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

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Abstract

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

Key Words

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

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

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

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

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

2. Materials and Methods

2.1. Strains and Culture Conditions

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

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

2.2. *Preparation of formaldehyde-inactivated BPZE1*

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

2.3. *Mouse infections*

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

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

2.4. Determination of SP1 load in blood and organs

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

2.5. Statistical Analyses

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

2.6. Ethics Statement

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

3. Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.7. BPZE1-mediated protection against mortality depends on MyD88

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

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

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,

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

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

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

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

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

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

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

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

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

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

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

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

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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

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

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

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

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

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

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

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

Figure 1

Figure 2

Figure 3

Figure 4

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.