 **Mouse model of chronic *P. aeruginosa* lung infection**

545 Immunocompetent C57BL/6NCrlBR male mice (8-10 weeks of age) were purchased
546 from Charles River (Calco, Italy), shipped in protective, filtered containers, transported
547 in climate-controlled trucks, and allowed to acclimatize for at least two days in the
548 stabulary before use. Mice were maintained in the biosafety level 3 (BSL3) facility at
549 San Raffaele Scientific Institute (Milano, Italia) where three-five mice per cage were
550 housed. Mice were maintained in sterile ventilated cages. Mice were fed with standard
551 rodent autoclaved chow (VRFI, Special Diets Services, UK) and autoclaved tap water.
552 Fluorescent lights were cycled 12h on, 12h off, and ambient temperature (23±1°C) and
553 relative humidity (40-60%) were regulated.

554 For infection experiments, mice were anesthetized by an intraperitoneal injection of a
555 solution of Avertin (2,2,2-tribromethanol, 97%) in 0.9% NaCl and administered at a
556 volume of 0.015 ml/g body weight. Mice were placed in a supine position. The trachea
557 was directly visualized by ventral midline, exposed and intubated with a sterile, flexible
558 22-g cannula attached to a 1 ml syringe. An inoculum of 50 µl of agar-bead suspension
559 was implanted via the cannula into the lung. After inoculation, all incisions were closed
560 by suture.

561 Infections and sacrifices were all performed in the late morning. Besides, in all the
562 experiments, mice had been subdivided according to the bodyweight to have similar
563 mean in all the groups of treatment.

564 Mice were monitored daily for coat quality, posture, attitude, ambulation, hydration
565 status, and bodyweight. Mice that lost >20% bodyweight and had evidence of severe
566 clinical diseases, such as scruffy coat, inactivity, loss of appetite, poor locomotion, or
567 painful posture, were sacrificed before the termination of the experiments with an
568 overdose of carbon dioxide. Gross lung pathology was checked. After 13 days post-
569 infection, bronchoalveolar lavage fluid (BALF) was collected and lungs were aseptically
570 excised.
571 BALF was extracted with a 22-gauge venous catheter, ligated to the trachea to prevent
572 backflow. The lungs were washed with three one ml of RPMI-1640 (Euroclone) with
573 protease inhibitors (Complete tablets, Roche Diagnostic) and pooled. Quantitative
574 bacteriology on BALF was performed by plating serial dilution on TSA. Total cells
575 present in the BALF were counted using an inverted light optical microscope after
576 diluting an aliquot of the BALF 1:2 with Tuerk solution in a disposable counting
577 chamber. BALF cells were centrifuged at 330 g for 8 min at 4°C. If the pellet was red,
578 erythrocytes were lysed by resuspending the pellet in 250-300 µl of RBC lysis buffer
579 diluted 1:10 in ultra-pure distilled water for 3 min. Then, 2-3 ml PBS were added and
580 cells were centrifuged at 330 g for 8 min at 4°C. The pellet was resuspended in RPMI
581 1640 10% fetal bovine serum (FBS) at a concentration of 1×10^6 cells/ml, and an aliquot
582 of 170 µl was pipetted into the appropriate wells of the cytospin and centrifuged at 300
583 g for 5 min medium brake. Slides were then stained by Diff-Quik staining using a
584 commercial kit (Medion Diagnostics, code: 726443), according to the manufacturer's
585 instructions. A differential cell count was performed at an inverted light optical
586 microscope.

587 Lungs were excised aseptically and homogenized in 2 ml PBS added with protease
588 inhibitors using the homogenizer gentleMACSTM Octo Dissociator. One-hundred µl of
589 the homogenates and 10-fold serial dilutions were spotted onto TSA. CFUs were
590 determined after overnight growth at 37°C. Recovery of ≥1000 CFU of *P. aeruginosa*
591 from lung + BALF cultures was considered as evidence of chronic infection.

592 **Quantification of murine chemokines**

593 Lung homogenates were centrifuged at 16,000 g for 30 min at 4°C, then supernatants
594 were collected and stored at -80°C. Murine KC and JE concentrations were determined
595 in the lung homogenate supernatants by DuoSet® ELISA Development Systems (R&D
596 Systems), according to manufacturer instructions.

597 **MIC measurement**

598 MICs of antibiotics were determined according to CLSI guidelines (53), as previously
599 described (54). The medium used for the MIC testing was cation-adjusted Mueller-Hinton
600 broth (MH-II broth). MIC testing was run in sterile 96-well Microtiter plates (polystyrene V
601 shape) and analyzed after 20 hrs.

602 **Statistics**

603 Statistical analyses were performed with GraphPad Prism. Survival curves and
604 incidences of chronic colonization were compared using the Mantel-Cox test and Fisher
605 exact test, respectively. Levels of chemokines, leukocytes, and CFUs were compared
606 using a nonparametric two-tailed Mann-Whitney U test. $P < 0.05$ was considered
607 significant. Significance of the differences in the levels of secreted IL-8 and converted

608 MTT by infected IB3-1 cells were determined using the Student t-test. $P < 0.05$ was
609 considered significant.
610

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616 of the Università degli Studi di Milano.
617

618 **Figure captions**

619 **Fig 1. Deletion of ErsA results in decreased *P. aeruginosa*–induced cytotoxicity of**
620 **pulmonary cells.** (A) Time course of cell death of CF bronchial epithelial cells after
621 bacterial infection with *P. aeruginosa* PA14 wild-type and Δ*ersA*. Viability of IB3-1 cells
622 uninfected (No Infection) or infected with a MOI=100 (PA14 wild-type and PA14 Δ*ersA*)
623 was analyzed by MTS assay. At each time point, results are plotted as the ratio of the
624 average values in infected (blank-subtracted) cells to the uninfected cells. The data are
625 pooled from three independent experiments and are represented as mean ± SEM.
626 Significance by one-way *ANOVA* with post-hoc Tukey's HSD is indicated as follows. PA14
627 wild-type *vs* Δ*ersA*: $^{*}p<0.05$ and $^{**}p<0.01$; PA14 wild-type *vs* No Infection: $^{\#}p<0.05$,
628 $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$; PA14 Δ*ersA* *vs* No Infection: $^{\S}p<0.05$ and $^{\S\S}p<0.01$. (B) Relative
629 viability percentage of IB3-1 cell after bacterial infection as measured by MTS assay.
630 Cytotoxicity attenuation (%) of PAO1 wild-type, PAO1 Δ*ersA*, and PA14 Δ*ersA* is shown

631 respect to the PA14 wild-type strain during infection of IB3-1 cells with a MOI=100. At
632 each time point after infection, results are plotted as the ratio of the average values in
633 infected (blank-subtracted) cells to the uninfected cells. At the indicated time points after
634 infection, the ratio of the average values in infected (blank-subtracted) cells to the
635 uninfected cells has been determined. Results are shown as the difference of the ratios
636 between each strain and PA14 wild-type. Data are pooled from three independent
637 experiments and are represented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 and
638 ****p<0.0001 in the one-way *ANOVA* with post-hoc Tukey's HSD. Significance of each
639 strain *vs* PA14 wild-type is indicated above single histograms.

640 **Fig 2. ErsA levels influence the pro-inflammatory response in pulmonary cells.**
641 Inflammatory response of CF bronchial epithelial cells after stimulation with (A) *P.*
642 *aeruginosa* PAO1 wild-type and PAO1 Δ*ersA* deleted mutant strains and (B) *P.*
643 *aeruginosa* PAO1 strain harboring the empty vector pGM931, or the sRNA
644 overexpressing vectors pGM-*ersA*. IL-8 was evaluated by ELISA in supernatants of IB3-
645 1 cells 24 hrs post-infection (MOI=0.1). Uninfected IB3-1 cells were used as control (Ctrl).
646 Data are represented as mean ± standard error of the mean (SEM). The data are pooled
647 from three independent experiments. *p<0.05, **p<0.01, ***p<0.001 in the Student's t-
648 test.

649 **Fig 3. Survival, the incidence of chronic colonization, bacterial burden, and**
650 **leukocyte recruitment after chronic lung infection by wild-type and Δ*ersA* *P.***
651 ***aeruginosa* PAO1.** C57BL/6NCrlBR mice were infected with 1×10^6 colony-forming
652 units/lung embedded in agar beads. At day 13 post-infection, mice were sacrificed,
653 bronchoalveolar lavage fluid (BALF) was collected and lungs were excised and

654 homogenized. (A) Survival was evaluated on challenged mice. (B) Clearance (<1000
655 CFU of *P. aeruginosa* from lung + BALF cultures) and capacity to establish chronic
656 airways infection (≥1000 CFU of *P. aeruginosa* from lung + BALF cultures) were
657 determined on surviving mice. (C) CFUs were evaluated in the lungs and BALF after
658 plating onto tryptic soy agar. Dots represent values of individual mice, and horizontal lines
659 represent median values. (D) Neutrophils, macrophages, and total cells were measured
660 in the BALF. Values represent the mean ± SEM. The data were pooled from at least three
661 independent experiments (n=20-28). ***p<0.001 in the Mantel-Cox test.

662 **Fig 4. Chemokine levels after lung infection by wild-type and Δ*ersA* *P. aeruginosa***
663 **PAO1.** C57BL/6NCrlBR mice were infected with 1×10^6 colony-forming units/lung
664 embedded in agar beads. At day 13 post-infection, mice were sacrificed, and lungs were
665 excised and homogenized. (A) KC and (B) JE levels were measured by ELISA in the
666 supernatant fluids of lung homogenates. Values represent the mean ± SEM. The data
667 were pooled from at least three independent experiments (n=14-20). *p<0.05, **p<0.01
668 in the nonparametric two-tailed Mann-Whitney U test.

669 **Fig 5. Dissemination of the *ersA* gene and its expression levels in a collection of *P.***
670 ***aeruginosa* isolates.** Bacterial strains from CF (gray pentagons), COPD patients (white
671 circles), and environmental isolates (black squares) are indicated on top. After overnight
672 growth at 37°C on BHI-agar plates, culture samples were processed for genomic DNA
673 extraction and total RNA purification and analysis by Northern blot, and for. PAO1 and
674 PA14 were used as control strains. The presence (+) or absence (-) of the *ersA* gene is
675 indicated below each Northern Blot. of Northern blot lane. The relative abundance of ErsA

676 in each isolate was calculated to the reference strain PAO1 after normalization to 5S
677 RNA.
678

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830

831 ## Supporting information

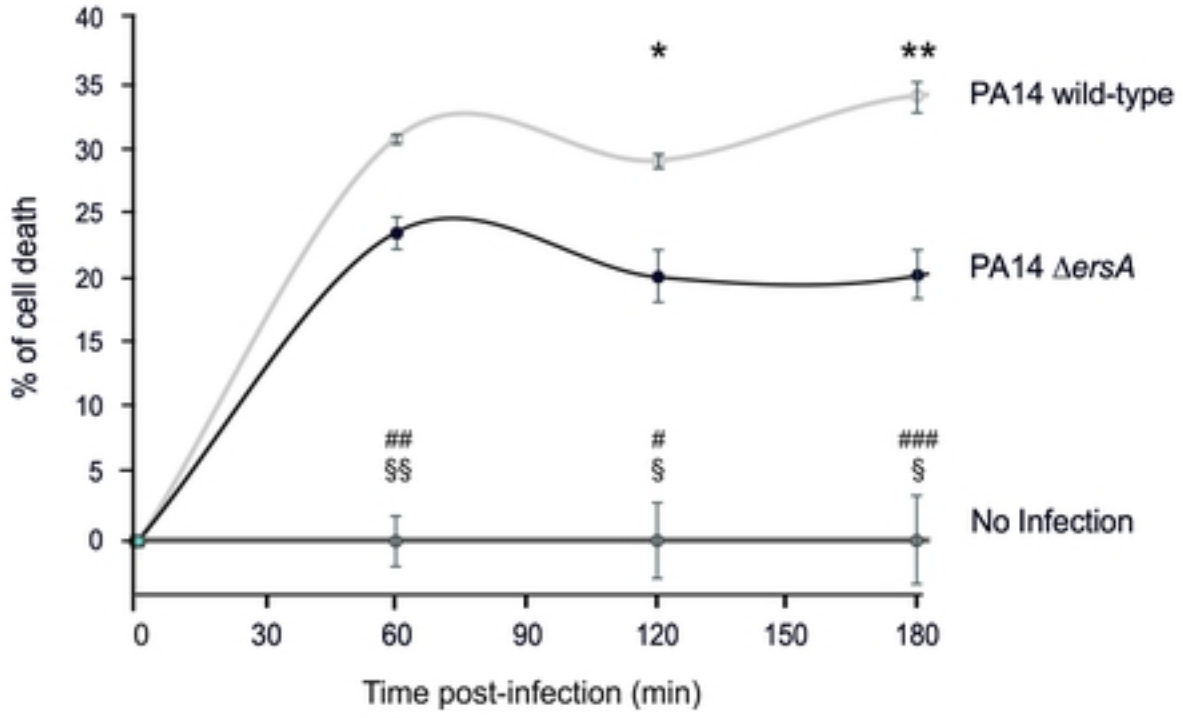
832 **S1 Fig. ErsA influences aggregation and adherence of the *P. aeruginosa* PAO1**
833 **strain.** *P. aeruginosa* PAO1 strains with wild-type, deleted, or overexpressed ErsA grown
834 in liquid T-Broth in presence of 40 µg/ml of Congo red. To observe aggregation, strains
835 were inoculated at OD_{600} of 0.05 using glass culture tubes with 10 ml of medium and
836 incubated overnight at 37°C in a roller drum. Carbenicillin and arabinose were added for
837 ectopic expression of ErsA from pGM-*ersA* and the growth of the control culture harboring
838 the pGM931 empty vector. Adherent biomass is noticeable on the culture tube of PAO1
839 wild-type strain. Conversely, the absence of adherence is shown on the Δ*ersA* strain tube.
840 More abundant aggregation and biofilm are present when ErsA is over-expressed by
841 pGM-*ersA* from wild-type background, compared to the empty vector-harboring control
842 strain PAO1/pGM931 grown in the same conditions.

843 **S1 Table. Analysis of ErsA expression in a panel of clinical and environmental**
844 **strains of *P. aeruginosa.***

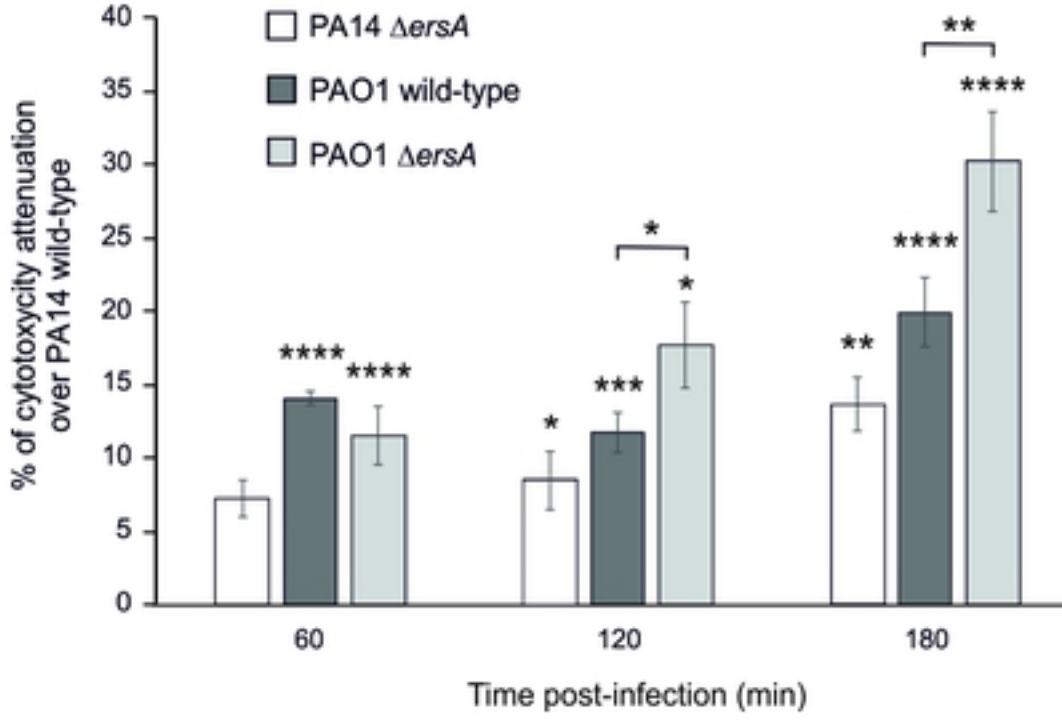
845

846

A

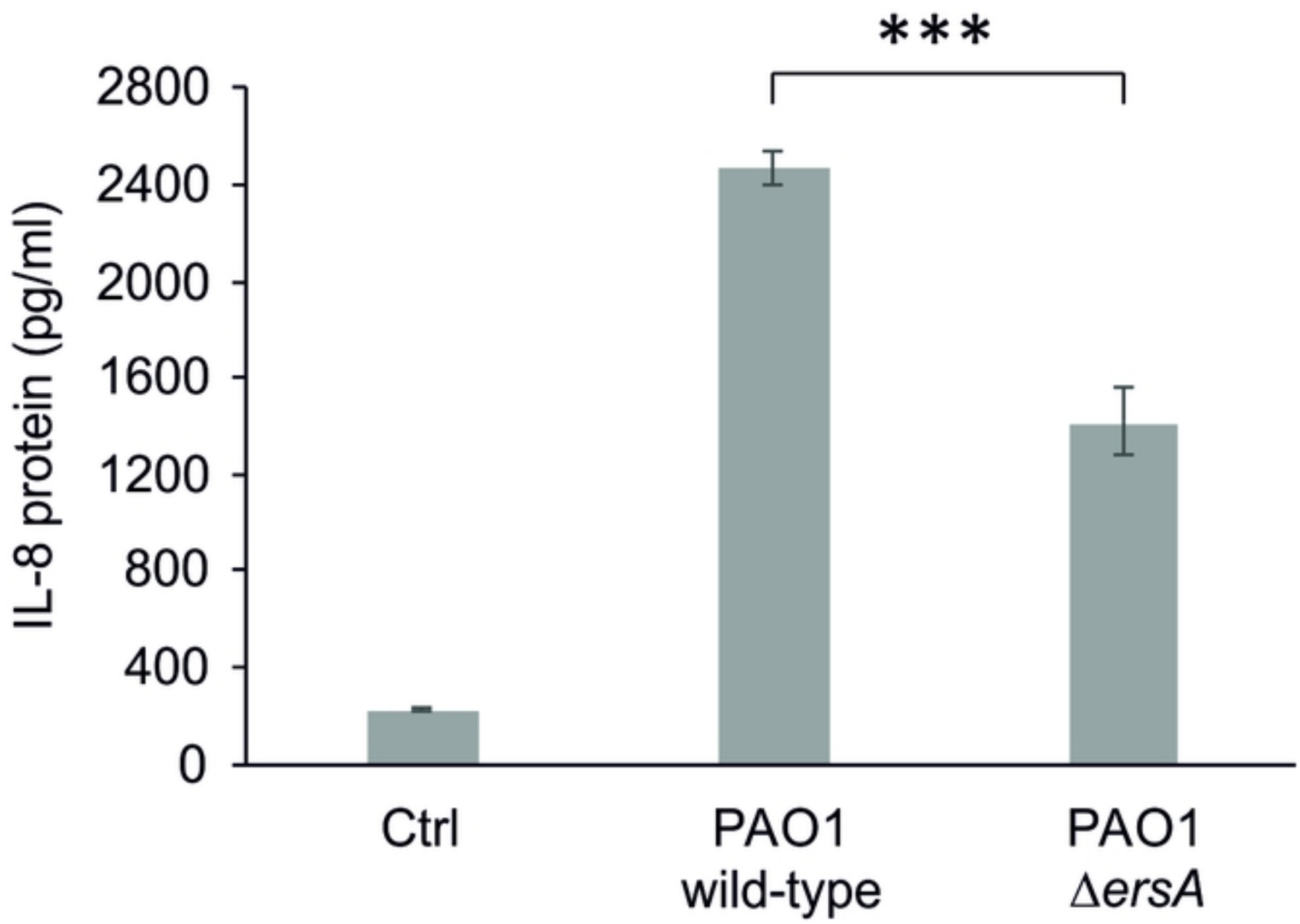

% of cell death
40
35
30
25
20
15
10
5
0
*
**
PA14 wild-type
PA14 ΔersA
##
§§
#
§
###
§
No Infection
0
30
60
90
120
150
180
Time post-infection (min)

B

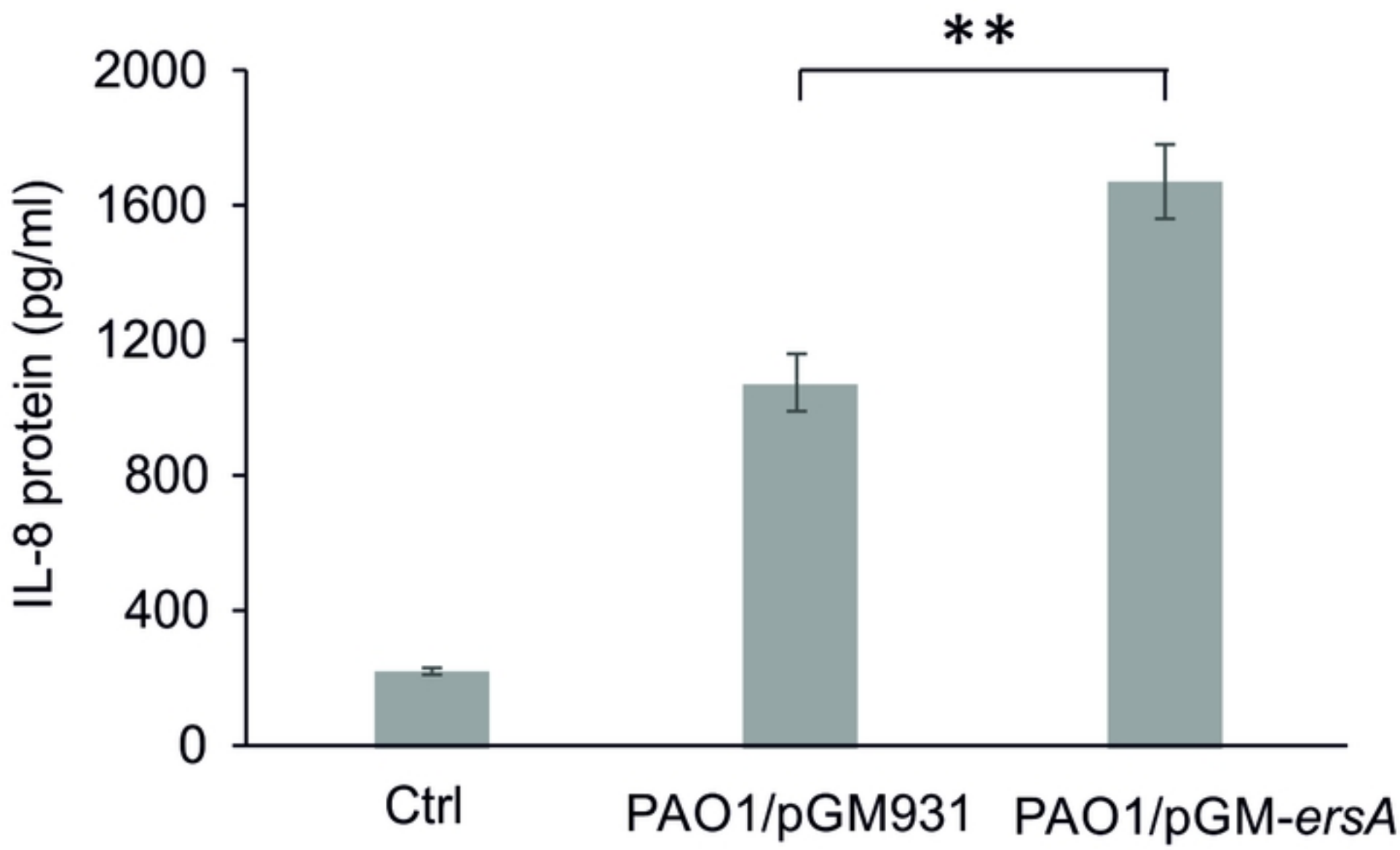

PA14 ΔersA
PAO1 wild-type
PAO1 ΔersA
% of cytotoxycity attenuation over PA14 wild-type
40
35
30
25
20
15
10
5
0
****
****
*
***
*
*
**
****
**
****
60
120
180
Time post-infection (min)

Figure

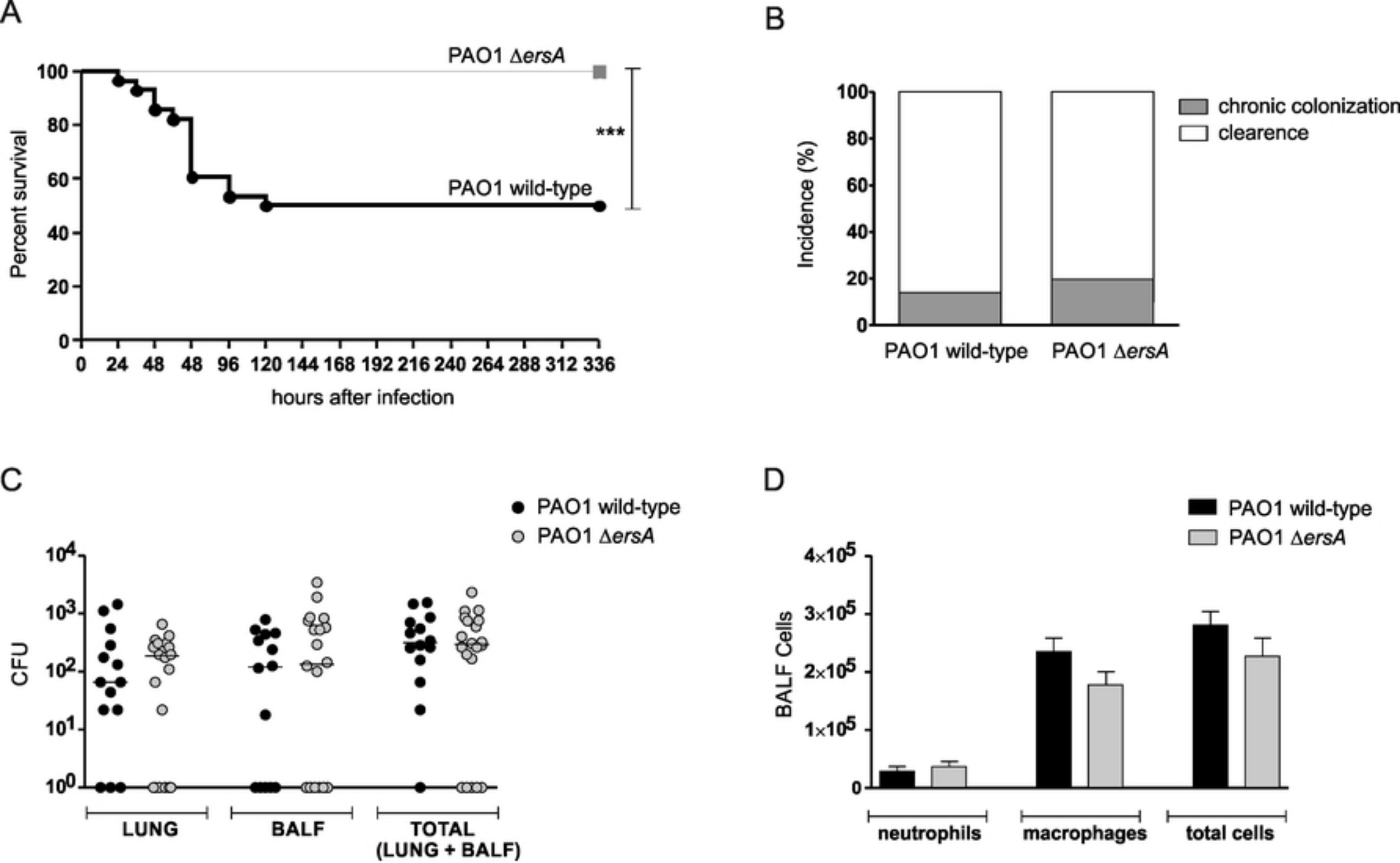
A


***
2800
2400
2000
1600
1200
800
400
0
IL-8 protein (pg/ml)
Ctrl
PAO1
wild-type
PAO1
ΔersA

B

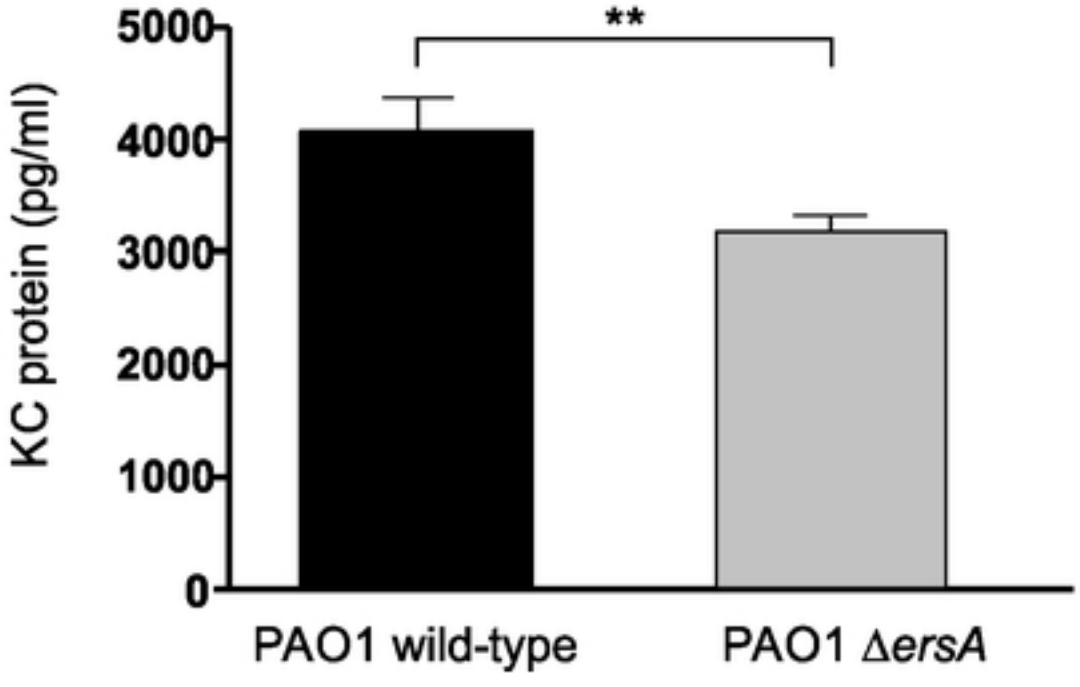

**
2000
1600
1200
800
400
0
IL-8 protein (pg/ml)
Ctrl
PAO1/pGM931
PAO1/pGM-ersA

Figure

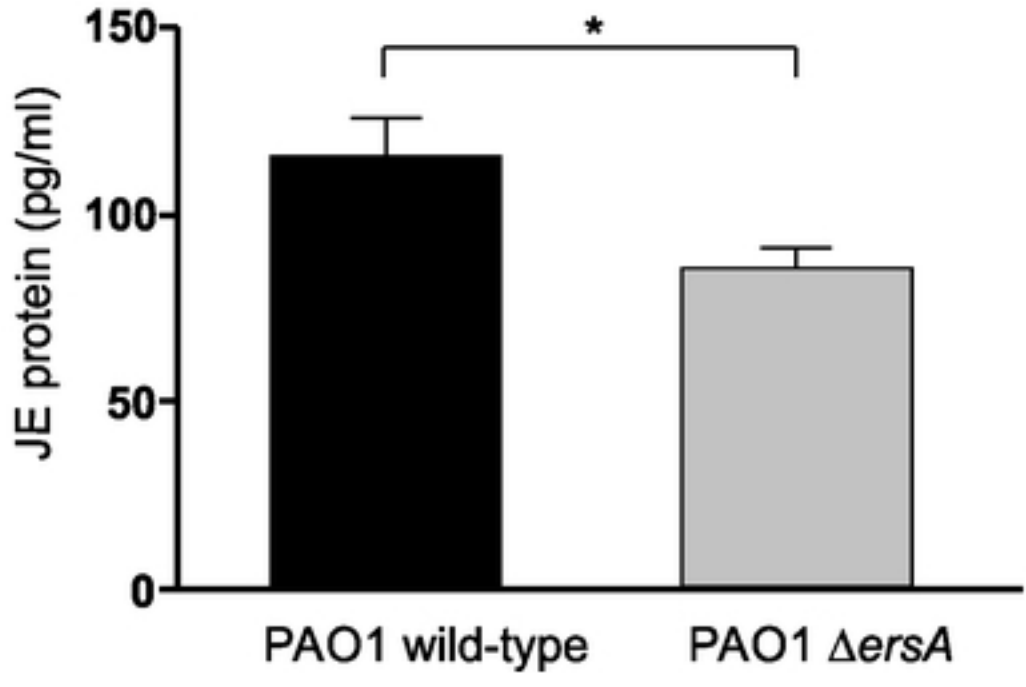

A
PAO1 ΔersA
PAO1 wild-type
***
Percent survival
100
80
60
40
20
0
0 24 48 48 96 120 144 168 192 216 240 264 288 312 336
hours after infection
B
chronic colonization
clearence
Incidence (%)
100
80
60
40
20
0
PAO1 wild-type
PAO1 ΔersA
C
PAO1 wild-type
PAO1 ΔersA
CFU
10^4
10^3
10^2
10^1
10^0
LUNG
BALF
TOTAL
(LUNG + BALF)
D
PAO1 wild-type
PAO1 ΔersA
BALF Cells
4×10^5
3×10^5
2×10^5
1×10^5
0
neutrophils
macrophages
total cells

Figure

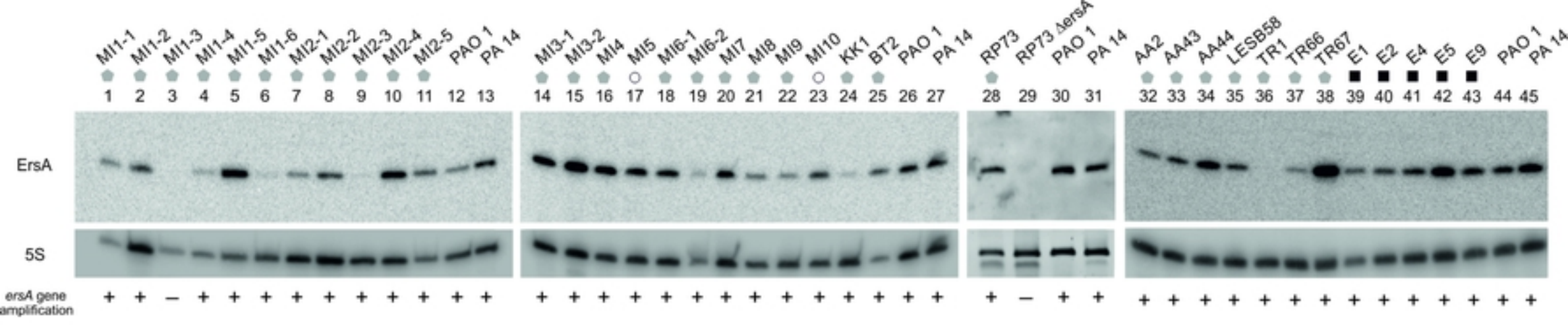
A


**
5000
4000
3000
2000
1000
0
KC protein (pg/ml)
PAO1 wild-type
PAO1 ΔersA

B


*
150
100
50
0
JE protein (pg/ml)
PAO1 wild-type
PAO1 ΔersA

Figure


MI1-1
MI1-2
MI1-3
MI1-4
MI1-5
MI1-6
MI2-1
MI2-2
MI2-3
MI2-4
MI2-5
PAO 1
PA 14
MI3-1
MI3-2
MI4
MI5
MI6-1
MI6-2
MI7
MI8
MI9
MI10
KK1
BT2
PAO 1
PA 14
RP73
RP73 ΔersA
PAO 1
PA 14
AA2
AA43
AA44
LESB58
TR1
TR66
TR67
E1
E2
E4
E5
E9
PAO 1
PA 14
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45
ErsA
5S
ersA gene amplification
+ + – + – + + + + + + + + + + + + +

Figure