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

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

HAL Id: hal-03372774
https://hal.science/hal-03372774

Submitted on 12 Oct 2021

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 Debrie, 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

1. Introduction

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

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

Streptococcus pneumoniae is a Gram-positive bacterium and the causative agent of invasive pneumococcal disease (IPD), which continues to be a significant health burden worldwide despite the widespread use of vaccines [14]. Pneumococci are mostly carried asymptomatically in the upper respiratory tract and carriage rates vary, but colonization is more frequent in infants, declining to about 5-10% of healthy adults [15-17]. Disease 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% CO_2 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

4

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% CO_2 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 µl PBS. For challenge,

115   anaesthetized mice were infected intranasally with a lethal dose (2×10^6 CFU) of SP1 in

116   30 µl 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 30µl 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 50µl 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% CO_2. 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 naive 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

4. Discussion

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

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

14

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

16

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

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.

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

Acknowledgements

428 **References**

429

430 [1] Mielcarek N, Debrie 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, Debrie AS, Mielcarek N, Locht 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, Debrie 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] Locht 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, Nasciemento-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 IFNγ and IL-17 responses

517   to *Bordetella pertussis* between BALB/c and C57BL/6 mice: role of γδT 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, Locht 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]   Debrie AS, Mielcarek N, Lecher S, Roux X, Sirard JC, Locht 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

Figure Legends

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

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

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

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

27

589   dose of SP1. Survival was followed daily; n=5 (all groups). (**B**) BALB/c mice were

590   primed with life 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
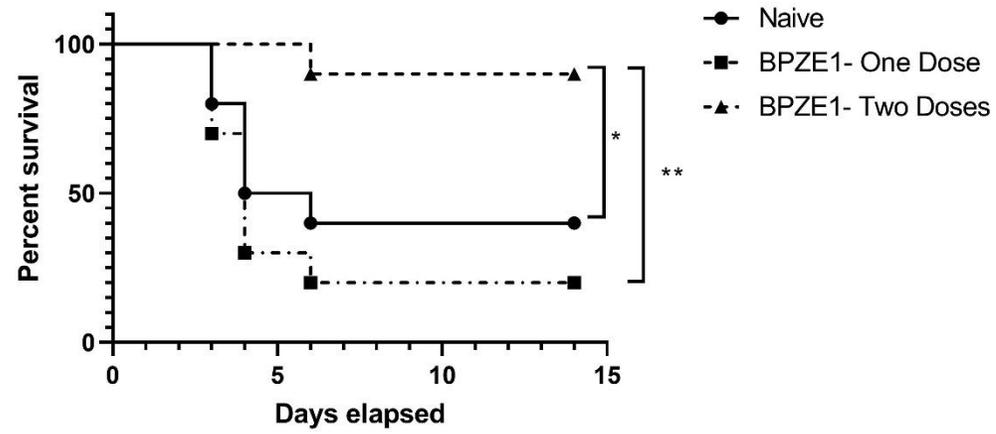
Figure 2
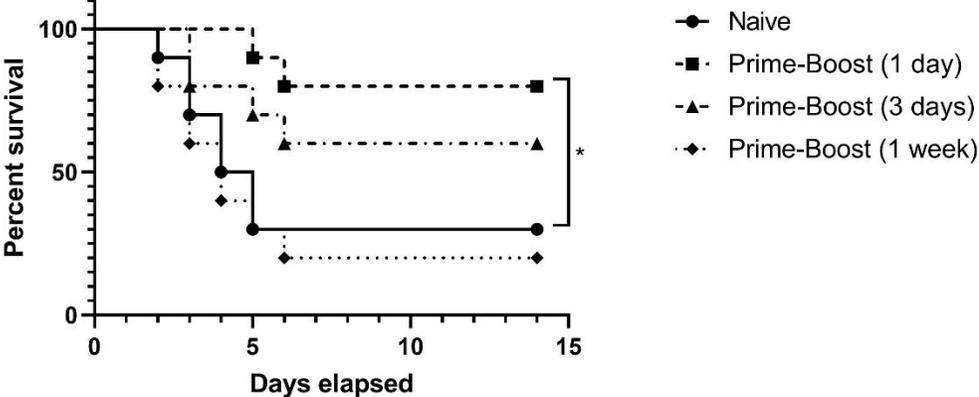
Figure 3
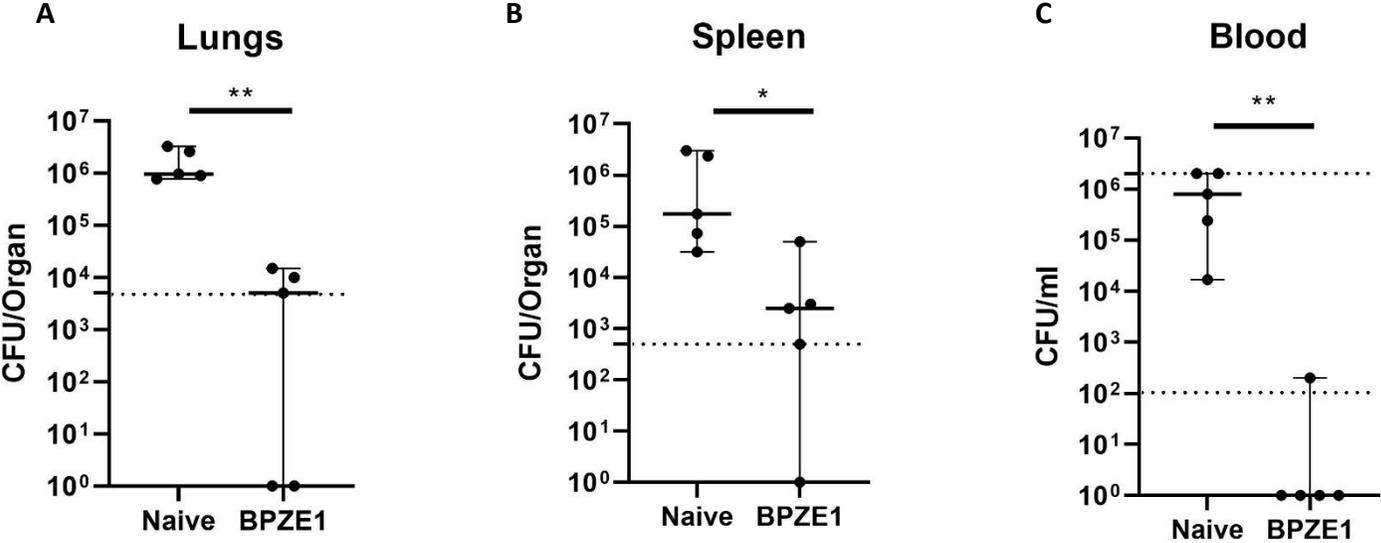
Figure 4

Figure 5

A

B
Blood Colonisation Naive

C
Blood Colonisation BPZE1
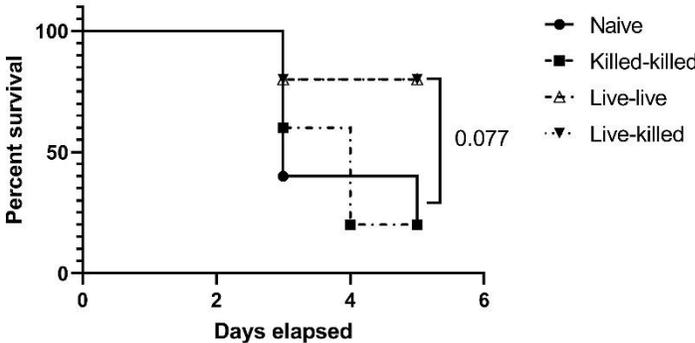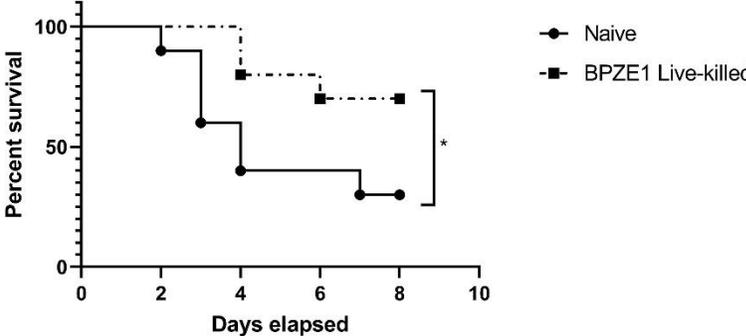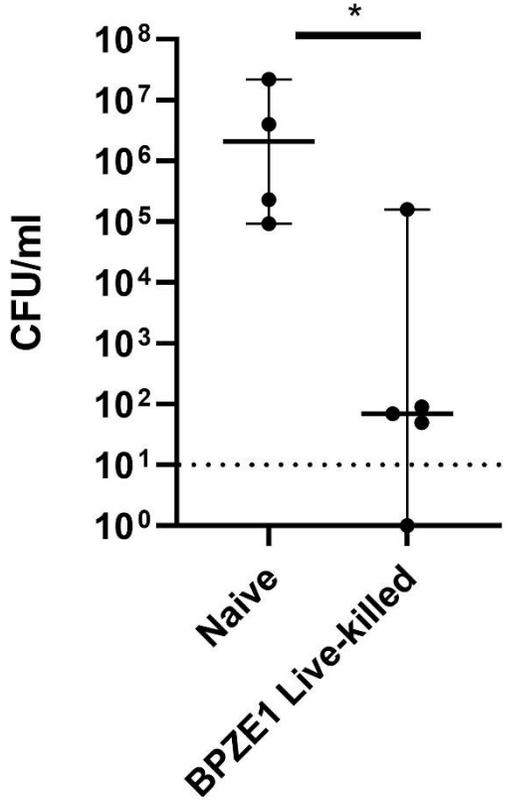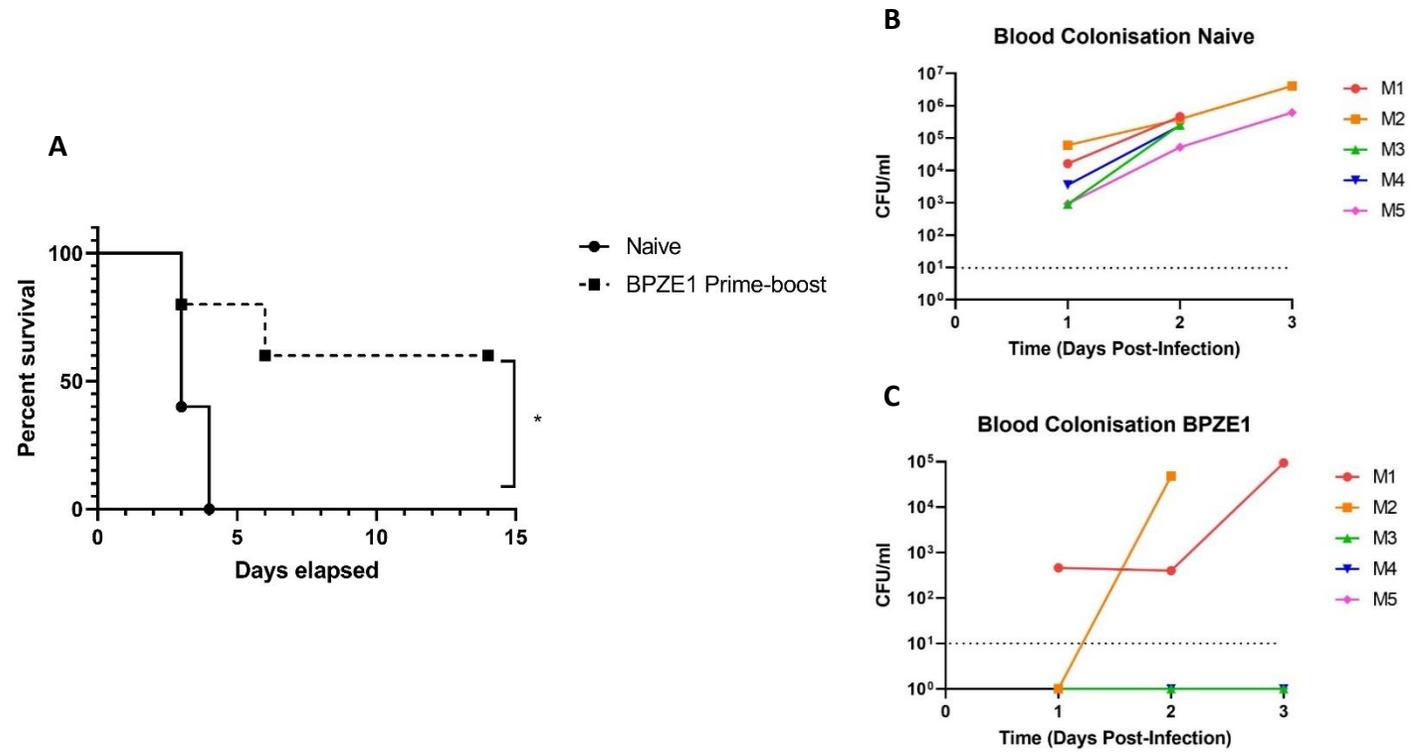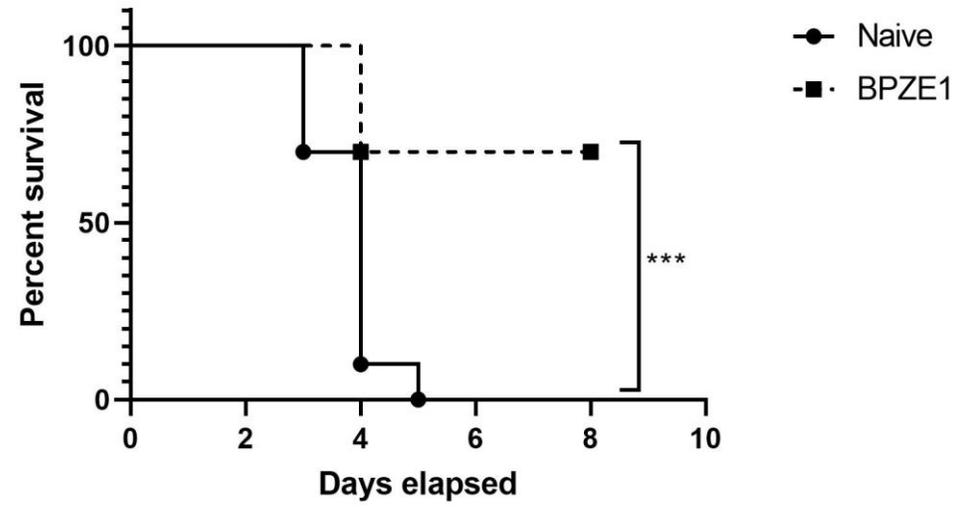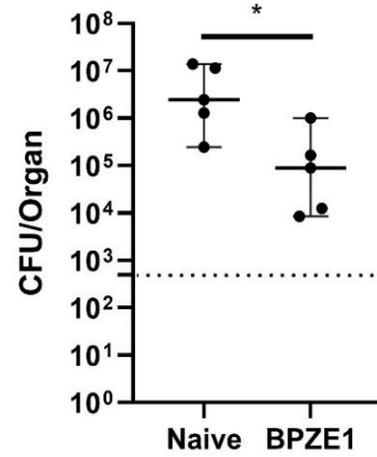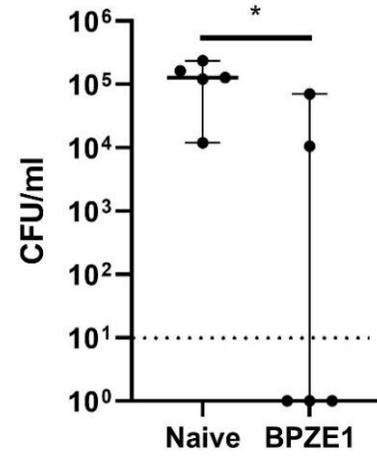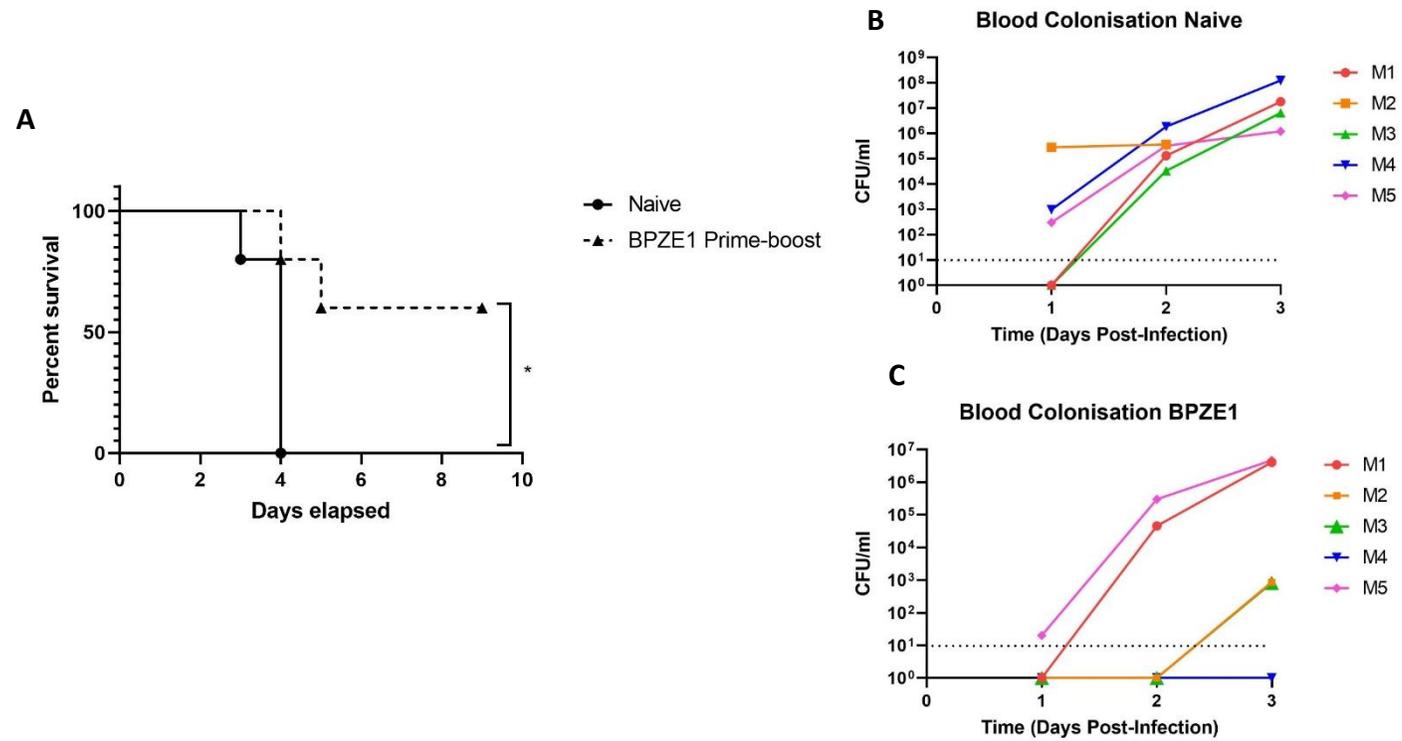
Figure 6

Figure 7

Figure 8
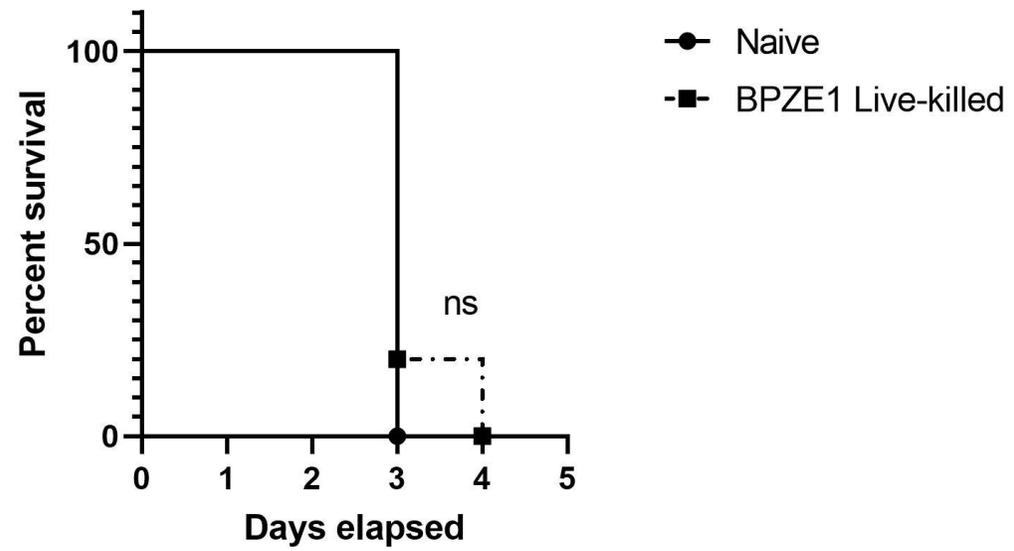
Conflict of Interest statement

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.