



HAL
open science

Live attenuated *Bordetella pertussis* vaccine candidate BPZE1 transiently protects against lethal pneumococcal disease in mice

Thomas Belcher, Hana Kammoun, Loïc Coutte, Anne-Sophie Debie, Nathalie Mielcarek, Jean-Claude Sirard, Stephane Cauchi, Camille Locht

► **To cite this version:**

Thomas Belcher, Hana Kammoun, Loïc Coutte, Anne-Sophie Debie, Nathalie Mielcarek, et al.. Live attenuated *Bordetella pertussis* vaccine candidate BPZE1 transiently protects against lethal pneumococcal disease in mice. *Vaccine*, In press, <10.1016/j.vaccine.2021.01.025>. <hal-03372774>

HAL Id: hal-03372774

<https://hal.science/hal-03372774v1>

Submitted on 12 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons CC BY-NC-ND 4.0 - Attribution - Non-commercial use - No Derivative Works - International License

1 **Live Attenuated *Bordetella pertussis* vaccine candidate BPZE1 protects against**
2 **lethal pneumococcal disease in mice**

3

4 Thomas Belcher⁺, Hana Kammoun^{+ #}, Loïc Coutte, Anne-Sophie Debie, Nathalie
5 Mielcarek, Jean-Claude Sirard, Stéphane Cauchi, Camille Locht*

6

7 Univ. Lille, CNRS, Inserm, CHU Lille, Institute Pasteur de Lille, U1019-UMR9017-CIIL-
8 Centre for Infection and Immunity of Lille, F-59000 Lille, France

9

10

11 *Correspondence : Institute Pasteur de Lille, 1, rue du Prof. Calmette, F-59019 Lille
12 Cedex, France ; e-mail : camille.locht@pasteur-lille.fr; tel. : +33 3 20 87 11 51

13

14 #Current address : Inserm U1117, Biology of Infection Unit, Institute Pasteur, 25 Rue du
15 Dr. Roux, 75015 Paris, France

16

17 ⁺TB and HK contributed equally to this work

18 **Abstract**

19 BPZE1 is a live attenuated vaccine against infection by *Bordetella pertussis*, the
20 causative agent of whooping cough. It was previously shown that BPZE1 provides
21 heterologous protection in mouse models of disease caused by unrelated pathogens,
22 such as influenza virus and respiratory syncytial virus. Protection was also observed in
23 mouse models of asthma and contact dermatitis. In this study, we demonstrate that
24 BPZE1 also displays protection against an unrelated bacterial pathogen in a mouse
25 model of invasive pneumococcal disease mediated by *Streptococcus pneumoniae*.
26 While a single administration of BPZE1 provided no protection, two doses of 10^6 colony-
27 forming units of BPZE1 given in a three-week interval protected against mortality, lung
28 colonization and dissemination in both BALB/c and C57BL/6 mice. Unlike for the
29 previously reported influenza challenge model, protection was short-lived, and waned
30 within days after booster vaccination. Formaldehyde-killed BPZE1 protected only when
31 administered following a live prime, indicating that priming requires live BPZE1 for
32 protection. Protection against mortality was directly linked to substantially decreased
33 bacterial dissemination in the blood and was lost in MyD88 knock-out mice,
34 demonstrating the role of the innate immune system in the mechanism of protection.
35 This is the first report on a heterologous protective effect of the live BPZE1 vaccine
36 candidate against an unrelated bacterial infection.

37

38 **Key Words**

39 Heterologous protection, BPZE1, live vaccine, *Bordetella pertussis*, invasive
40 pneumococcal disease, *Streptococcus pneumonia*

41 **1. Introduction**

42

43 BPZE1 is a live attenuated vaccine candidate against infection by *Bordetella*
44 *pertussis* [1], the causative agent of whooping cough, which still causes more than
45 160,000 infant deaths per year worldwide [2]. BPZE1 was developed to raise long-
46 lasting immunity against *B. pertussis* [1, 3, 4] and was shown to prevent *B. pertussis*
47 infection in the nasal cavity in murine [5] and baboon models [6]. The vaccine has
48 completed several clinical studies and was shown to be safe and immunogenic in
49 humans after a single nasal administration [7, 8].

50 In addition to providing protection against pertussis disease and colonization by *B.*
51 *pertussis*, nonspecific off-target effects have been observed [9]. These include
52 protection against other infectious pulmonary diseases such as influenza and
53 respiratory syncytial virus disease [10, 11]. This heterologous protection is not a result
54 of B- or T-cell cross-reaction, but rather of a dampening of the immunopathology, which
55 is a key feature of these diseases. Furthermore, BPZE1 was found to attenuate disease
56 in models of non-infectious inflammation, including asthma [12] and contact dermatitis
57 at a site distant from that of BPZE1 administration [13].

58 *Streptococcus pneumoniae* is a Gram-positive bacterium and the causative agent of
59 invasive pneumococcal disease (IPD), which continues to be a significant health burden
60 worldwide despite the widespread use of vaccines [14]. Pneumococci are mostly carried
61 asymptotically in the upper respiratory tract and carriage rates vary, but colonization
62 is more frequent in infants, declining to about 5-10% of healthy adults [15-17]. Disease
63 is caused when the bacteria move to other parts of the airways, manifesting in otitis

64 media and pneumonia. Further invasion may result in septicemia, which is associated
65 with the highest level of mortality due to pneumococcal disease, of around 20% [18].

66 There are more than 90 different pneumococcal serotypes, defined by the structure
67 of the polysaccharide capsule surrounding the bacteria [19, 20]. Current vaccines
68 against *S. pneumoniae* consist of serotype-specific conjugated polysaccharide antigens
69 representing the most prevalent serotypes. Despite the ability of these vaccines to
70 provide immunity to the serotypes which they cover [21, 22], there still exists a burden
71 of pneumococcal disease worldwide due to the phenomenon known as serotype
72 replacement [23-25].

73 Thus, due to the continuing problem of serotype replacement and persistence of
74 antibiotic-resistant strains of *S. pneumoniae* [26, 27] there is a need for new ways to
75 protect against IPD. We show here that BPZE1 protects mice against IPD.

76

77 **2. Materials and Methods**

78

79 *2.1. Strains and Culture Conditions*

80

81 BPZE1, an attenuated *B. pertussis* Tohama I derivative [1], was cultured on Bordet-
82 Gengou (BG) agar (Difco) supplemented with 10% defibrinated sheep blood for 48
83 hours at 37 °C. SP1, a *S. pneumoniae* serotype 1 clinical isolate (E1586) obtained from
84 the National Reference Laboratory, Ministry of Health, Montevideo, Uruguay was grown
85 overnight at 37 °C with 5% CO₂ on tryptic soy agar (TSA) consisting of tryptic soy broth
86 (TSB) base (Sigma-Aldrich), 1.5% w/v agar (Euromedex) and 5% defibrinated sheep

87 blood. SP1 working stocks were prepared by culturing colonies grown on TSA in Todd-
88 Hewitt yeast broth (THYB), consisting of Todd-Hewitt broth (THB, Sigma-Aldrich) and
89 0.5% w/v yeast extract (Becton Dickinson) statically at 37 °C with 5% CO₂ for 4-6 hours.
90 Working stocks were stored in THYB containing 12% v/v glycerol at -80 °C for a
91 maximum of 3 months. Numbers of viable bacteria in the stocks were confirmed by
92 plating serial dilutions onto TSA blood plates. For mouse infections, working stocks
93 were thawed, washed with sterile phosphate-buffered saline (PBS), and diluted with
94 PBS to the desired concentration.

95

96 2.2. *Preparation of formaldehyde-inactivated BPZE1*

97

98 BPZE1 cells were harvested from BG agar and grown in modified Stainer-Scholte
99 (MSS) medium [28] at 37 °C under constant shaking. At mid-log phase, after about 24
100 hours of growth, formaldehyde was added to a final concentration of 0.2%, and
101 incubated at 37 °C overnight. Inactivated bacteria were harvested by centrifugation and
102 adjusted to the desired concentration with PBS.

103

104 2.3. *Mouse infections*

105

106 6-8 week-old female BALB/c ByJ or C57BL/6J mice were obtained from Charles
107 River and were maintained under specific pathogen-free (SPF) conditions at the Institut
108 Pasteur de Lille animal facility. *Myd88*^{-/-} mice backcrossed on C57BL/6J mice were bred
109 at the animal facilities of the Institut Pasteur de Lille and maintained under SPF

110 conditions. For infections, the animals were maintained in individually ventilated cages
111 and were handled in a vertical laminar flow cabinet (biosafety level 2). Mice were
112 anaesthetized with a cocktail of ketamine, atropine and valium (BALB/c); or ketamine
113 and xylazine (C57BL/6) administered by intraperitoneal injection. Anaesthetized mice
114 were infected intranasally with 10^6 CFU BPZE1 in 20 μ l PBS. For challenge,
115 anaesthetized mice were infected intranasally with a lethal dose (2×10^6 CFU) of SP1 in
116 30 μ l of PBS.

117

118 *2.4. Determination of SP1 load in blood and organs*

119

120 48 hours post-challenge with SP1, mice were anaesthetized as described above and
121 blood was collected from the eye by a capillary lined with heparin into a 1.5ml
122 Eppendorf tube containing 30 μ l 0.1M EDTA. Alternatively, mice challenged with SP1
123 were shaved on both cheeks. Blood was then taken daily starting at day 1 until day 3
124 post-challenge. The cheek was cleaned with a cotton swab soaked in ethanol and then
125 pricked with a sterile needle to take roughly 50 μ l of blood by capillary into a tube pre-
126 coated with heparin. Blood was serially diluted in PBS and plated on TSA blood agar for
127 assessing CFU counts following incubation overnight at 37 °C with 5% CO₂. Mice were
128 sacrificed by cervical dislocation and lungs and spleens were aseptically collected and
129 placed in 5ml sterile PBS before homogenization using a sterile blade. Homogenized
130 organs were serially diluted in PBS and plated onto TSA blood plates for CFU counting.

131

132 *2.5. Statistical Analyses*

133

134 The significance between two groups was analyzed by non-parametric unpaired
135 two-tailed *t*-test or log-rank test (survival curves) and was calculated using Prism
136 Graphpad. Differences were considered significantly different at $p < 0.05$. Group sizes (n)
137 are indicated in the figure legends.

138

139 2.6. *Ethics Statement*

140

141 All animal experiments were carried out in accordance with the guidelines of the
142 French Ministry of Research regarding animal experiments and with institutional
143 regulations and ethical guidelines (B59-350009; Institut Pasteur de Lille, Lille, France).
144 The protocols were approved by the Ethical Committees of the Region Nord Pas de
145 Calais and the Ministry of Research (agreement number APAFIS#9107 _
146 201603311654342 V3). Experiments were conducted by qualified, accredited
147 personnel.

148

149 **3. Results**

150

151 3.1. *BPZE1 protects against mortality in a murine model of pneumococcal* 152 *challenge*

153

154 BALB/c mice were immunized twice with BPZE1 three weeks apart and challenged
155 with a lethal dose of *S. pneumoniae* SP1 one day later. A second group was immunized

156 once with BPZE1 one day prior to challenge, and a naïve control group was challenged
157 without prior BPZE1 vaccination. Mortality was followed daily.

158 Two doses of BPZE1 protected significantly against mortality with 90% survival
159 compared to 30% for the naïve group (figure 1, $p=0.016$). Mice died between three and
160 six days post challenge. The group that received only one vaccine dose showed no
161 protection and was not significantly different from the naïve group ($p=0.34$), suggesting
162 that two doses of BPZE1 are required for significant protection against mortality.

163

164 3.2. *The BPZE1-induced protection against pneumococcal disease is short-lived*

165

166 To assess the duration of BPZE1-mediated protection, three groups of BALB/c mice
167 received two doses of BPZE1 three weeks apart while a fourth group was not
168 immunized. One of the immunized groups was challenged with a lethal dose of SP1 one
169 day after the second inoculation of BPZE1, another one was challenged three days after
170 the second inoculation and the third one was challenged one week after the second
171 inoculation. The group challenged one day after the second inoculation of BPZE1 was
172 significantly protected compared to the naïve group (figure 2, $p=0.012$). The group
173 challenged three days after the second inoculation was less well protected and when
174 the challenge was administered one week after vaccination, no protection was observed
175 ($p=0.59$). These data indicate that protection is short lived, waning within only a few
176 days following the second dose of BPZE1 and completely lost after one week.

177

178 3.3. *BPZE1 vaccination decreases the pneumococcal bacterial load in lungs,*
179 *spleen and blood*

180

181 In order to investigate whether the protection against mortality by SP1 challenge is
182 linked to a decrease in SP1 load in the vaccinated mice, one group of BALB/c mice was
183 inoculated twice with BPZE1 and challenged with a lethal dose of SP1 one day after the
184 second dose of BPZE1. A control group did not receive BPZE1 prior to SP1 challenge.
185 Two days after the SP1 challenge mice from both groups were sacrificed and lungs,
186 spleens and blood were collected in order to count SP1 colony-forming units (CFU) of
187 SP1 in these organs.

188 Two doses of BPZE1 significantly reduced the numbers of SP1 CFU in the lungs
189 (figure 3A, $p=0.0079$), spleens (figure 3B, $p=0.016$) and blood (figure 3C, $p=0.0079$) of
190 vaccinated mice, as compared to the naïve group, indicating that two doses of BPZE1
191 protect against lung colonization by SP1 and decrease dissemination, thereby
192 protecting against IPD.

193

194 3.4. *Boosting with killed BPZE1 protects against mortality and reduces SP1*
195 *bacterial load*

196

197 Heterologous protection is a known feature of live vaccines [29]. To determine
198 whether live BPZE1 is required for protection against pneumococcal disease, three
199 groups of BALB/c mice were vaccinated either with two doses of live BPZE1 given three
200 weeks apart (live-live group), with live BPZE1, boosted with formaldehyde-killed BPZE1

201 (live-killed group) or with two doses of killed BPZE1 (killed-killed group), while a fourth
202 group was not vaccinated. All groups were challenged with a lethal dose of SP1 one
203 day after the final administration of BPZE1 and were monitored daily for mortality. The
204 live-live group presented with an increased survival rate as compared to the naïve
205 group (80% survival vs. 20% survival, $p=0.077$) (figure 4A). The live-killed group was
206 also protected as compared to the naïve group ($p=0.077$), indicating that the second
207 dose of BPZE1 did not have to be live to provide protection. In contrast, the killed-killed
208 group was not protected when compared to the naïve group ($p=0.98$), demonstrating
209 that in order to mediate protection live BPZE1 is required as the first dose.

210 In order to link protection and septicemia, mice were vaccinated with live BPZE1,
211 boosted with formaldehyde-killed BPZE1 (live-killed) and challenged with SP1 one day
212 after the boost. Blood was recovered two days post-challenge. As expected, compared
213 to non-vaccinated mice live-killed BPZE1 vaccination provided protection against lethal
214 SP1 challenge (figure 4B, $p=0.048$) and resulted in a significant decrease in SP1
215 CFU/ml in blood (figure 4C, $p=0.032$). Thus, while the first dose of BPZE1 must consist
216 of live bacteria, the booster dose can be given with live or formaldehyde-killed bacteria
217 in order to protect against both disseminated infection and death.

218

219 *3.5. BPZE1-mediated protection against mortality likely comes from lowering*
220 *levels of septicemia*

221

222 In order to link mortality to level of septicemia, BALB/c mice were inoculated twice
223 with live BPZE1 three weeks apart or left unvaccinated, and then challenged with a

224 lethal dose of SP1 one day after the second dose. Blood was recovered daily from each
225 mouse for three days following the SP1 challenge.

226 Three BPZE1-treated mice out of five survived for the entire duration of the
227 experiment, while non-vaccinated mice died within four days post-challenge (figure 5A).
228 At day one post-challenge, detectable levels of SP1 were recovered from the blood of
229 all five naïve mice (figure 5B), and only in one of the five BPZE1-treated mice (figure
230 5C). In the naïve group, all mice showed increased levels of SP1 day-on-day. By day
231 three post-challenge three of the naïve mice had died (mice 1, 3 and 4) and by day four
232 post-challenge all five mice from the naïve group had died (figure 5B). For the BPZE1-
233 treated group, the mouse with detectable SP1 in the blood on day one (mouse 1) had
234 similar levels at day two, increased levels at day three (figure 5C) and died at day six.
235 Another mouse in the BPZE1-treated group had detectable levels of SP1 in the blood at
236 day two and died by day three (figure 5C). None of the other BPZE1-treated mice had
237 detectable levels of SP1 in blood at any time point and they all survived.

238 These results strongly link mortality to detectable septicemia, as all mice with
239 septicemia, regardless of BPZE1 vaccination, died, while those without detectable
240 septicemia survived. Thus, BPZE1 likely prevented death due to SP1 disease by
241 preventing lethal septicemia.

242

243 3.6. *BPZE1 protects against mortality and colonization by SP1 in C57BL/6 mice*

244

245 Since the immune responses to *B. pertussis* infection differ between BALB/c and
246 C57BL/6 mice [30], we investigated whether BPZE1-mediated protection against IPD

247 was also observed in C57BL/6 mice. C57BL/6 mice were inoculated twice with live
248 BPZE1 three weeks apart and then challenged with a lethal dose of SP1 one day
249 following the second dose of BPZE1. 70% of the BPZE1-treated mice survived the
250 challenge, while all control mice died within five days after challenge (figure 6A,
251 $p=0.0006$). These data show that, as for BALB/c mice, C57BL/6 mice were protected
252 from death caused by lethal SP1 challenge when vaccinated with two doses of BPZE1.

253 Lungs and blood were collected to measure SP1 load two days post-challenge.
254 BPZE1 vaccination led to a significant reduction of SP1 CFU counts in both the lungs
255 (figure 6B, $p=0.016$) and the blood (figure 6C, $p=0.016$). Thus, BPZE1 protected against
256 mortality and lung colonization as well as disseminated disease in C57BL/6 mice,
257 demonstrating that the BPZE1-mediated protection against SP1 disease is not
258 influenced by the genetic background of the animals.

259 In order to link mortality and the level of septicemia in C57BL/6 mice, mice
260 vaccinated with two doses of live BPZE1 were compared to naïve mice as described
261 above for the BALB/c mice. As expected, two doses of BPZE1 significantly protected
262 against mortality (figure 7A, $p=0.015$). On day one post-challenge three out of the five
263 naïve mice had detectable levels of SP1 in the blood (mice 2, 4 and 5, figure 7B), while
264 SP1 was detectable at relatively low levels in the blood of only one BPZE1-vaccinated
265 mouse (mouse 5, figure 7C). By day two post-challenge, all naïve mice had high levels
266 of SP1 in the blood. By day three, one mouse from the naïve group had died (mouse 2,
267 figure 7B) while all of the mice had died by day four. In the BPZE1 vaccinated group,
268 two mice had detectable levels of SP1 in the blood at day two post-challenge (mouse 1
269 and mouse 5, figure 7C). Both of these mice had increased levels of SP1 on day three

270 and died at day four (mouse 1) and day 5 (mouse 5). Two further mice from the
271 vaccinated group showed detectable levels of SP1 in the blood on day 3 (mice 2 and 3)
272 but they survived at least until day nine, when the experiment was terminated. The
273 levels of SP1 detected in these two mice were relatively low compared to mice that
274 died. The remaining mouse from the vaccinated group did not show any detectable level
275 of SP1 in the blood and survived. Therefore, while vaccinated C57BL/6 mice seem to be
276 more susceptible to developing SP1 septicemia, the BPZE1 treatment, as seen in
277 BALB/c mice, prevents high levels of septicemia and death.

278

279 *3.7. BPZE1-mediated protection against mortality depends on MyD88*

280

281 The BPZE1-mediated protection is induced rapidly after vaccination and may
282 therefore be mediated by innate immunity rather than adaptive immunity. To determine
283 the role of innate immunity in BPZE1-mediated protection against IPD, MyD88 knock-
284 out mice were used in a BPZE1 protection experiment, as these mice are defective in
285 TLR signaling, an essential pathway in the stimulation of innate immunity.

286 One group of MyD88 knock-out mice was vaccinated with the BPZE1 live-killed
287 regimen while another group remain naïve. All mice were challenged with a lethal dose
288 of SP1. Unlike wild-type C57BL/6 mice (figure 6A), MyD88 knock-out mice were not
289 protected by BPZE1 vaccination (figure 8). All mice from both groups died upon SP1
290 challenge within four days. These observations indicate that MyD88 is critical for
291 mediating the protection by BPZE1 against IPD.

292

293 4. Discussion

294

295 It was previously shown that BPZE1 induces heterologous protection in mouse
296 models of inflammation caused by both infectious and non-infectious agents [10-13].
297 This study is the first report of BPZE1-induced protection against disease caused by a
298 bacterium other than *Bordetella*. We show here that BPZE1 is able to protect against
299 mortality as well as lung colonization and septicemia caused by the unrelated bacterial
300 pathogen *S. pneumoniae*. While one dose was not sufficient, two doses of BPZE1
301 provided significant protection, suggesting a booster effect. Booster effects have been
302 described for other models of BPZE1-mediated protection, in which enhanced
303 protection was observed with two doses of BPZE1 in the case of influenza [10], while a
304 second dose was required to observe protection in a model of contact dermatitis [13]. A
305 booster effect is suggestive of innate immune memory, i.e. the stimulation of the innate
306 immune cells to respond more quickly and robustly to subsequent stimuli, even when
307 the agents used to prime and challenge are not the same [9, 31, 32].

308 In addition, we show that priming with live BPZE1 is necessary in order to observe
309 significant protection, presumably to allow for robust colonization, since no protective
310 effect was observed when mice were primed with formaldehyde-killed BPZE1. This is in
311 agreement with well-known heterologous protective effects specifically of live vaccines
312 [29, 33, 34], and in agreement with the model of BPZE1-mediated protection against
313 influenza in which killed BPZE1 did not induce protection [10]. On the other hand, in this
314 model of protection against pneumococcal pneumonia, a formaldehyde-killed BPZE1
315 boost produced protection, provided that priming was done using live bacteria,

316 indicating that for boosting of mice that had already been exposed to live BPZE1
317 colonization was not necessary.

318 There are important differences between the observations made in this study and
319 that of previously published work. Unlike BPZE1-mediated protection against viral
320 diseases, namely influenza [10] and respiratory syncytial virus (RSV) disease [11],
321 protection against *S. pneumoniae* was short-lived. BPZE1-mediated protection against
322 influenza was observed several weeks after vaccination and lasted for at least up to 12
323 weeks after BPZE1 was given [10], while protection against *S. pneumoniae* was
324 significant 24 hours after boosting and then waned in the following days, suggesting that
325 the protective mechanism in this model is different from that of other published models.

326 Various mechanisms have been suggested for the heterologous protection by
327 BPZE1, including epigenetic reprogramming of innate immune cells, referred to as
328 trained innate immunity, as well as the induction of regulatory Tr1 cells or
329 CD4⁺CD25⁺FoxP3⁺ T_{reg} cells [9, 35]. However, the short-lived protection observed in the
330 pneumococcal model suggests a rapid induction of cells that likely already inhabit the
331 lung rather than a more lengthy specific recruitment, expansion or differentiation. The
332 short-lived phenotype seen here likely reflects a need for recent stimulation by BPZE1,
333 which appears to be sufficient to affect colonization of SP1 in a subsequent challenge.

334 Another important difference to previously published work on BPZE1 is that here we
335 show a significant effect by BPZE1 on the bacterial load of *S. pneumoniae* in the lungs.
336 This is in contrast to the lack of significant change in viral load seen in the influenza
337 model [10], and suggests that the mechanism of protection against pneumococcal
338 disease was a result of decreasing pathogen burden rather than merely dampening the

339 inflammatory response as has been suggested for influenza [10]. What we report here
340 better reflects resistance to infection rather than anti-inflammatory effects, in contrast to
341 other models of BPZE1-mediated protection. We found that BPZE1 protected against
342 mortality from a lethal challenge of SP1 by decreasing colonization of the blood and
343 disseminated infection, and were able to tightly link blood colonization to mortality. Two
344 doses of BPZE1 reduced numbers of SP1 in the lungs 48 hours after challenge,
345 suggesting that lack of dissemination was likely due to reduced pathogen burden in the
346 lungs. Only in the model of protection against RSV has BPZE1 been shown to reduce
347 burden of an unrelated pathogen, but this protection was evident 14 days after single
348 BPZE1 administration [11], suggesting a different mechanism.

349 Two doses of BPZE1 protected against lethal pneumococcal disease in both BALB/c
350 and C57BL/6 strains of mice, demonstrating that this was not restricted to Th2-proned
351 BALB/c mice but was independent of the genetic background. BPZE1 did not protect
352 MyD88-deficient mice against lethal SP1 challenge, demonstrating a role for innate
353 immunity and signaling through toll-like receptors (TLRs) in the mechanism of
354 protection. This is reminiscent of previous findings showing a role of MyD88 signaling in
355 early protection against *B. pertussis* itself, before the adaptive immune response kicks
356 in [36]. In this latter case, TLR4 played a major role in protection. Whether a similar
357 system is at play against IPD awaits further studies.

358 Interestingly, Clement et al. [37] reported that administering a lysate of
359 *Haemophilus influenzae* to mice induced short-lasting protection from disease caused
360 by *S. pneumoniae*, similar to what we report here. In that model survival also correlated
361 with a decrease in bacterial load, while protection was not caused by any particular

362 immune cell, but was shown to be the result of epithelial cells which had been
363 stimulated to kill the pathogen of challenge [38]. Protection likely depended on TLR
364 signaling, since MyD88 knock-out mice were not protected. However, knock-out mice of
365 any individual TLR were still protected, suggesting redundancy among TLR signaling.
366 Furthermore, administration of combinations of ligands for different TLRs, such as
367 TLR2/6 and TLR9 induced significant protection [39, 40]. These TLRs are expressed by
368 epithelial cells, and their stimulation induces the production of anti-microbial peptides
369 able to kill pathogens, as well as chemokines that can mobilize both resident and blood
370 phagocytes, such as monocytes and neutrophils. Similarly, the intranasal administration
371 of the TLR5 agonist flagellin promotes stimulation of the airway epithelium and induces
372 protection against IPD in mice [41]. However, BPZE1 does not produce flagellin.
373 Nevertheless, although we have not investigated the role of epithelial TLR signaling in
374 BPZE1-mediated protection, given the similarities between the observations with
375 BPZE1 and those with the *H. influenzae* lysate and flagellin in protection against IPD it
376 is tempting to suggest that the mechanisms share similarities. During infection *B.*
377 *pertussis* preferentially targets respiratory epithelial cells [42-46]. It would therefore not
378 be surprising that epithelial cells play an important role in protection provided by BPZE1.
379 In line with this hypothesis bronchial epithelial cells have been shown to induce the
380 expression of a number of genes within hours after incubation with *B. pertussis*,
381 including chemokine/cytokine-encoding genes, which may attract neutrophils able to kill
382 the bacteria [46]. This provides scope for further investigation.

383 However, there are a number of notable differences between the BPZE1 model and
384 that reported by Clement et al. We found here that two doses of BPZE1 are required for

385 strong, robust protection against mortality, while a single administration of the *H.*
386 *influenzae* extract was sufficient for protection. The other difference is the requirement
387 for the first dose of BPZE1 to be alive, while protection was observed with inactivated *H.*
388 *influenzae* [37]. However, this latter difference may be explained by the differences
389 between the methods of killing: formaldehyde killing in this study, as opposed to UV-
390 and sonicated-inactivation. Alternatively, the difference may be due to the utilization of
391 different bacterial species that behave differently in the respective models.

392 In summary, we present here the first evidence that BPZE1 can protect against
393 disease caused by a bacterial species that is unrelated to *Bordetella spp.* This
394 mechanism of protection appears to be quite different from those of already published
395 models of BPZE1 heterologous protection and is characterized by reduced survival of
396 pneumococci in the lungs, which reduced invasion and death. Although vaccines
397 against pneumococci presently exist, they are serotype specific, and serotypes that are
398 not covered by the vaccines are replacing those that are covered [23, 24]. New
399 preventative measures are therefore needed, and while the protection provided by
400 BPZE1 is short-lived, it may still have prophylactic potential in a setting where *S.*
401 *pneumoniae* actively circulates, such as in hospital outbreaks. BPZE1 is currently
402 undergoing clinical development for an indication against pertussis and was shown to
403 be safe in healthy adults [7, 8]. This opens up the possibility of testing the vaccine in a
404 short-term prophylactic strategy against IPD.

405

406

407

408 **Author Contributions**

409

410 Conceived and designed the experiments: TB, HK, LC, SC and CL. Funding acquisition:

411 CL, HK. Performed the experiments: TB, LC, HK, ASD and SC. Analyzed the data: TB,

412 HK, NM, CL and SC. Contributed reagents/materials and helped with SP1 culturing:

413 JCS. Wrote the paper: TB, LC and CL.

414

415

416

417 **Conflict of Interest statement**

418

419 ASD, NM and CL are co-inventors of patents on BPZE1, which were licensed to ILiAD

420 Biotechnologies. No conflict of interest is declared by the other authors

421

422 **Acknowledgements**

423

424 This work was funded in part by Région Nord/Pas-de-Calais and Institut Pasteur de Lille

425 through a pre-doctoral fellowship to HK.

426

427

428 **References**

429

- 430 [1] Mielcarek N, Debie AS, Raze D, et al. Live attenuated *B. pertussis* as a single-
431 dose nasal vaccine against whooping cough. PLoS Pathog 2006;2(7):e65.
- 432 [2] Yeung KHT, Duclos P, Nelson EAS, Hutubessy RCW. An update of the global
433 burden of pertussis in children younger than 5 years: a modelling study. Lancet
434 Infect Dis 2017;17(9):974-80.
- 435 [3] Feunou PF, Kammoun H, Debie AS, Mielcarek N, Loch C. Long-term immunity
436 against pertussis induced by a single nasal administration of live attenuated *B.*
437 *pertussis* BPZE1. Vaccine 2010;28(43):7047-53.
- 438 [4] Skerry CM, Mahon BP. A live, attenuated *Bordetella pertussis* vaccine provides
439 long-term protection against virulent challenge in a murine model. Clin Vaccine
440 Immunol 2011;18(2):187-93.
- 441 [5] Solans, Debie AS, Borkner L, et al. IL-17-dependent SIgA-mediated protection
442 against nasal *Bordetella pertussis* infection by live attenuated BPZE1 vaccine.
443 Mucosal Immunol 2018;11(6):1753-62.
- 444 [6] Loch C, Papin JF, Lecher S, et al. Live attenuated pertussis vaccine BPZE1
445 protects baboons against *Bordetella pertussis* disease and infection. J Infect Dis
446 2017;216(1):117-24.
- 447 [7] Thorstensson R, Trollfors B, Al-Tawil N, et al. A phase I clinical study of a live
448 attenuated *Bordetella pertussis* vaccine--BPZE1; a single centre, double-blind,
449 placebo-controlled, dose-escalating study of BPZE1 given intranasally to healthy
450 adult male volunteers. PLoS One 2014;9(1):e83449.

- 451 [8] Jahnmatz M, Richert L, Al-Tawil N, et al. Safety and immunogenicity of the live
452 attenuated intranasal pertussis vaccine BPZE1: a phase 1b, double-blind,
453 randomised, placebo-controlled dose-escalation study. *Lancet Infect Dis* 2020;in
454 press.
- 455 [9] Cauchi S, Locht C. Non-specific effects of live attenuated pertussis vaccine
456 against heterologous infectious and inflammatory diseases. *Front Immunol* 2018;
457 9:2872.
- 458 [10] Li R, Lim A, Phoon MC, et al. Attenuated *Bordetella pertussis* protects against
459 highly pathogenic influenza A viruses by dampening the cytokine storm. *J Virol*
460 2010;84(14):7105-13.
- 461 [11] Schnoeller C, Roux X, Sawant D, et al. Attenuated *Bordetella pertussis* vaccine
462 protects against respiratory syncytial virus disease via an IL-17-dependent
463 mechanism. *Am J Respir Crit Care Med* 2014;189(2):194-202.
- 464 [12] Kavanagh H, Noone C, Cahill E, English K, Locht C, Mahon BP. Attenuated
465 *Bordetella pertussis* vaccine strain BPZE1 modulates allergen-induced immunity
466 and prevents allergic pulmonary pathology in a murine model. *Clin Exp Allergy*
467 2010;40(6):933-41.
- 468 [13] Li R, Cheng C, Chong SZ, et al. Attenuated *Bordetella pertussis* BPZE1 protects
469 against allergic airway inflammation and contact dermatitis in mouse models.
470 *Allergy* 2012;67(10):1250-8.
- 471 [14] O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by
472 *Streptococcus pneumoniae* in children younger than 5 years: global estimates.
473 *Lancet* 2009;374(9693):893-902.

- 474 [15] Regev-Yochay G, Raz M, Dagan R, et al. Nasopharyngeal carriage of
475 *Streptococcus pneumoniae* by adults and children in community and family
476 settings. Clin Infect Dis 2004;38(5):632-9.
- 477 [16] Cardozo DM, Nascimento-Carvalho CM, Andrade ASS, et al. Prevalence and
478 risk factors for nasopharyngeal carriage of *Streptococcus pneumoniae* among
479 adolescents. J Med Microbiol 2008;57(2):185-9.
- 480 [17] Wyllie AL, Rümke LW, Arp K, et al. Molecular surveillance on *Streptococcus*
481 *pneumoniae* carriage in non-elderly adults; little evidence for pneumococcal
482 circulation independent from the reservoir in children. Sci Rep 2016;6:34888.
- 483 [18] Balakrishnan I, Crook P, Morris R, Gillespie SH. Early predictors of mortality in
484 pneumococcal bacteraemia. J Infect 2000;40(3):256-61.
- 485 [19] Bentley SD, Aanensen DM, Mavroidi A, et al. Genetic analysis of the capsular
486 biosynthetic locus from all 90 pneumococcal serotypes. PLoS Genet
487 2006;2(3):e31.
- 488 [20] Loughran AJ, Orihuela CJ, Tuomanen EI. *Streptococcus pneumoniae*: Invasion
489 and Inflammation. Microbiol Spectr 2019;7(2): doi: 10.1128/microbiolspec.GPP3-
490 0004-2018.
- 491 [21] Berical AC, Harris D, Dela Cruz CS, Possick JD. Pneumococcal vaccination
492 strategies. An update and perspective. Ann Am Thorac Soc 2016;13(6):933-44.
- 493 [22] Papadatou I, Tzovara I, Licciardi PV. The role of serotype-specific immunological
494 memory in pneumococcal vaccination: current knowledge and future prospects.
495 Vaccines 2019;7(1):13.

- 496 [23] Feikin DR, Kagucia EW, Loo JD, et al. Serotype-specific changes in invasive
497 pneumococcal disease after pneumococcal conjugate vaccine introduction: a
498 pooled analysis of multiple surveillance sites. PLoS Med 2013;10(9):e1001517.
- 499 [24] Weinberger DM, Malley R, Lipsitch M. Serotype replacement in disease after
500 pneumococcal vaccination. Lancet 2011;378(9807):1962-73.
- 501 [25] Izurieta P, Bahety P, Adegbola R, Clarke C, Hoet B. Public health impact of
502 pneumococcal conjugate vaccine infant immunization programs: assessment of
503 invasive pneumococcal disease burden and serotype distribution. Expert Rev
504 Vaccines 2018;17(6):479-93.
- 505 [26] Kim L, McGee L, Tomczyk S, Beall B. Biological and epidemiological features of
506 antibiotic-resistant *Streptococcus pneumoniae* in pre- and post-conjugate
507 vaccine eras: a United States perspective. Clin Microbiol Rev 2016;29(3):525-52.
- 508 [27] Liñares J, Ardanuy C, Pallares R, Fenoll A. Changes in antimicrobial resistance,
509 serotypes and genotypes in *Streptococcus pneumoniae* over a 30-year period.
510 Clin Microbiol Infect 2010;16(5):402-10.
- 511 [28] Imaizumi A, Suzuki Y, Ono S, Sato H, Sato Y. Effect of heptakis (2,6-O-dimethyl)
512 beta-cyclodextrin on the production of pertussis toxin by *Bordetella pertussis*.
513 Infect Immun 1983;41(3):1138-43.
- 514 [29] Saadatian-Elahi M, Aaby P, Shann F, et al. Heterologous vaccine effects.
515 Vaccine 2016;34(34):3923-30.
- 516 [30] Mosley YC, Lu F, HogenEsch H. Differences in innate IFN γ and IL-17 responses
517 to *Bordetella pertussis* between BALB/c and C57BL/6 mice: role of $\gamma\delta$ T cells, NK
518 cells, and dendritic cells. Immunol Res 2017;65(6):1139-49.

- 519 [31] Álvarez-Errico D, Vento-Tormo R, Sieweke M, Ballestar E. Epigenetic control of
520 myeloid cell differentiation, identity and function. *Nat Rev Immunol* 2015;15(1):7-
521 17.
- 522 [32] Netea MG, Latz E, Mills KH, O'Neill LA. Innate immune memory: a paradigm shift
523 in understanding host defense. *Nat Immunol* 2015;16(7):675-9.
- 524 [33] Aaby P, Mogensen SW, Rodrigues A, Benn CS. Evidence of increase in mortality
525 after the introduction of diphtheria-tetanus-pertussis vaccine to children aged 6-
526 35 months in Guinea-Bissau: a time for reflection? *Front Public Health* 2018;6:79.
- 527 [34] Kowalewicz-Kulbat M, Loch C. BCG and protection against inflammatory and
528 auto-immune diseases. *Expert Rev Vaccines* 2017;16(7):1-10.
- 529 [35] Fedele G, Sanseverino I, D'Agostino K, et al. Unconventional, adenosine-
530 producing suppressor T cells induced by dendritic cells exposed to BPZE1
531 pertussis vaccine. *J Leukoc Biol* 2015;98(4):631-9.
- 532 [36] Debie AS, Mielcarek N, Lecher S, Roux X, Sirard JC, Loch C. Early protection
533 against pertussis induced by live attenuated *Bordetella pertussis* BPZE1
534 depends on TLR4. *J Immunol* 2019;203(12):3293-300.
- 535 [37] Clement CG, Evans SE, Evans CM, et al. Stimulation of lung innate immunity
536 protects against lethal pneumococcal pneumonia in mice. *Am J Respir Crit Care*
537 *Med* 2008;177(12):1322-30.
- 538 [38] Evans SE, Scott BL, Clement CG, et al. Stimulated innate resistance of lung
539 epithelium protects mice broadly against bacteria and fungi. *Am J Respir Cell Mol*
540 *Biol* 2010;42(1):40-50.

- 541 [39] Evans SE, Xu Y, Tuvim MJ, Dickey BF. Inducible innate resistance of lung
542 epithelium to infection. *Annu Rev Physiol* 2010;72:413-35.
- 543 [40] Evans SE, Tuvim MJ, Fox CJ, Sachdev N, Gibiansky L, Dickey BF. Inhaled
544 innate immune ligands to prevent pneumonia. *Br J Pharmacol* 2011;163(1):195-
545 206.
- 546 [41] Munoz N, Van Maele L, Marqués JM, Rial A, Sirard JC, Chabalgoity JA. Mucosal
547 administration of flagellin protects mice from *Streptococcus pneumoniae* lung
548 infection. *Infect Immun* 2010;78(10):4226-4233.
- 549 [42] van den Berg BM, Beekhuizen H, Willems RJ, Mooi FR, van Furth R. Role of
550 *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived
551 from the human respiratory tract. *Infect Immun* 1999;67(3):1056-62.
- 552 [43] Rodríguez ME, Hellwig SM, Pérez Vidakovics ML, Berbers GA, van de Winkel
553 JG. *Bordetella pertussis* attachment to respiratory epithelial cells can be impaired
554 by fimbriae-specific antibodies. *FEMS Immunol Med Microbiol* 2006;46(1):39-47.
- 555 [44] Lamberti Y, Alvarez Hayes J, Perez Vidakovics ML, Rodriguez ME. Cholesterol-
556 dependent attachment of human respiratory cells by *Bordetella pertussis*. *FEMS*
557 *Immunol Med Microbiol* 2009;56(2):143-50.
- 558 [45] Guevara C, Zhang C, Gaddy JA, et al. Highly differentiated human airway
559 epithelial cells: a model to study host cell-parasite interactions in pertussis. *Infect*
560 *Dis* 2016;48(3):177-88.
- 561 [46] Belcher CE, Drenkow J, Kehoe B, et al. The transcriptional responses of
562 respiratory epithelial cells to *Bordetella pertussis* reveal host defensive and

563 pathogen counter-defensive strategies. Proc Natl Acad Sci U S A

564 2000;97(25):13847-52.

565

566 **Figure Legends**

567

568 **Fig. 1.** Survival of BPZE1-treated BALB/c mice following a lethal intranasal challenge
569 with SP1. Groups were either naïve (circles), or had received one (squares) or two
570 doses (triangles) of BPZE1. Survival was monitored daily; n=10 (all groups). (*p<0.05,
571 **p<0.01).

572

573 **Fig. 2.** Longevity of BPZE1-mediated protection against lethal challenge with SP1 in
574 BALB/c mice. Groups were challenged either 1 day (squares), 3 days (triangles) or 1
575 week (diamonds) after the second BPZE1 administration, while the naïve group (circles)
576 was not vaccinated with BPZE1. Survival was monitored daily; n=10 (all groups).
577 (*p<0.05).

578

579 **Fig. 3.** Colonization of SP1 in organs of BPZE1-treated BALB/c mice. Mice were naïve
580 or treated twice with BPZE1 as indicated. Organs and blood were collected 2 days after
581 SP1 challenge, homogenized and plated for counts of CFUs in the (A) lungs, (B)
582 spleens and (C) blood of mice; n=5 (all groups). The dotted lines represent the lower
583 and upper (in C) thresholds of detection. (*p<0.05, **p<0.01).

584

585 **Fig. 4.** Protection against SP1 by either live or formaldehyde-killed BPZE1. (A) BALB/c
586 mice were either naïve (circles) or treated with two doses of killed BPZE1 (squares,
587 killed-killed), two doses of live BPZE1 (open triangles, live-live), or primed with live and
588 boosted with killed BPZE1 (inversed triangles, live-killed), before challenge with a lethal

589 dose of SP1. Survival was followed daily; n=5 (all groups). **(B)** BALB/c mice were
590 primed with live BPZE1 and boosted with killed (squares, BPZE1 live-killed), or were
591 naïve (circles) before a lethal challenge with SP1. Survival was monitored daily; n=10
592 (all groups); **(C)** blood was collected 48 hours later for measurement of SP1 levels in
593 different mice; n=4 (naïve) n=5 (BPZE1 live-killed). The dotted line represents a
594 threshold of detection (*p<0.05).

595

596 **Fig. 5.** BPZE1 protection against lethal septicemia in individual BALB/c mice. Mice were
597 naïve (circles) or treated twice with BPZE1 (squares) and challenged with a lethal dose
598 of SP1. **(A)** Survival was followed daily; n=5 (all groups). **(B and C)** SP1 counts in the
599 blood were monitored daily in individual mice (M1-M5) in the naïve group **(B)** and the
600 BPZE1-treated group **(C)**. The dotted lines represent the threshold of detection.
601 (*p<0.05).

602

603 **Fig. 6.** BPZE1-induced protection against lethal SP1 challenge in C57BL/6 mice. Mice
604 were treated with two doses of BPZE1 (squares) or left naïve (circles) before challenge
605 with a lethal dose of SP1. **(A)** Survival was monitored daily; n=10. **(B and C)** SP1 counts
606 in the lungs **(B)** and blood **(C)** were measured 48 hours after challenge in different mice;
607 n=5 (all groups). The dotted lines represent a threshold of detection. (*p<0.05,
608 ***p<0.001).

609

610 **Fig. 7.** BPZE1 protection against lethal septicemia in individual C57BL/6 mice. Mice
611 were treated twice with BPZE1 (triangles, BPZE1 prime-boost) or left naïve (circles)

612 before challenge with a lethal dose of SP1. **(A)** Survival was followed daily, n=5 (all
613 groups). **(B and C)** SP1 counts in the blood were monitored daily in individual mice (M1-
614 M5) in the naïve group **(B)** and the BPZE1-treated group **(C)**. The dotted lines represent
615 the threshold of detection. (*p<0.05).

616

617 **Fig. 8.** Loss of BPZE1-induced protection against SP1 in MyD88 knock-out mice.

618 MyD88 knock-out mice were primed with live BPZE1 and boosted with killed BPZE1
619 (squares, BPZE1 live-killed) or left naïve (circles). Survival was monitored daily, n=4
620 (naïve) n=5 (BPZE1 live-killed). ns, not significant.

621

622

623

624

625

626

627

628

629

630

631

632

633

634

Figure 1

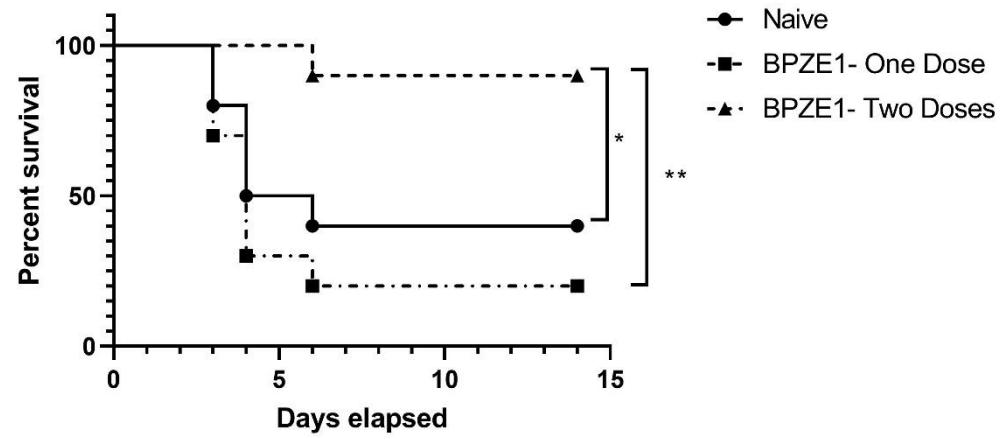


Figure 2

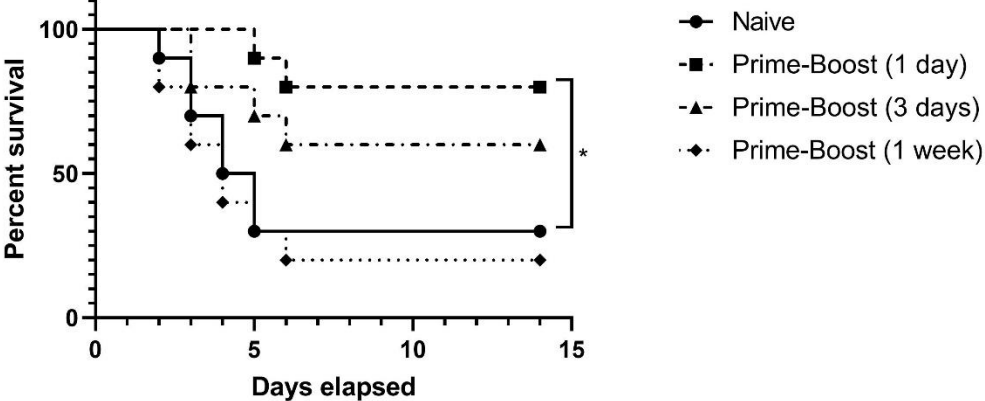


Figure 3

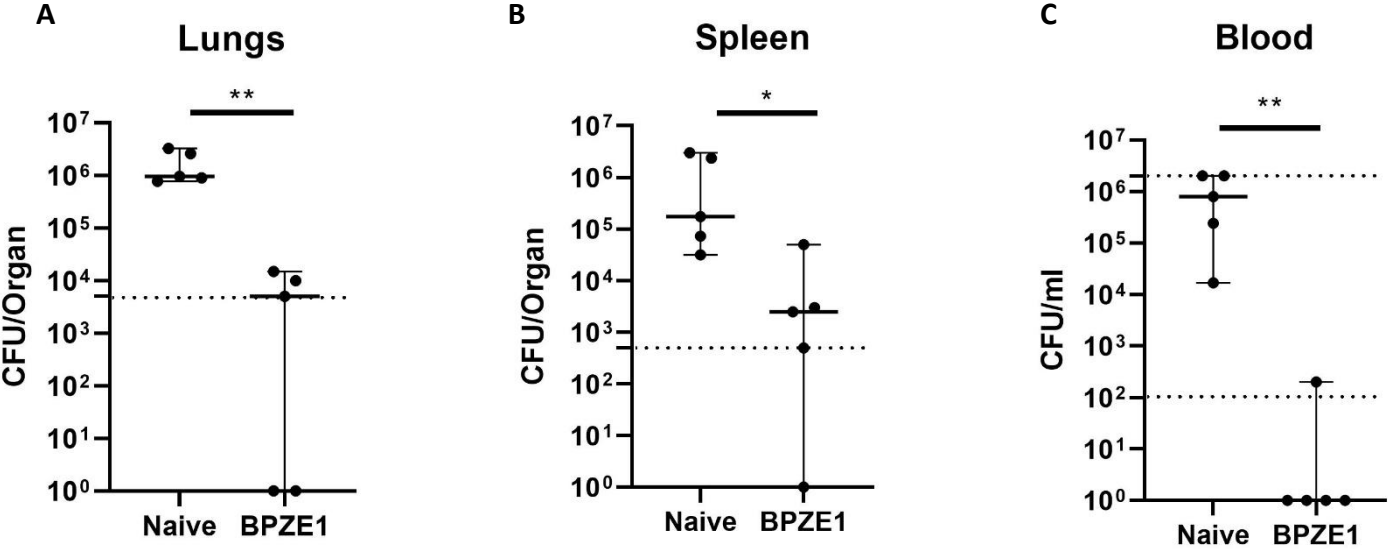
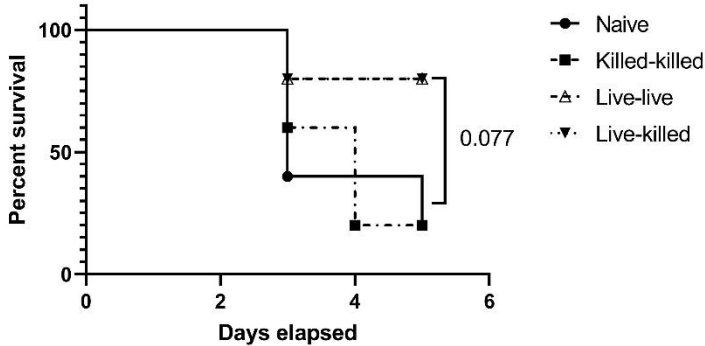
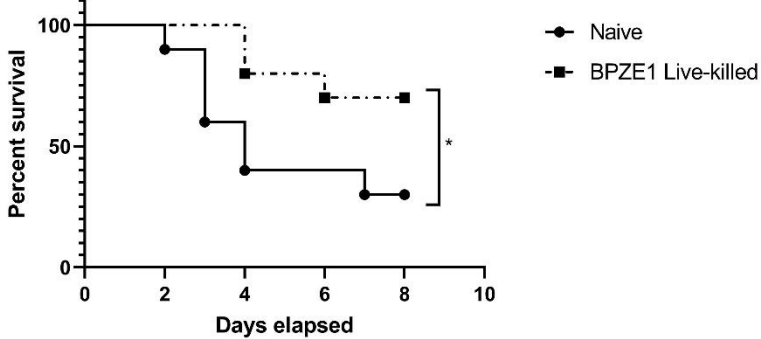


Figure 4

A



B



C

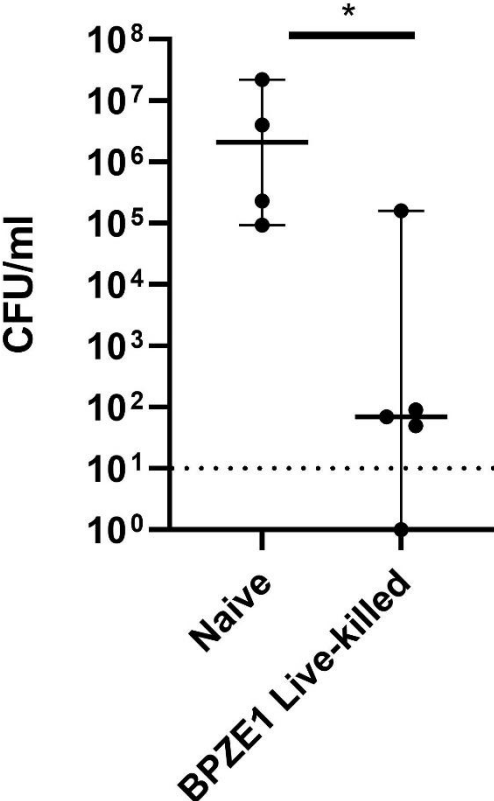


Figure 5

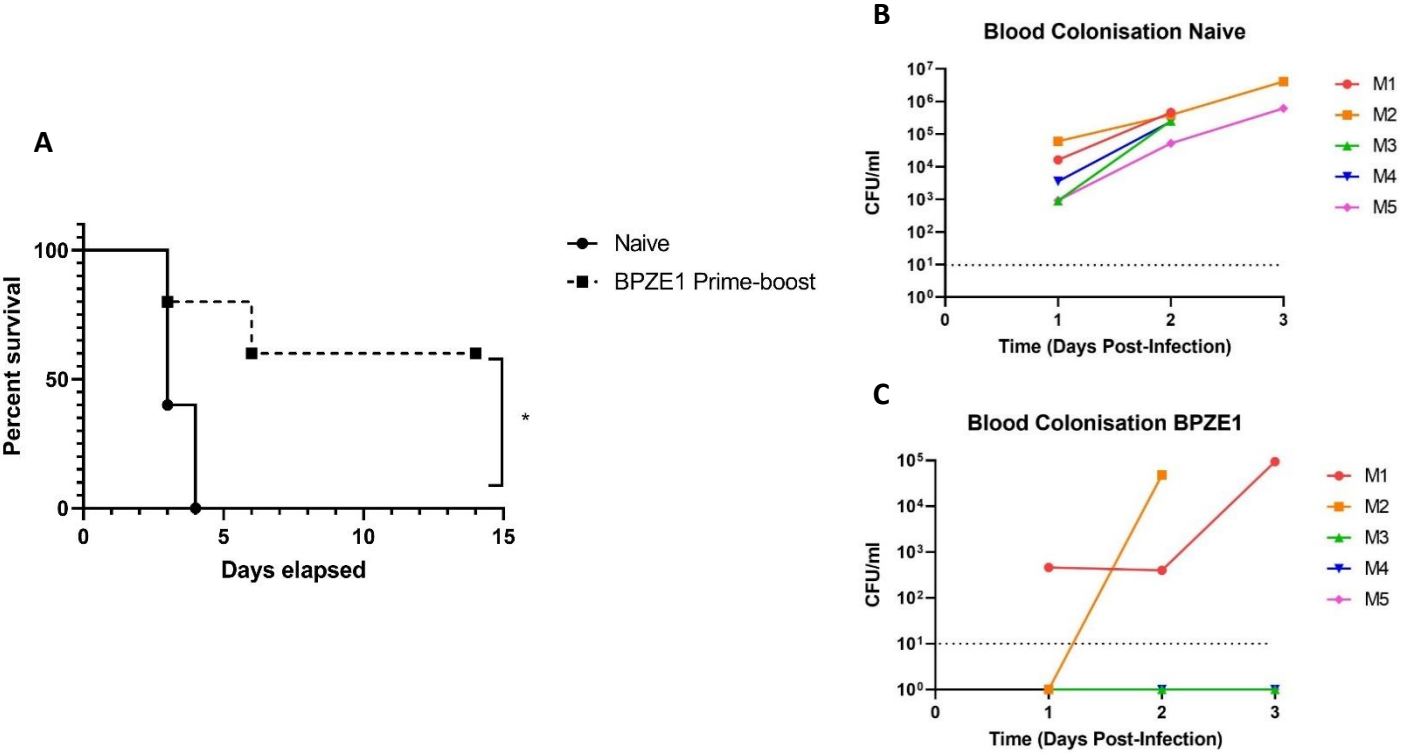
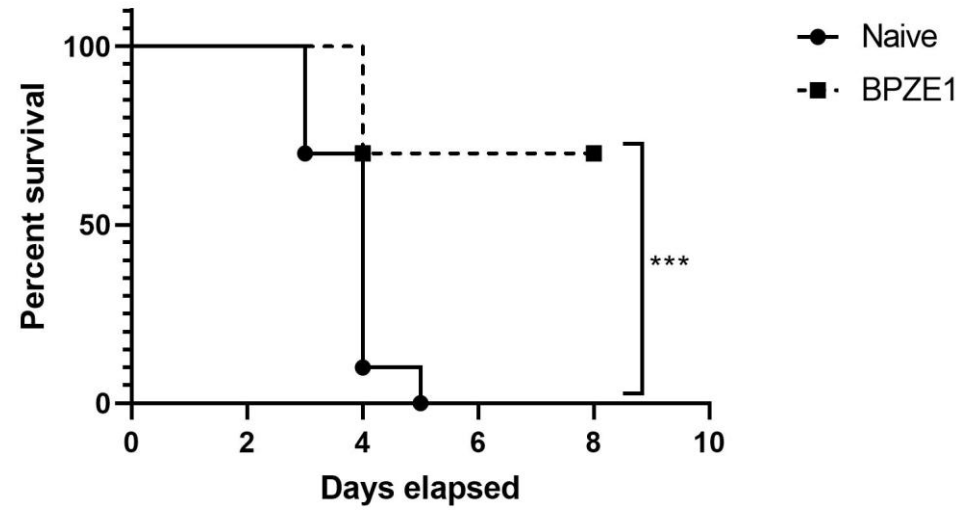


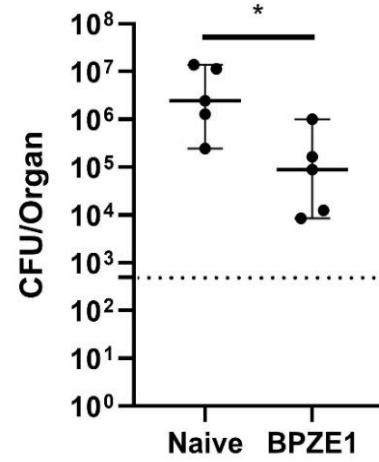
Figure 6

A



B

SP1 Colonisation Lungs



C

SP1 Colonisation Blood

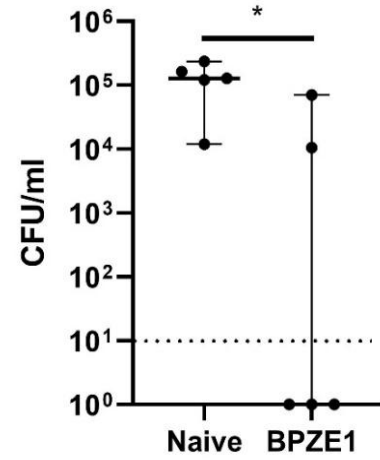


Figure 7

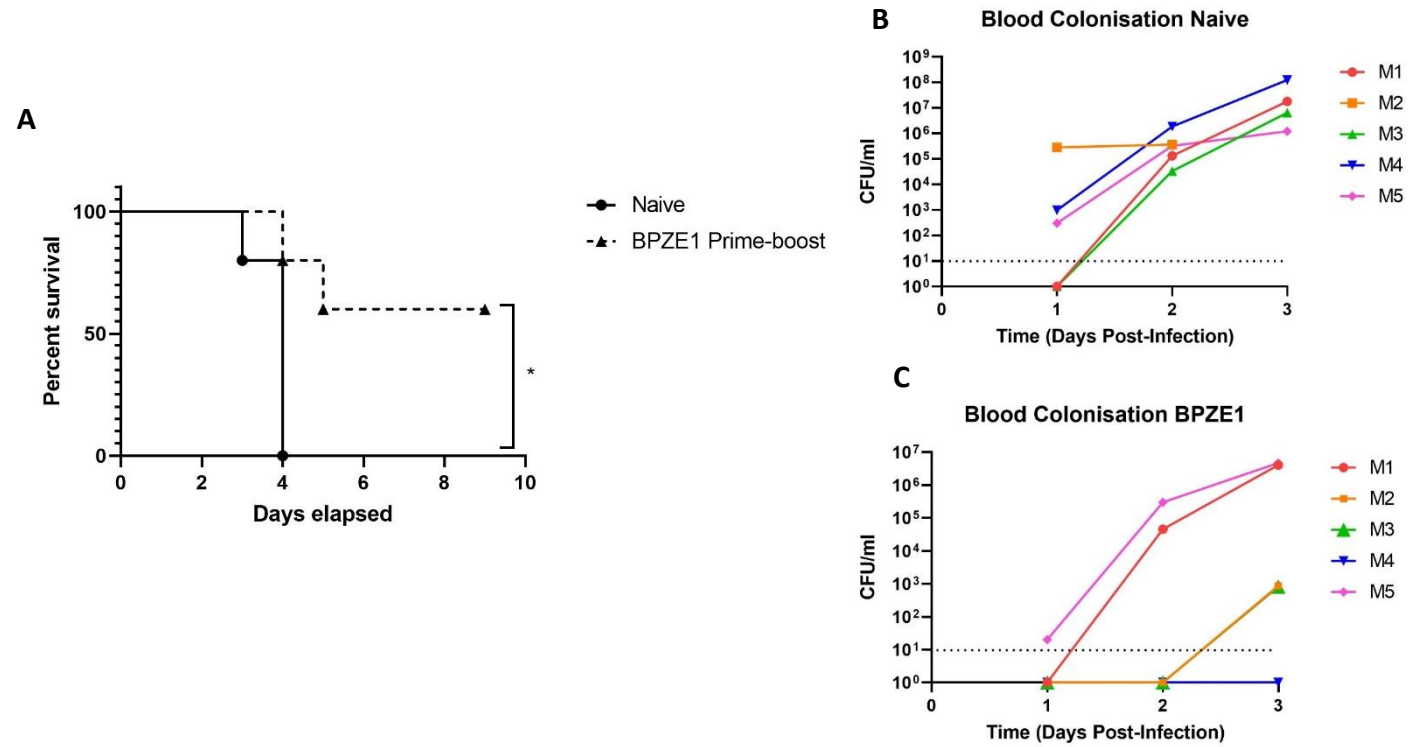
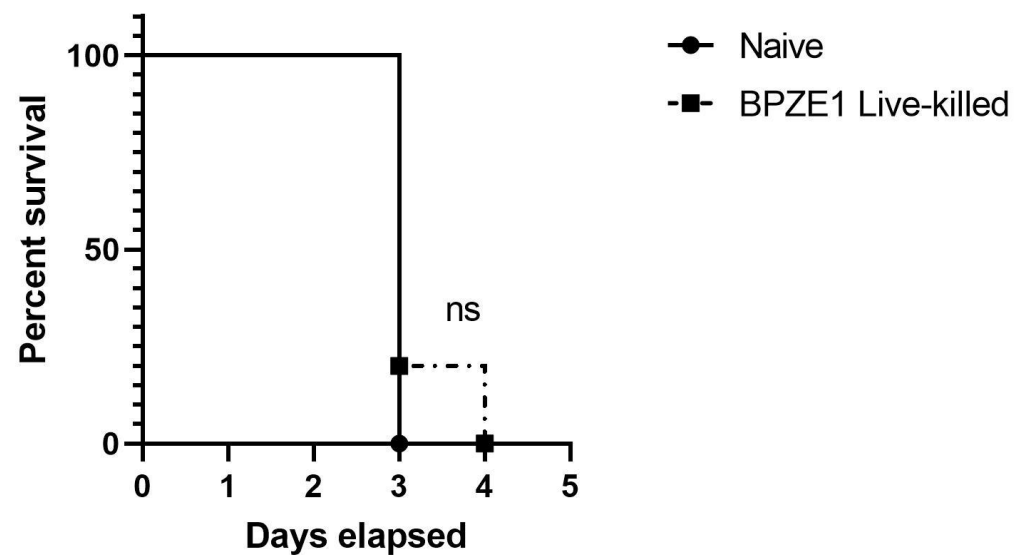


Figure 8



Conflict of Interest statement

ASD, NM and CL are co-inventors of patents on BPZE1, which were licensed to ILiAD Biotechnologies. No conflict of interest is declared by the other authors

Author Contributions

Conceived and designed the experiments: TB, HK, LC, SC and CL. Funding acquisition: CL, HK. Performed the experiments: TB, LC, HK, ASD and SC. Analyzed the data: TB, HK, NM, CL and SC. Contributed reagents/materials and helped with SP1 culturing: JCS. Wrote the paper: TB, LC and CL.