Vaccine 29 (2011) 5502–5511

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Development of live attenuated *Bordetella pertussis* strains expressing the universal influenza vaccine candidate M2e

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ARTICLE INFO

Article history:
Received 25 March 2011
Received in revised form 11 May 2011
Accepted 14 May 2011
Available online 30 May 2011

Keywords:
Bordetella pertussis
BPZE1
Influenza vaccine
M2e
Filamentous hemagglutinin

ABSTRACT

The attenuated *Bordetella pertussis* BPZE1 vaccine strain represents an attractive platform for the delivery of heterologous vaccine candidates via the nasal route. The filamentous hemagglutinin (FHA) has been used to secrete or expose the foreign antigens at the bacterial surface. In this study, one, two and three copies of the Cys-containing ectodomain of matrix protein 2 (M2e) from influenza A virus were genetically fused to full length FHA and expressed in BPZE1. The secretion efficacy of the FHA-$(M2e)_{1,2,3}$ chimera in the extracellular milieu and the ability of the recombinant bacteria to colonize the mouse lungs inversely correlated with the number of M2e copies fused to FHA. Nevertheless FHA-$(M2e)_3$-producing bacteria (BPLR3) triggered the highest systemic anti-M2e antibody response upon nasal administration to BALB/c mice. Nasal immunization with BPLR3 bacteria resulted in a significant reduction in the viral loads upon challenge with H1N1/PR8 influenza A virus, but did not improve the survival rate compared to BPZE1-immunized mice. Furthermore, since previous work reported that disulfide bond formation in Cys-containing passenger antigens affects the secretion efficacy of the FHA chimera, the *dsbA* gene encoding a periplasmic disulfide isomerase was deleted in the FHA-$(M2e)_3$-producing strain. Despite improving significantly the secretion efficacy of the FHA-$(M2e)_3$ chimera, the *dsbA* deletion did not result in higher anti-M2e antibody titers in mice, due to impaired bacterial fitness and colonization ability.

1. Introduction

Bordetella pertussis, a strict human pathogen, is the causative agent of whooping cough. Despite the availability and intensive use of efficacious vaccines for several decades, pertussis has not been eradicated and *B. pertussis* strains keep circulating in many countries with high vaccine coverage. In fact, reports of increasing incidence of pertussis infection worldwide have been accumulating for the past 20 years [1,2]. The re-emergence of pertussis cases has been attributed partially to the waning immunity conferred by current acellular pertussis vaccines and to other factors [3–7]. Since natural infection with *B. pertussis* induces strong and long-lasting immunity that wanes later than vaccine-induced immunity [8], live attenuated *B. pertussis* may represent an interesting approach for early (ideally at birth) and effective pertussis vaccination.

To this attempt, a highly attenuated *B. pertussis* strain, BPZE1, has been engineered [9] and has recently entered a phase I clinical trial in human as a live nasal pertussis vaccine (http://www.child-innovac.org). BPZE1 is deleted for the dermonecrotic (DNT)-encoding gene, produces an enzymatically inactive pertussis toxin (iPTX) and overexpresses the *Escherichia coli ampG* gene to reduce tracheal cytotoxin (TCT) release in the extracellular milieu [9]. Although highly attenuated as evidenced by a markedly reduced lung inflammation in the infected mice, BPZE1 bacteria still retain the ability to colonize the respiratory tract efficiently and induce effective protective immunity against pertussis infection [9]. Furthermore, high stability *in vitro* and *in vivo* [10], and good safety profile in immunocompromised animals [11] have been demonstrated for this vaccine strain.

In addition, the attenuated *B. pertussis* BPZE1 strain represents an appealing platform for the development of live recombinant vac-

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doi:10.1016/j.vaccine.2011.05.052

Table 1
B. pertussis strains used in this study.

Strains	Relevant features	Reference
BPZE1	BPSM-derivative lacking the *dnt* gene, and producing inactive PTX and reduced TCT	[9]
BPLR1	BPZE1-derivative expressing FHA-$(M2e)_1$ chimera	This study
BPLR2	BPZE1-derivative expressing FHA-$(M2e)_2$ chimera	This study
BPLR3	BPZE1-derivative expressing FHA-$(M2e)_3$ chimera	This study
BPST6	BPLR3-derivative deleted for *dsb*A	This study

cines that would confer simultaneous protection against pertussis and another infectious disease. The use of BPZE1 as live vaccine delivery vehicle was first reported by us where a B-cell epitope from Enterovirus 71 (EV71) was fused to the filamentous hemagglutinin (FHA); a strong EV71 neutralizing systemic antibody response was induced in mice upon nasal administration of the live recombinant bacteria [12].

Here, the objective of this work was to express an influenza antigen candidate in BPZE1. Targeting a respiratory disease such as influenza is particularly relevant since both *B. pertussis* bacteria and influenza virus primarily infect the respiratory tract. This approach makes even more sense when considering that BPZE1 confers cross-protection against influenza A viruses by dampening the virus-induced cytokine storm [13]. The construction of a recombinant BPZE1 strain expressing an influenza antigen vaccine candidate would thus combine the anti-inflammatory properties of BPZE1 with the ability to induce anti-influenza protective antibodies, thereby resulting in both non-specific and specific protection against influenza A virus.

In this work, we investigated the feasibility to express in BPZE1 the ectodomain of the matrix protein 2 (M2e) from influenza A virus. M2e consists of 24 non-glycosylated amino acids with 2 Cys residues, and has been remarkably conserved in all human influenza strains over the past 80 years [14,15]. M2e has been proposed as a universal influenza vaccine candidate inducing antibody-mediated protection against lethal influenza challenges in mice, ferrets and rhesus monkeys [16]. Recently, phase I clinical studies with M2e-vaccine conjugates have been completed, demonstrating their safety and immunogenicity [17,18].

The filamentous hemagglutinin (FHA) has been chosen as carrier to express M2e in BPZE1. Three heterologous antigens have been previously successfully fused to FHA, including the *Schistosoma mansoni* 28-kDa glutathione S-transferase (Sm28GST) [19], *Haemophilus influenzae* HtrA [20] and SP70 from *Enterovirus* 71 [12]. FHA is one of the major adhesins produced by *B. pertussis*. This 220-kDa protein is both secreted and exposed at the bacterial surface. FHA is highly immunogenic, inducing high levels of mucosal and systemic protective antibodies upon *B. pertussis* infection in mice [21] and humans [22]. This has led to the inclusion of FHA in most acellular pertussis vaccines. In addition, its different functional domains play important roles in adherence to the respiratory mucosa and stimulation of the host immune responses [23–27]. Thus, heterologous antigens genetically fused to FHA may also benefit from the various binding activities and stimulatory properties of the carrier protein. Targeting antigens to mononuclear phagocytes including macrophages would be expected to improve antigen presentation to T helper cells thereby facilitating the development of antibody responses. Together with its adjuvant properties [28,29], FHA is thus an attractive candidate carrier to secrete foreign antigens into the extracellular milieu or to present them at the bacterial surface of *B. pertussis*-based live recombinant vaccines.

Here, one, two and three copies of M2e were genetically fused to full length FHA and expressed in BPZE1. The secretion efficacy of the FHA-$(M2e)_{1,2,3}$ chimera in the extracellular milieu was studied and the ability of the recombinant strains to colonize the mouse lungs was determined. Immunogenicity and protection studies were subsequently carried out. Furthermore, we investigated the effect of the deletion of the *dsbA* gene, encoding a periplasmic disulfide isomerase, on the secretion efficacy of the FHA-$(M2e)_3$ chimera and on the immunogenicity of the recombinant strain.

2. Materials and methods

2.1. Bacterial and viral strains, and growth conditions

The bacterial strains used in this study are listed in Table 1. BPLR1, BPLR2, BPLR3 and BPST6 were derived from *B. pertussis* BPZE1, a streptomycin-resistant TohamaI derivative producing inactivated pertussis toxin (PTX), no dermonecrotic toxin (DNT) and background levels of tracheal cytotoxin (TCT) [9]. All *B. pertussis* strains were grown at 37 °C for 72 h on Bordet–Gengou (BG) agar (Difco, Detroit, MI) supplemented with 1% glycerol, 10% defibrinated sheep blood and 100 μg/ml streptomycin (Sigma Chemical CO., St. Louis, Mo.). Liquid cultures were performed as described previously [30] in Stainer–Scholte (SS) medium containing 1 g/l heptakis(2,6-di-o-methyl) β-cyclodextrin (Sigma).

For *in vitro* growth kinetic of B. pertussis strains, exponential liquid pre-cultures in SS medium were used to inoculate a fresh culture medium at an initial $OD_{600\,nm}$ of 0.5. Absorbance at 600 nm of the cultures was monitored over time at the indicated time points.

To prepare a BPZE1 total cell lysate for ELISA, 10 ml of exponentially grown BPZE1 liquid culture were centrifuged at 7000 rpm for 15 min at room temperature. The pellet was washed three times with 5 ml sterile PBS and resuspended in 1 ml PBS. The suspension was sonicated on ice 15 times for 15 s each, with intervals of 15 s (Model 150 V/T Ultrasonic Homogenizer, BioLogics Inc., Manassas, VA). Total protein concentration of the lysate was measured using the NanoDrop® ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

All DNA manipulations were carried out in chemically competent One-Shot® TOP10 *E. coli* (Invitrogen). The bacteria were grown at 37 °C overnight on Luria–Bertani (LB) agar or in LB broth with vigorous shaking. When appropriate, 100 μg/ml ampicillin, 50 μg/ml ampicillin or 10 μg/ml gentamicin were added to select for antibiotic-resistant strains.

H1N1 A/PR/8/34 virus was purchased from the ATCC (#VR-95) and amplified in egg following ATCC's recommendations.

2.2. Oligonucleotides, peptides and antibodies

Selected M2e peptide covers nucleotides 689–750 of the M2 protein from influenza A virus H5N1 strain (A/chicken/Nakorn-Patom/Thailand/CU-K2/2004). To circumvent any potential problems in protein translation due to poor codon usage, the original *m2e* DNA sequence was optimized to *B. pertussis* codon usage preference. For generating the FHA-$(M2e)_{1,2,3}$ construct, the upper and lower DNA strands of optimized *m2e* 5′-GATCAGCCTGCTGACCGAGGTGGAGACCCCGACCCGCAACGAGTGG-GAGTGCCGCTGCAGCGACTCGAGCGACGA-3′ and 5′-GATCTCGTC-GCTCGAGTCGCTGCAGCGGCACTCCCACTCGTTGCGGGTCGGGGTCT-CCACCTCGGTCAGCAGGCT-3′ were chemically synthesized and annealed, generating cohesive *Bgl*II-compatible ends.

Unconjugated M2e peptide and KLH-conjugated M2e peptide (KLH-M2e) were chemically synthesized at Mimotopes Pty Ltd. (Clayton, Victoria, Australia).

An anti-M2e polyclonal immune serum was raised in 10 mice upon two intraperitoneal administrations at a 2-week interval of 50 μg KLH-M2e peptide mixed with complete and incomplete Freund adjuvant, respectively. Blood was obtained by cardiac puncture on euthanized animals two weeks after the last immunization. Sera were pooled and the anti-M2e antibody titer was determined by ELISA.

2.3. Construction of recombinant B. pertussis strains

To construct the recombinant BPLR1, BPLR2 and BPLR3 *B. pertussis* strains expressing FHA-$(M2e)_1$, FHA-$(M2e)_2$ and FHA-$(M2e)_3$ chimera, respectively, a 1620-bp *Hind*III PCR fragment was amplified from the BPZE1 chromosomal DNA using the primers 5′-TTAAGCTTGCGAACGCGCTGCTGTGGG-3′ and 5′-TTAAGCTTCGCATCGGCGCTGCCCAGC-3′ (*Hind*III sites are underlined) and cloned into *Hind*III-opened pBR322 plasmid [31], yielding pBRSY0. The PCR fragment corresponds to nt5221 to nt6840 of the *fhaB* open reading frame (ORF) and contains its unique *Bgl*II site. The insertion of one copy of *m2e* DNA into *Bgl*II-digested pBRSY0 restored a *Bgl*II site only at the 3′end of the *m2e* DNA fragment, allowing the insertion of a second then a third *m2e* copy, yielding pBRLR1, pBRLR2 and pBRLR3, respectively. The *Hind*III fragments from pBRLR1, pBRLR2 and pBRLR3 were then cloned into *Hind*III-opened pJQmp200rpsL18 suicide plasmid [32] giving pJQLR1, pJQLR2 and pJQLR3, respectively. BPZE1 bacteria were electroporated with pJQLR1, pJQLR2 and pJQLR3, allowing the *fha-(m2e)*$_1$, *fha-(m2e)*$_2$, *fha-(m2e)*$_3$ constructs to integrate into the chromosomal DNA by allelic exchange [33] at the *fhaB* locus.

To construct the *dsbA*-knockout BPST6 strain, a 732-bp PCR1 fragment including the 703-bp sequence upstream the first nucleotide of the *dsbA* ORF and the first 29-bp of *dsbA*, and a 731-bp PCR2 fragment including the 702-bp sequence downstream the STOP codon of *dsbA* ORF and the last 29-bp of *dsbA*, were amplified by PCR. Both PCR1 and PCR2 fragments were cloned into pJQmp200rpsL18 to obtain the pJQ-PCR2-PCR1 plasmid construct using *Eco*RI and *Hind*III restriction sites. BPLR3 bacteria were electroporated with pJQ-PCR2-PCR1 for integration via double homologous recombination at the *dsbA* locus to obtain the recombinant *B. pertussis* BPST6.

2.4. Whole cell extracts and culture supernatant concentration

Mid- to late exponentially grown bacteria in SSAB medium (10 ml) were centrifuged at 7000 rpm for 15 min at room temperature. The supernatant was concentrated 10 times using the 30 kDa cut-off Ultra-4 Centrifugal Filter Device (Amicon) following the manufacturer's protocol. The bacterial pellet was re-suspended in 500 μl of ultrapure water. An equal volume of 2× loading buffer [0.125 M Tris–HCl (pH 6.8), 10% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 1 drop of Bromophenol blue] was added before heating at 95 °C for 10 min. Chromosomal DNA was sheared by passing the suspension 10 times through a 27G needle followed by heating at 95 °C for 15 min prior to loading 30 μl onto sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel for western blot analysis.

2.5. Immunodetection of the FHA-$(M2e)_{1,2,3}$ chimera

Concentrated (10×) culture supernatants and whole cell extracts of the *B. pertussis* strains were analyzed by SDS-PAGE using 8% or 12% polyacrylamide gels. The proteins were electrotransferred onto nitrocellulose membranes and incubated with mouse anti-M2e polyclonal antibodies (this study) diluted at 1:1000, anti-FHA monoclonal antibodies diluted at 1:250 [34], or anti-PTX monoclonal antibodies (National Institute for Biological Standards and Control, UK) diluted at 1:5000 in Tris-buffered saline containing 0.1% Tween 20 and 2% bovine serum albumin (BSA). Alkaline phosphatase (AP)-conjugated goat anti-mouse IgG secondary antibodies (Sigma) diluted at 1:3000 were used for chromogenic detection of the proteins upon adding the AP substrate (NBT and BCIP reagents, Sigma).

2.6. Mouse experiments

The mice were kept under specific-pathogen free conditions in Individual Ventilated Cages, and all the experiments were carried out under the guidelines and upon approval of the National University of Singapore animal study board. All the non-terminal procedures described below were performed under anaesthesia to minimize pain and distress incurred to the animals.

For colonization studies, 6–8-week-old BALB/c mice (Biopolis Research Center (BRC), Singapore) were intranasally (i.n.) infected with 5×10^6 colony-forming units (CFU) in 20 μl of the different *B. pertussis* strains as described previously [35]. At the indicated time points, four mice per group were sacrificed, and their lungs were aseptically removed and homogenized in PBS. Serial dilutions from individual lung homogenates were plated onto BG agar and the total CFU per lung was determined after 4–5 days incubation at 37 °C.

For immunization studies, groups ($n = 6$) of 6–8-week-old BALB/c mice (BRC, Singapore) were i.n. infected with 5×10^6 CFU in 20 μl of the different *B. pertussis* strains, and then boosted twice at 3-week intervals with equal amount of bacteria. An additional group ($n = 6$) was intraperitoneally (i.p.) injected twice at a 4-week interval with 50 μg of KLH-M2e in complete and incomplete Freund's adjuvant, respectively.

Broncho-alveolar lavage fluids (BALFs) were recovered two weeks after the last boost from euthanized mice by flushing twice 1 ml sterile PBS into the lungs. BALFs were centrifuged at 2000 rpm for 10 min at 4 °C and the supernatants were kept at −80 °C until further analysis. Blood was also collected from the euthanized mice by cardiac puncture and was allowed to clot overnight at 4 °C. The sera were obtained after centrifugation at 4000 rpm for 10 min and stored at −80 °C until further use.

For influenza infection, 6–8-week-old BALB/c mice were i.n. administered with 500 PFU of H1N1 A/PR/8/34 in sterile PBS supplemented with penicillin and streptomycin. Ten mice per group were used to determine the survival rates based on body weight loss; the mice were euthanized when body weight loss exceeded 20% of the original body weight. For viral load determination, 5 mice per group were used.

2.7. Antibody detection

The anti-*B. pertussis* and anti-M2e antibody responses were measured by enzyme-linked immunosorbent assay (ELISA) using BPZE1 total cell lysate and unconjugated M2e peptide (Mimotopes Pte Ltd., Clayton, Victoria, Australia) as coating antigens, respectively.

Flat 96-well microtiter plates (Corning NUNC) were coated overnight at 4 °C with 100 μl of coating buffer (0.1 M Na_2CO_3–$NaHCO_3$, pH 9.6) containing 5 μg/ml of BPZE1 total cell lysate and 3 μg/ml of unconjugated M2e peptide. The wells were washed three times with wash buffer (0.1% Tween 20 in PBS), before blocking with blocking buffer (0.1% Tween 20, 2% BSA in PBS) for 1 h at 37 °C. The wells were then washed three times with wash buffer and 100 μl of diluted mouse serum in blocking buffer or neat BALFs were added to each well. After incubation at 37 °C for 2 h, the plates were washed thrice before adding 100 μl

of 1:3000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L) (Bio-rad) or 1:2000 diluted HRP-conjugated goat anti-mouse IgA (Chemicon) secondary antibodies. The plates were again incubated at 37 °C for 1 h followed by three washes. The ELISA was developed by adding 100 μl of *o*-phenylenediamine dihydrochloride (OPD) substrate (Sigma) to each well and incubating at room temperature for 20 min in the dark. To stop the reaction, 50 μl of 1 M sulfuric acid was added to each well and absorbance at 490 nm was measured using the Biorad model 680 Microplate Reader and recorded with the Microplate Manager 5.2 software (Biorad).

2.8. Determination of the viral titers

Mouse lungs were harvested and homogenized using mechanical disruption (Omni homogenizer), and tested for the presence of viable virus by tissue culture infectious dose 50 ($TCID_{50}$) assay using a modified method reported by the WHO and as described previously [13]. Briefly, 90% confluent Madin–Darby canine kidney (MDCK) cells in 96-well plates were inoculated with 100 μl of 10-fold serially diluted lung homogenates. Plates were incubated at 35 °C in a humidified incubator (5% CO_2) for 3 days. $TCID_{50}$ was determined by a reduction in cytopathic effect (CPE) of 50%, and the log $TCID_{50}$/lung was derived. Five mice per group per time point were individually assessed.

2.9. Statistical analysis

Bars represent the means ± standard deviations (SD), and averages were compared using a bidirectional unpaired Student's *t* test with a 5% significance level with $^{*}P \leq 0.05$, $^{**}P \leq 0.01$ and $^{***}P \leq 0.001$.

3. Results

3.1. Construction of the recombinant B. pertussis strains producing the FHA-$(M2e)_{1,2,3}$ chimera

One, two or three copies of the 24-amino acid M2e peptide were fused in frame to full-length FHA. Each DNA construct was introduced by allelic exchange into the chromosomal *fhaB* locus of attenuated *B. pertussis* BPZE1, giving rise to recombinant strains BPLR1, BPLR2 and BPLR3, respectively. The production and secretion of the FHA-M2e, FHA-$(M2e)_2$ and FHA-$(M2e)_3$ chimera in the whole bacterial lysates and culture supernatants, respectively, was analyzed for each recombinant strain by Western blot analysis using monoclonal anti-FHA antibodies or polyclonal anti-M2e immune serum. Western blot using anti-PTX antibodies was also performed in order to confirm equal loading of each bacterial lysate, since the recombinant strains are expected to produce comparable amounts of PTX to the parental BPZE1 strain. The results showed that the FHA-$(M2e)_{1,2\text{ or }3}$ chimera are secreted into the external milieu but not as efficiently as wild type FHA (Fig. 1A and B). Furthermore, the secretion efficacy of the chimera inversely correlated with the number of M2e copies fused to the carrier FHA. In contrast, comparable signal intensities were obtained for the chimera and wild type FHA detected from the whole bacterial lysates (Fig. 1C and D), thus indicating that the intracellular production of the chimera is as effective as the production of wild type FHA and only their secretion into the external environment is impaired.

3.2. Lung colonization efficacy and in vitro growth kinetic of the recombinant B. pertussis strains

To investigate the ability of the recombinant *B. pertussis* strains to colonize the mouse respiratory tract, adult BALB/c mice were intranasally (i.n.) infected with each of them. The lung colonization profiles of BPLR1, BPLR2 and BPLR3 were compared to that of the parental BPZE1 strain. As described before [9], BPZE1 displayed a slight peak of multiplication at day 7 followed by a progressive clearance of the bacteria from the lungs (Fig. 2A). BPLR1 which produces the FHA-M2e chimera colonized the mouse respiratory tract almost as efficiently as the parental BPZE1 strain. In contrast, BPLR2 and BPLR3 which produce the FHA-$(M2e)_2$ and FHA-$(M2e)_3$ chimera respectively, were significantly impaired in their ability to colonize the mouse lungs; no peak of multiplication was observed and instead, the bacteria were rapidly cleared from the lungs. By day 17 post-infection, no BPLR2 and BPLR3 bacteria were detected whereas about 3 logs of bacteria were still present in the lungs of mice infected with either BPZE1 or BPLR1 strain (Fig. 2A).

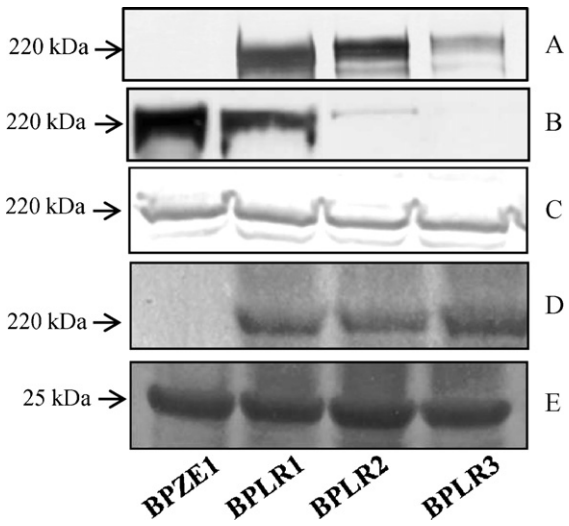

Fig. 1. Production and secretion of the FHA-$(M2e)_{1,2,3}$ chimera by recombinant *B. pertussis*. Western blot analysis was performed on 10× concentrated culture supernatants (A and B) or whole bacterial lysates (C–E) from parental BPZE1, or recombinant BPLR1, BPLR2 and BPLR3 strains (as indicated) using anti-M2e polyclonal immune serum (A and D), anti-FHA (B and C) or anti-PTX (E) monoclonal antibodies.

These results thus suggested that the lung colonization ability of the recombinant *B. pertussis* strains correlates with the secretion efficacy of the FHA-(M2e) chimera into the extracellular environment. Alternatively, the lower colonization efficacy displayed by BPLR2 and BPLR3 strains may result from an impaired general bacterial fitness. To test this hypothesis, the *in vitro* kinetic profiles were determined. The results indicated that BPLR1 bacteria grew as well as the parental BPZE1 bacteria, whereas BPLR2 and to a greater extent BPLR3 displayed some impairment in their growth kinetic with $OD_{600\,nm}$ readings substantially lower than those obtained for the parental BPZE1 strain over the course of the experiment (Fig. 2B).

Together, this set of data indicated that the lower secretion efficacy of the FHA-$(M2e)_{2,3}$ chimera correlates with impaired bacterial fitness and lung colonization ability.

3.3. Specific antibody responses elicited by the recombinant B. pertussis strains

To study the antibody responses, adult BALB/c mice were i.n. administered with live recombinant BPLR1, BPLR2 and BPLR3, or parental BPZE1 bacteria. Three administrations were performed at 3-week intervals. The sera and broncho-alveolar lavages fluids (BALFs) were collected 2 weeks after the last boost, and the anti-*B. pertussis* and anti-M2e antibody responses were analyzed.

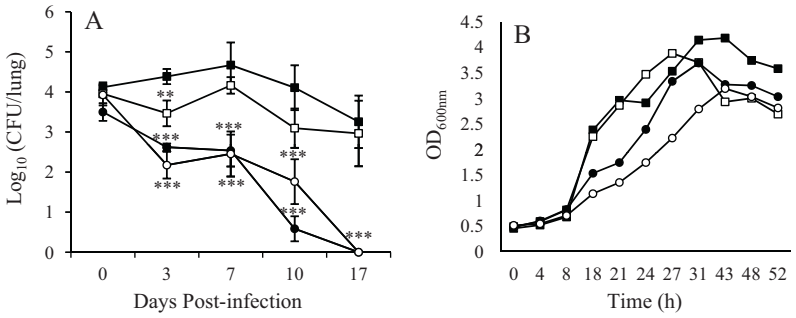

Fig. 2. Lung colonization and *in vitro* growth kinetic profiles of FHA-$(M2e)_{1,2,3}$-producing *B. pertussis*. Lung colonization. BALB/c mice were infected intranasally (i.n.) with 5×10^6 CFU of parental BPZE1 (solid square) or recombinant BPLR1 (open square), BPLR2 (solid circle) and BPLR3 (open circle) strains. The lungs were harvested at the indicated time points and homogenized. Appropriate dilutions of the lung homogenates were plated onto blood agar plates and the number of CFU was counted after 4 days incubation at 37 °C. Four mice per group per time point were assessed individually. Results are expressed the average ± standard deviations (SD) of the Log_{10} CFU/lung calculated for each individual mouse. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$, compared with the values obtained with BPZE1 at the same time points. (B) *In vitro* growth kinetic. Exponential liquid pre-cultures of the parental BPZE1 or recombinant BPLR1, BPLR2 and BPLR3 strains were used to inoculate a fresh culture medium at an initial $OD_{600\,nm}$ of 0.5. The absorbance at 600 nm of the cultures was monitored over time at the indicated time points. Same legend as in (A). The data are representative of three independent experiments.

All the *B. pertussis* strains triggered very strong and comparable systemic (IgG) and local (IgG and IgA) antibody responses against *B. pertussis* (Fig. 3A–C). This observation indicated that despite weaker colonization ability, BPLR2 and BPLR3 bacteria efficiently primed the host's immune system. In addition, these two strains were found better at inducing local anti-M2e antibody responses (IgA and IgG) compared to BPLR1 (Fig. 3E and F). Remarkably, BPLR3 which produces the FHA-$(M2e)_3$ chimera triggered the highest systemic anti-M2e IgG response (Fig. 3D). Surprisingly, the sera and BALFs obtained from BPZE1-immunized mice consistently gave significant absorbance readings in ELISA when using purified M2e peptide as coating antigen. This may suggest the presence of cross-reacting epitopes shared between M2e from influenza A viruses and *B. pertussis*. Alternatively, aspecific background noise may also account for this observation.

Taken together, our data indicated that BPLR3 recombinant strain which produces the FHA-$(M2e)_3$ chimera induced anti-pertussis antibody responses as effectively as the parental BPZE1 strain, and triggered the strongest systemic anti-M2e antibody response upon nasal vaccination. This finding suggested that the number of M2e copies fused to FHA is a key parameter to induce a strong specific immune response, even though the secretion efficacy of the chimera and the colonization ability of the recombinant strain may be sub-optimal.

3.4. Protective efficacy of the FHA-$(M2e)_3$ producing strain against influenza virus

Since BPLR3 strain triggered the strongest systemic anti-M2e antibody response in mice, its ability to protect against an influenza challenge was assayed. BPLR3-immunized BALB/c mice were i.n. challenged with H1N1/PR8 influenza A virus and the survival rate was determined based on the body weight loss. An additional control group immunized with KLH-M2e was also challenged. 100% of the non-treated challenged mice succumbed to H1N1 infection within 7–8 days (Fig. 4A and B). As reported before [13], BPZE1 nasal pre-treatment conferred 50% protection against H1N1/PR8 virus (Fig. 4A and B); this protective effect was shown to be due to the dampening of the virus-induced cytokine storm and did not result in reduction in the viral load [13] (Fig. 4C). No significant

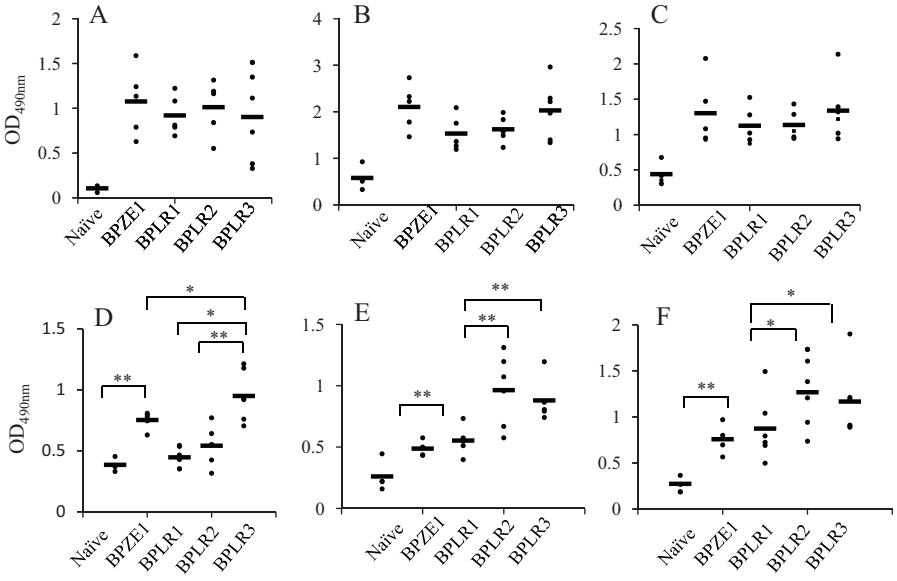

Fig. 3. Local and systemic specific antibody responses in mice nasally immunized with FHA-$(M2e)_{1,2,3}$-producing *B. pertussis* strains. Groups of 6 adult BALB/c mice were i.n. administered thrice at 3-week intervals with 5×10^6 CFU of BPZE1, BPLR1, BPLR2, or BPLR3. Two weeks after the last administration, sera were collected and broncho-alveolar lavages (BAL) were performed. Systemic IgG (A and D), local IgG (B and E) and local IgA (C and F) anti-*B. pertussis* (A–C) or anti-M2e (D–F) antibody responses were measured by ELISA on individual sera diluted 1/1,000 (A–C) or 1/40 (D–F) and neat BALFs, using BPZE1 whole cell lysate (A–C) or synthesized M2e peptide (D–F) as coating antigens. The results are representative of at least two independent experiments. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

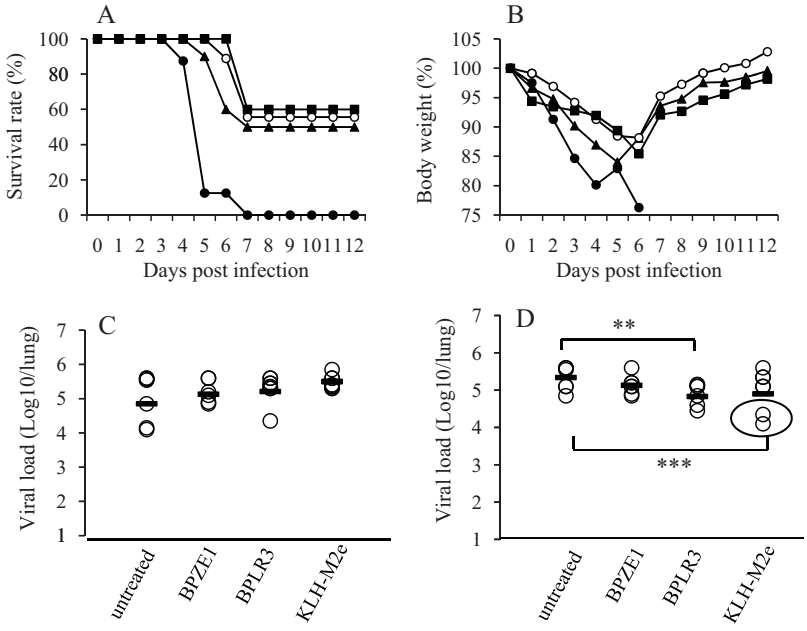

Fig. 4. Influenza challenge in BPLR3-immunized mice. Groups of adult BALB/c mice were nasally administered thrice at 3-week intervals with 5×10^6 CFU of BPZE1 (solid triangle) or BPLR3 (open circle), or left untreated (solid circle). A fourth group was immunized twice at a 2-week interval with 50 μg of KLH-M2e (solid square). Two weeks after the last administration, the mice were challenged with 500 PFU of H1N1/A/PR8 virus via the nasal route. (A and B) The animals were daily monitored for body weight loss. Mice were euthanized when the body weight was below 80% of the original body weight. Ten animals per group were used. (C and D) The viral load was determined in the lungs from the infected animals at day 3 (panel C) and 5 (panel D) post-influenza challenge. The results are expressed for each individual mouse in $TCID_{50}$. Five animals per group per time point were analyzed.

difference in survival rate between BPLR3- and BPZE1-immunized animals was observed (Fig. 4A). However, the body weight loss profile indicated that the BPLR3-immunized mice did not lose weight as much as the BPZE1-immunized group (compare values at day 5 post-challenge in Fig. 4B), suggesting that imunization with recombinant BPLR3 bacteria may confer some additional protection against H1N1/PR8 virus compared to the vehicle BPZE1 alone. This was further supported by the significant reduction in the viral load at day 5 post-challenge in the BPLR3-immunized mice compared to the non-treated group (Fig. 4D). Surprisingly, despite the induction of high systemic anti-M2e IgG antibody response (OD_{490nm} 3.8 of pooled serum diluted at 1/5000), KLH-M2e immunization resulted in only 50% survival rate upon challenge with H1N1/PR8 virus (Fig. 4A and B). This partial protection translated into no significant difference in the average viral load compared to the untreated infected control group (Fig. 4C and D). However, when analyzed individually, two KLH-M2e immunized mice displayed significantly lower viral loads at day 5 post-influenza challenge (Fig. 4D).

Overall, the results indicated that immunization with BPLR3 bacteria limited body weight loss and led to a significant reduction in the viral loads but this did not translated into a greater survival rate when compared to BPZE1.

3.5. Expression of the FHA-$(M2e)_3$ chimera in a dsbA knockout background

We hypothesized that the weak level of antibody-mediated protection against influenza in BPLR3-immunized mice may partly result from too low anti-M2e antibody titers. We thus postulated that a greater secretion efficacy of the FHA-$(M2e)_3$ chimera may lead to a higher anti-M2e antibody response. The presence of Cys residues in the passenger domain fused to FHA has been previously shown to impair the secretion of the FHA-chimera [36]. The *dsbA* gene encoding a periplasmic disulfide isomerase [37], was thus deleted in the BPLR3 strain, resulting in the recombinant *B. pertussis* strain BPST6. Western blot analysis using anti-FHA or anti-M2e antibodies showed that the secretion efficacy of the FHA-$(M2e)_3$ chimera was significantly greater in BPST6 compared to BPLR3 but still significantly lower than the secretion of wild type FHA (Fig. 5A and B). In addition, comparable amount of chimera and wild type

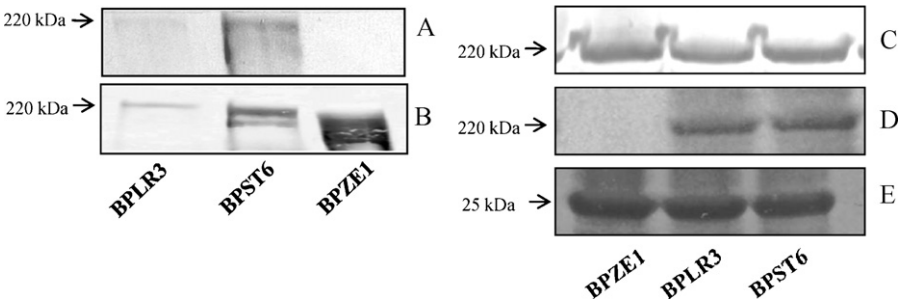

Fig. 5. Effect of dsbA deletion on the production and secretion of the FHA-$(M2e)_3$ chimera. Western blot analysis using anti-M2e (A and D), anti-FHA (B and C) or anti-PTX (E) antibodies was performed on 10× concentrated culture supernatants (A and B) or on whole bacterial lysates (C–E) from BPLR3, BPST6 and parental BPZE1 strains as indicated.

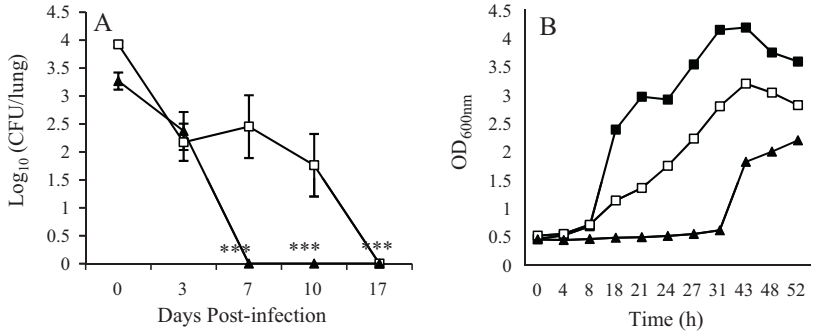

Fig. 6. Lung colonization and *in vitro* growth profile of BPST6 strain. Lung colonization. BALB/c mice were i.n. inoculated once with 5×10^6 CFU of recombinant BPLR3 (open square) or BPST6 (solid triangle) bacteria. The lungs were processed as described in the legend of Fig. 2. Four mice per group per time point were assessed individually. Results are expressed the average ± SD of the Log_{10} CFU/lung calculated for each individual mouse. $^{***}p \leq 0.001$, compared with the values obtained with BPZE1 at the same time point. (B) *In vitro* growth kinetic. Exponential liquid pre-cultures of BPZE1 (solid square), BPLR3 (open square) and BPST6 (solid triangle) strains were used to inoculate a fresh culture medium at an initial $OD_{600\,nm}$ of 0.5. The absorbance at 600 nm of the cultures was monitored over time at the indicated time points. The data are representative of two independent experiments.

FHA were detected from the bacterial lysates (Fig. 5C and D). These results thus indicated that *dsbA* deletion improved the secretion efficacy of the FHA-$(Me)_3$ chimera and supported previous observations that disulfide bond formation between Cys residues in the foreign antigen impairs the secretion efficacy of the FHA chimera [36].

3.6. Colonization efficacy, bacterial fitness and immunogenicity of the ΔdsbA BPST6 mutant

The ability of BPST6 bacteria to colonize the mouse lungs was then investigated and compared to parental BPLR3, the *dsbA*$^+$ counterpart. By day 7 post-infection, BPST6 bacteria were totally cleared from the lungs whereas 2.5 Log_{10} of BPLR3 bacteria were still detected (Fig. 6A). We tested whether the drastic impairment in colonizing the mouse lungs was related to the overall fitness of BPST6 strain. The *in vitro* kinetic profile indeed revealed that BPST6 bacteria are severely impaired in their growth ability (Fig. 6B).

The immunogenicity of BPST6 strain was further evaluated in mice upon nasal administration and compared to the immunogenicity induced upon administration of BPLR3 and BPZE1 strains. As described in the previous section, 3 nasal administrations were performed at 3 week-intervals. The sera and BALFs were collected 2 weeks after the last boost and the anti-pertussis and anti-M2e antibody responses were analyzed by ELISA. Although high, the anti-pertussis antibody responses obtained in the BPST6-immunized mice were significantly lower than the responses measured in the BPLR3- and BPZE1-immunized animals (Fig. 7A–C). Moreover, both systemic and local anti-M2e IgG and IgA antibody responses in the BPST6-immunized group were significantly lower than those measured in the BPLR3-immunized mice (Fig. 7D–F).

Altogether, these data indicate that *dsbA* deletion in the BPLR3 strain resulted in increased secretion efficacy of the FHA-$(M2e)_3$ chimera but impaired the overall fitness of the bacteria which correlated with poor colonization ability and lower immunogenicity in mice.

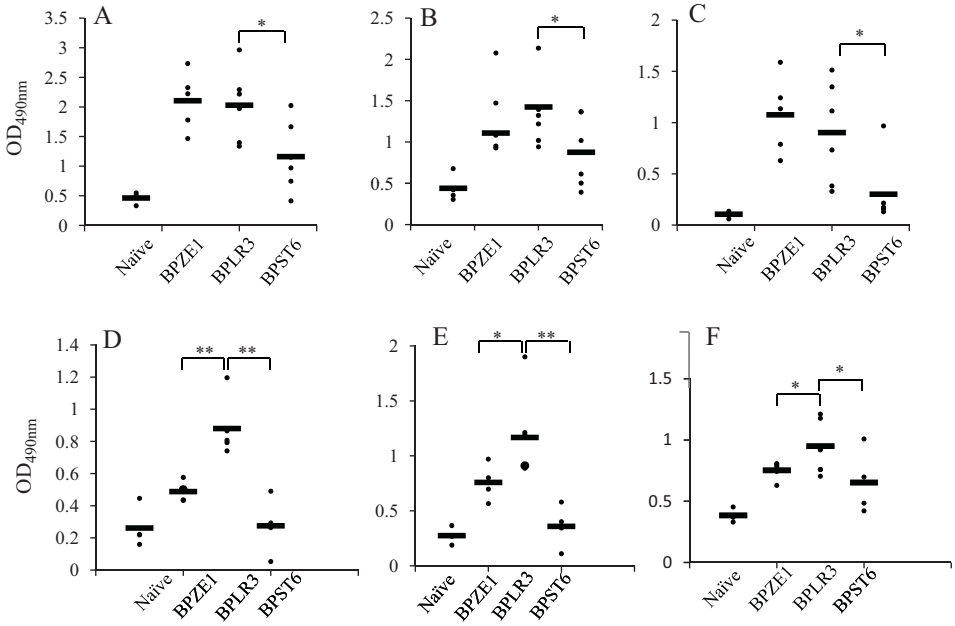

Fig. 7. Local and systemic specific antibody responses in mice immunized with BPST6 strain. Groups of 6 adult BALB/c mice were nasally administered thrice at 3-week intervals with 5×10^6 CFU of BPZE1, BPLR3 or BPST6. Two weeks after the last administration, sera were collected and broncho-alveolar lavages (BAL) were performed. The anti-pertussis and anti-M2e antibody responses were measured by ELISA. Systemic IgG (A and D), local IgG (B and E) and local IgA (C and F) anti-*B. pertussis* (A–C) or anti-M2e (D–F) antibody responses were measured by ELISA on individual sera diluted 1/1000 (A–C) or 1/40 (D–F) and neat BALFs, using BPZE1 whole cell lysate (A–C) or synthesized M2e peptide (D–F) as coating antigens. $^*p \leq 0.05$; $^{**}p \leq 0.01$.

4. Discussion

Heterologous expression in *B. pertussis* using FHA as carrier has been reported before by us and others [19,20,38,39]. However, to be efficiently secreted, FHA and FHA chimera should remain in an extended, secretion-compatible conformation prior to FhaC-mediated export [40]. Thus, FHA chimera containing heterologous antigens with one or several disulfide bond-forming cysteine (Cys) residues are likely to adopt globular conformations incompatible with FhaC-dependent secretion, as they will become the substrates of periplasmic disulfide oxidases/isomerases. Not surprisingly, Cys-containing passenger proteins like the tetanus toxin fragment C (TTFC) was poorly secreted when fused to FHA [36].

Here we have investigated the feasibility to express in the BPZE1 vaccine strain, one, two and three copies of the M2e universal influenza vaccine candidate fused to FHA. M2e is a 24-amino acid peptide which contains 2 Cys residues, thus allowing the formation of intra and/or intermolecular disulfide bonds. We found that the secretion efficacy of the chimera inversely correlated with the number of M2e copies fused to FHA, thus further supporting the idea that the presence of Cys residues in the foreign passenger domain impairs the secretion of the FHA-chimera. Consistently, we showed that deletion of the periplasmic disulfide isomerase encoding gene *dsbA* significantly improved the secretion of the FHA-$(M2e)_3$ chimera.

In the mouse model of pertussis infection, the ability of the recombinant bacteria to colonize the respiratory tract correlated with the secretion efficacy of the FHA-chimera. This finding is consistent with a previous study in which we reported that FHA release, which relies on SphB-mediated cleavage, is critical for optimal colonization of *B. pertussis* bacteria [41]. Furthermore, the colonization ability of BPZE1 is likely to be even more FHA-dependent when compared to a virulent *B. pertussis* strain because BPZE1 produces enzymatically inactive PTX; indeed we showed previously that the production of enzymatically active PTX is necessary to allow FHA-deficient *B. pertussis* bacteria to colonize effectively the mouse respiratory tract [41].

In addition, we showed that the overall fitness of the recombinant bacteria expressing FHA-$(M2e)_{2,3}$ chimera was impaired as evidenced by the *in vitro* growth profiles, which is likely to play a role also in the attenuated phenotype observed *in vivo*. Since the secretion efficacy of the FHA chimera is impaired in the recombinant BPLR2 and BPLR3 strains, it is conceivable that a substantial amount of FHA chimera would accumulate in the plasma membrane and/or in the periplasmic space thereby impairing some important membrane- or cell wall-associated functions, thus resulting in impaired bacterial fitness. However, comparable intracellular levels of the FHA chimera and wild type FHA were detected, which argues against this hypothesis. One would have thus to assume that the FHA chimera that are not effectively secreted and accumulate into the periplasmic space are readily degraded by proteolytic activity [42]. Since proteolytic activity usually requires energy [42,43], it may be to the expense of the bacterial multiplication and overall fitness.

Altered bacterial fitness and poor colonization efficacy were observed upon deletion of the *dsbA* gene in spite of increased secretion of the FHA-$(M2e)_3$ chimera. Indeed, it has been well characterized that DsbA plays a critical role in the correct folding and function of many secreted virulence factors of a variety of pathogenic bacteria; DsbA activity is required for production of functional type IV pili (also called fimbriae) that mediate adherence to host surfaces in *Vibrio cholerae*, *Neisseria meningitidis*, enteropathogenic and uropathogenic *E. coli* [44–47]. Toxin production or secretion is defective in many *dsbA* mutants, such as cholera toxin from *V. cholera* [45], heat-labile enterotoxin from enterotoxigenic *E. coli* [48]. In particular, deletion of the *dsbA* gene in *B. pertussis* has previously been reported to affect the production of other virulence factors critical for an efficient colonization, such as pertussis toxin [37]. However, impaired *in vitro* fitness of the *dsbA* KO *B. pertussis* mutant was not reported. Tinsley et al. revealed that *dsbA* deletion in *N. meningitidis* resulted in complete loss of its growth at 37 °C [46]. Moreover, as proposed recently by Powers and Balch commenting on the study of Quan et al., protein folding is probably an evolutionary mechanism for the host to protect against stresses from the outside [49,50]. Thus, the absence of DsbA protein in *B. pertussis* may result in the inability to cope with environmental stresses, thereby affecting the overall fitness of the bacterium.

Despite significant impairment in its secretion efficacy and colonization ability, BPLR3 expressing FHA-$(M2e)_3$ induced an anti-pertussis antibody response as strong as the parental BPZE1 strain, and triggered a stronger systemic IgG specific anti-M2e antibody response than BPLR1 and BPLR2 upon nasal administration to the mice. This observation is in agreement with previous studies which reported that high epitope density in fusion proteins are more immunogenic and significantly enhanced protective immunity [51–55]. Tandem repeats of epitopes in fusion proteins were shown to be more effective at stimulating T-cell immunity thanks to better antigen-processing and presentation [51,52,55]. Specifically, epitope density in M2e fusion proteins was shown to impact on B cell recognition and the higher epitope density resulted in M2e-specific antibodies with higher average avidity, which is likely due to stronger helper function of M2e-specific $CD4^+$ T-cells [53].

In contrast, although the secretion of the FHA-$(M2e)_3$ chimera was greatly improved upon *dsbA* deletion, both anti-pertussis and anti-M2e specific immune responses triggered by BPST6 were significantly lower than those triggered by BPLR3, the *dsbA*$^+$ parental counterpart. We thus concluded that the weak immunogenicity of BPST6 likely results from an inefficient priming of the host immune system due to the dramatic impairment in BPST6 colonization ability. This work thus indicates that *dsbA* deletion does not represent a promising approach to overcome the low secretion efficacy of Cys-containing FHA chimera.

We showed in a previous work that nasal pre-treatment with live BPZE1 bacteria confers non-specific protection against influenza A virus by dampening the production of inflammatory cytokines and chemokines; the protection efficacy was influenza A virus strain-dependent and did not lead to the reduction in the viral loads [13]. We thus reasoned that immunization with a BPZE1 strain expressing the M2e vaccine candidate should provide both non-specific and specific (anti-M2e antibody-mediated) protection against influenza. Since BPZE1 confers only 50% protection rate against H1N1/PR8 virus [13], we decided to use this virus for the challenge experiment, expecting an increase in the survival rate and decrease in the viral loads with the BPLR3-immunized mice compared to the BPZE1-treated group. Whereas a significant reduction in the viral loads was observed in the BPLR3-immunized mice, it did not translate into a greater survival rate compared to the BPZE1-treated group. These data thus suggested that immunization with BPLR3 bacteria conferred some level of antibody-mediated protection. Such weak protective efficacy might be due to the very low anti-M2e antibody responses triggered upon immunization with BPLR3 bacteria. Alternatively or concurrently, the divergence in the M2e amino acid sequence between BPLR3 and H1N1/PR8 virus may account for the absence of specific protection. Indeed, a 4-amino acid difference exists between the M2e sequence expressed in BPLR3 (MSLLTEVETPTRNEWECRCSDSSD) and the one expressed by H1N1/PR8 virus (MSLLTEVETPIRNEWGCRCNGSSD) which may be responsible for the production of anti-M2e antibodies of poor cross-neutralizing capacity. This hypothesis is further supported by the partial protection (50%) observed with the KLH-M2e immunized group despite a very high systemic anti-M2e IgG titer. In this group, only two animals out of five displayed reduced viral loads

in their lungs. Instead, a previous study using the M2e consensus sequence (MSLLTEVETPIRNEWGCRCNDSSD) which only differs by one amino acid from the H1N1/PR8 M2e sequence reported 100% protection rate whereas the viral loads were not determined [56].

In conclusion, the data presented in this study indicate that the ability of (FHA-M2e) expressing-BPZE1 bacteria to trigger an anti-M2e antibody response depends on several parameters including secretion efficiency of the chimera, M2e epitope density, and lung colonization ability, and that a delicate balance between all these factors must be achieved when Cys-containing foreign antigens such as M2e are to be expressed in *B. pertussis* using FHA as a carrier. In addition, we show that despite improving FHA-chimera secretion efficacy, *dsbA* deletion is not the appropriate approach to improve the immunogenicity of the recombinant bacteria. Alternatively, replacing the Cys residues with another amino acid such as Ser should be considered and is in progress in our laboratory. Finally, our work highlights the limitation of M2e as a universal influenza vaccine candidate, and underscores the necessity to use the M2e consensus sequence to achieve cross-protection against heterologous influenza A viruses. This study is part of a rational approach to characterize the various factors that may influence the immunogenicity of recombinant BPZE1 bacteria, in order to optimize this promising nasal delivery system.

Acknowledgment

This work was funded by the National Medical Research Council of Singapore (IRG NMRC/1135/2007).

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