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Adenoviral-vectored vaccine against *Bordetella pertussis* fimbrial antigen induces partial protection in a mouse model

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ABSTRACT

Pertussis has been resurgent in many countries worldwide despite good vaccine coverage. One hypothesis for this resurgence is that the current acellular pertussis (aP) vaccines, used in most developed countries, induce short-term protection, and do not prevent asymptomatic infection and transmission of pertussis infection. As a first step to address these issues we developed novel *Bordetella pertussis* vaccine candidates using viral-vectored vaccine technology with the aim of producing durable functional antibodies that prevent nasal colonization. Fimbrial antigens Fim2 and Fim3 are protective in mouse models of *B. pertussis* disease and are included in some aP vaccines. Fim2, Fim3 and FimD were selected, and their genes cloned into entry plasmids for the creation of the corresponding human adenovirus serotype 5 (AdHu5) vectors. Groups of mice were vaccinated with a single dose of either of the three AdHu5 vaccines, or a mixture of them, or control vaccines, which consisted of one or two reduced doses of a whole-cell pertussis vaccine or 5-component aP vaccine. The Fim2 and Fim3 adenovirus-based vaccines and their combinations alone or with FimD induced antigen-specific antibodies, as assessed by whole cell ELISA assay. Strong IgG binding to a Fim3-expressing strain was observed using flow cytometry and these antibodies also mediated complement deposition onto this strain. The AdHu5 Fim3 vaccine induced partial protection against lung infection following aerosol exposure of mice to *B. pertussis* expressing Fim3. These results indicate that adenovirus vectors have the potential to be effective vaccine platforms for bacterial disease.

1. Introduction

Pertussis is a highly infectious respiratory disease (also known as whooping cough) caused by the Gram-negative bacterium *Bordetella pertussis*. The clinical manifestations may vary from asymptomatic or mild respiratory symptoms to violent, long-lasting coughing episodes with post-tussive vomiting and an inspiratory whoop, depending on the age of the patient and pre-existing immunity [1]. Pertussis outbreaks were controlled following the introduction of whole cell pertussis (wP) vaccines starting in the 1940s [2]. However, there has been a resurgence of pertussis disease primarily in older children and adolescents, with transmission of the infection to vulnerable infants in many developed

Abbreviations: ADCD, antibody-dependent complement deposition; AdHu, human adenovirus; aP, acellular pertussis vaccine; ChHu, chimpanzee adenovirus; DPBS, Dulbecco's phosphate buffered saline; FHA, filamentous haemagglutinin; SHD, single human dose; wP, whole cell pertussis vaccine.

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countries [3].

Although resurgence is multifactorial, one of the reasons for increased pertussis cases is the replacement of the wP vaccine with the less reactogenic acellular pertussis (aP) vaccine [4]. It has been shown in a baboon model that aP vaccines are unable to prevent nasopharyngeal colonization and therefore transmission of the organsim [4]. Studies in mice also suggest that clearance of *B. pertussis* from nasopharyngeal mucosa is compromised by priming with aP vaccines as the induction of *B. pertussis*-specific IL-17/IFN-y-secreting tissue-resident memory CD4+ T-cells is impaired [5–7]. Furthermore, according to several studies in humans, current aP vaccines fail to induce a long-lived immune response, characterized by a relatively rapid decline in pertussis antibodies, with greatly increased risk of pertussis as soon as one year after childhood vaccination [8].

Strategies considered to prevent pertussis resurgence include the boosting of populations at risk and of epidemiologic importance with aP vaccine (such as young infants, adolescents, pregnant woman, adults, elderly people) [9,10], vaccination of close contacts and health-care workers, and continued use of high-efficacy wP vaccines [11]. An alternative approach is the development of novel vaccines. Recombinant viral vectors are of particular interest due to their ability to enter cells and express the selected target antigens, and consequently to induce innate and adaptive immune responses, including effective T-cell responses [12]. Adenoviruses, most commonly human (AdHu) or chimpanzee (ChAd) represent the most advanced viral vectors for vaccine delivery. Several adenoviral-vectored vaccines were licensed and proven to be effective against SARS-CoV-2 (e.g., AZD1222/ChAdOx; Jcovden/Ad26; Convidecia/Ad5) [13].

We created three *B. pertussis* vaccine constructs using the adenoviral-vectored technology. *B. pertussis* Fim2, Fim3 and FimD were selected as target antigens. Serotype 2 and 3 fimbriae are long, multimeric, helical and filamentous surface-localized structures comprised of Fim2 or Fim3 subunits, which have molecular weights of 19.2 kDa each [14–16]. They play important roles in adhesion of the bacteria to the respiratory epithelium, colonization of the nasal cavity, and shedding and transmission of *B. pertussis* [6]. Household studies in the 1990s found an association between the presence of antibodies to Fim2 and Fim3, and protection against pertussis infection [17–19], and they are components of a 5-component aP vaccine.

In addition to the major subunits, the fimbriae contain a single minor fimbrial subunit, designated as FimD, possibly located at the tip of the fimbrial structure, which has a molecular weight of 36.7 kDa [20]. FimD has been shown to be involved in binding to human cells via the integrin very late activation antigen-5 which activates complement regulator 3, allowing stronger adhesion of *B. pertussis* via filamentous haemagglutinin (FHA) complement regulator 3 binding [21]. Studies in mice suggested an important role for FimD in the colonization of the respiratory epithelium [22].

In this study, mice were vaccinated with wP, aP or combinations of AdHu5 coding for Fim2, Fim3 or FimD and immune responses, antibody-mediated complement deposition, and protection following aerosol challenge of mice were determined.

2. Materials and methods

2.1. Plasmid design and production of viral vectors

Three adenoviral-based vaccines were produced expressing Fim2, Fim3 or FimD. The *B. pertussis* strain Tohama I was used for the sequences of all three antigens. Gene sequences of the *B. pertussis* antigens were obtained using Basic Local Alignment Service Tool (BLAST) from the GenBank sequence database for Fim2 (aa 27–207, NCBI accession number CAE41417.1, UniProt accession number P05788), Fim3 (aa 26–204, CAE41857.1, P17835), and FimD (aa 1–365, CAE42166.1 (equivalent to aa Met,13–376 Q00879)). The degree of identity between sequences were obtained using the Clusta Omega program on UniProt.org; molecular weights and pIs were from the ProtParam tool on ExPAsy.org.

The cloning was performed as described before [23] using a replication-deficient, E1-deleted human adenovirus serotype 5 (AdHu5) as the vector. Production, purification and titration were performed as described previously [24].

2.2. Traditional vaccines and monoclonal antibodies

The *B. pertussis* wP vaccine used was that available as a reference reagent from the National Institute for Biological Standards and Control (NIBSC, 94/532 4th WHO International Standard). The 5aP vaccine used was a commercially available dTaP-IPV vaccine containing pertussis toxoid, 2.5 µg; filamentous haemagglutinin, 5 µg; pertactin, 3 µg; and fimbriae 2/3, 5 µg, stored at 2 °C to 8 °C and diluted in phosphate buffered saline (PBS).

Monoclonal antibodies against Fim2 and Fim3 are WHO International Standards for serotyping *B. pertussis* fimbrial antigen 2 and 3, NIBSC codes: 06/124 and 06/128, respectively [25].

2.3. B. pertussis strains

B. pertussis strains UK22 [26], B1917 [27], and the B1917 single gene knock-out strains, B1917 KO Fim3 and B1917 KO BvgS, were used in this study (Table 1). B1917 derivative strains were generated as previously described [28,29]. *B. pertussis* strains were cultured on blood charcoal agar plates (Oxoid) for 2 days at 35 °C prior to seeding into THIJS medium [30] supplemented with 0.75 mM heptakis-(2,6-di-*O*-methyl)-β-cyclodextrin (Daito Pharmaceutical Company) at OD of 0.1 at 600 nm and cultured for 16 h at 35 °C with orbital shaking. Mid-exponential phase bacteria were harvested, and stocks were made in THIJS medium with 10 % glycerol and stored at −80 °C.

2.4. Immunization of mice

All animal experiments were performed in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines and EU Directive 2010/63/EU for animal experiments. Procedures were either approved by the University of Oxford or MHRA/NIBSC Animal Welfare Ethical Review Committees.

For immunogenicity studies seven groups of six-to-eight week old NIH Swiss mice (n = 6/group) were vaccinated intramuscularly (i.m.) into the thigh muscle (maximum 50 µl/limb) as shown in Table 2. One mouse, vaccinated with the wP vaccine, became unwell after the vaccination and was culled. Mice were terminally bled six weeks after immunization.

In a second immunization experiment, one group of six-to-eight week old NIH Swiss mice (n = 6/group) were vaccinated with two doses (at a three-week interval) at 1/10th of a single human dose (SHD) of 5aP. Mice were terminally bled three weeks following boost.

For the aerosol challenge study, six-to-eight weeks old BALB/c mice (Charles Rivers, UK) were randomly allocated to four different groups (n = 10/group) and each mouse was immunized via the intramuscular route as shown in Table 3. Vaccines were diluted in Dulbecco's

Table 1
Fimbriae serotype composition of *B. pertussis* strains used in the study. Fimbriae serotype composition of strains containing Fim2 and 3 was determined from whole cell ELISA and flow cytometry.

Strain	Fim2	Fim3
UK22	✓	–
B1917	–	✓
B1917 KO Fim3	–	–
B1917 KO BvgS	–	–

✓ indicates the positive expression of the antigen.

Table 2
Immunization schedule with AdHu5 Fim and pertussis vaccines for assessment of immunogenicity. Blood sampling was performed at six weeks following immunization.

Group[1]	Vaccine	Dose/mouse	One dose
1	Naïve	–	–
2	wP vaccine	1/5th human dose (0.8 IU)	✔
3	AdHu5 Fim2	1×10^9 IU/100 µl	✔
4	AdHu5 Fim3	1×10^9 IU/100 µl	✔
5	AdHu5 FimD	1×10^9 IU/100 µl	✔
6	AdHu5 Fim2, AdHu5 Fim3	5×10^8 IU/50 µl of both	✔
7	AdHu5 Fim2, AdHu5 Fim3, AdHu5 FimD	3.33×10^8 IU/33.3 µl of each	✔

[1] Another group of mice ($n = 6$) was vaccinated with two doses (at a three-week interval) with 1/10th of a single human dose (SHD) of 5aP. Mice were terminally bled three weeks following boost.

Table 3
Immunization schedule with AdHu5 Fim3 and pertussis vaccines prior to challenge with *B. pertussis* B1917 strain which was performed six weeks following immunization.

Group	Vaccine	Dose/mouse	One dose
1	Naïve	–	–
2	AdHu5 Fim3	1×10^9 IU/50 µl	✔
3	5aP vaccine	1/10th human dose	✔
4	wP vaccine	1/10th human dose	✔

phosphate buffered saline (DPBS).

2.5. Evaluation of anti-B. pertussis IgG by whole cell ELISA

96-well plates (Nalgene Nunc International, Rochester, NY) were coated with heat-killed *B. pertussis* strain B1917 or *B. pertussis* strain UK22 at an OD of 0.1 at 600 nm and incubated at 37 °C overnight until dry. Plates were washed with PBS 0.05 % Tween 20 before blocking with 1 % BSA in PBS (all Sigma Aldrich, MO, USA) for 2 h at 37 °C. Doubling dilutions of sera from mice immunized as outlined above and serum of naïve mice diluted in PBS 1 % BSA were added to each well. A pool of naïve mice sera was used as a negative control. The ELISA was optimized and standardized using pooled sera of mice vaccinated with wP vaccine as quality control (QC). Each test sample and all controls were analyzed in duplicate.

The plates were kept overnight at a temperature of 4 °C and after another washing cycle, 100 µl of diluted (0.1 % BSA in PBS) goat anti-mouse IgG (Jackson Laboratory) was added to the wells, and incubated at room temperature for 2 h. The plates were developed with Tetramethylbenzidine (TMB) and the reaction stopped after 10 min with 2 M H_2SO_4. Optical densities (O.D.) were measured at 450 nm with a reduction at 630 nm and analyzed using BioTek Gen 5 software (Agilent, U.S).

2.6. Evaluation of IgG binding to B. pertussis by flow cytometry

Heat-inactivated (56 °C for 30 min) mouse sera (2 µl) were incubated in duplicate with 198 µl of bacteria at an OD of 0.1 at 600 nm in PBS 2 % BSA for 30 min at 25 °C with shaking (900 rpm). Monoclonal antibodies specific for Fim2 (NIBSC 06/124) or Fim3 (NIBSC 06/128) were used for confirming expression of Fim2 and Fim3 by *B. pertussis* strains. A centrifugation (5 min, 3060 *g*) was then performed, and the bacteria were washed with 200 µl of PBS. After a second centrifugation step, the bacteria were resuspended in 200 µl of anti-mouse IgG-FITC conjugate (Jackson Immunochemicals) at 1:500 and incubated at room temperature in the dark for 20 min. A washing step was then performed and bacteria resuspended in 2 % formaldehyde in PBS for one hour. After another washing step, the bacteria were labelled with 100 µl of live/dead Violet stain (Invitrogen 1:500) for 30 min at room temperature in the dark. Following a further washing step, the bacteria were resuspended with 200 µl PBS and the samples were analyzed using a flow cytometer (CytoFLEX S, Beckman, Coulter, United Kingdom). Median fluorescence intensities (MFI) were calculated for each individual or control samples using CytExpert software. The median background fluorescence intensity, corresponding to the fluorescence obtained for the bacteria and conjugate, was removed from each sample value.

2.7. Antibody-dependent complement deposition assay (ADCD)

Sera were heat-inactivated at 56 °C for 30 min. 5 µl of serum was incubated in duplicate with 85 µl of bacteria at an OD of 0.1 at 600 nm in PBS with 2 % BSA and with 10 µl of IgG- and IgM-depleted human plasma [31] as an exogenous complement source. After 45 min at 37 °C and 900 rpm, the samples were centrifuged at 3060 *g* for 5 min and washed with PBS. Bacteria were resuspended in 200 µl anti-human anti-C3c coupled to FITC (Abcam) at 1:500 and anti-SC5b-9 coupled to AF647 (Quidel) at 1:4000. After 20 min at room temperature in the dark, a washing step was performed. The bacteria were resuspended in 200 µl PBS with 2 % formaldehyde and incubated for 1 h. After a washing step, bacteria were labelled with 100 µl Live/Dead Violet stain (Invitrogen) at 1:500 for 30 min at room temperature in the dark. Following a further washing step, pellets were resuspended in 200 µl PBS and analyzed on a Flow Cytometer (CytoFLEX S, Beckman, Coulter, United Kingdom). Median fluorescence intensities were calculated for each sample with CytExpert software. The median background fluorescence, corresponding to the fluorescence obtained for the bacteria, complement and conjugate, was removed from each sample value.

2.8. Aerosol infection of mice with B. pertussis

B. pertussis B1917 was grown on charcoal agar plates supplemented with 10 % (*v*/v) defibrinated horse blood (Thermo Scientific Oxoid) at 37 °C for 18–24 h. The bacteria were resuspended in 6 ml of sterile 0.9 % saline containing 1 % casein. The density of bacterial suspension was adjusted to an OD of 0.340 at 625 nm which corresponded to 1×10^9 bacteria/ml and kept on ice for 1–2 h prior to infection.

Six weeks following immunization, as outlined above, aerosol challenge of vaccinated mice was performed as outlined previously [32–34]. The mice were placed inside an aerosol apparatus, their noses were fitted into the nose ports of the apparatus and were then exposed to *B. pertussis* administered as aerosol, using a nebulizer containing 1×10^9 bacteria/ml, for five minutes. Two to three hours after the challenge, five out of 10 non-immunized mice, which serve as control, were anesthetized, and sacrificed to remove lungs and trachea for colony counting to assess if the challenged mice were infected during the procedure. The lungs and trachea from the remaining five non-immunized mice and immunized mice were harvested seven days after the challenge as this was found to be the optimum time point in a previous study [34]. The lungs and trachea were homogenized in 1 ml 1 % casein in saline and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} of the original culture. Four drops (25 µl / drop) of each diluted homogenate were plated onto charcoal agar supplemented with 10 % (vol/vol) defibrinated horse blood (TCS Biosciences, UK) and the corresponding viable counts for each mouse were determined after incubation at 37 °C for five to seven days.

2.9. Analysis and statistics

Data was analyzed using GraphPad PRISM, version 8.2.1 and IgG binding, ADCD and ELISA data were presented showing MFI and log endpoint titre respectively, with a geometric mean and 95 % confidence interval (ELISA) and a mean standard deviation (IgG binding and ADCD). Distribution of the data was assessed before analysis by one-way

ANOVA followed by a multi-comparison test (Tukey or Dunn). A p-value of <0.05 was considered statistically significant.

3. Results

3.1. Characterization of Fim2 and Fim3 expression by B. pertussis strains

Serotyping of *B. pertussis* strains used for evaluation of the immunogenicity of the vaccine candidates and for the challenge experiments was performed using WHO International Standards for anti-Fim2 and anti-Fim3 by flow cytometry. Expected results were obtained for each strain [26,27] (Table 1). The Fim2-specific mAb bound to strain UK22, the Fim2-expressing strain (Fig. 1A). Similarly, Fim3-specific mAb bound to strain B1917, which expresses Fim3 (Fig. 1B). The Fim2 and Fim3 mAbs did not bind to B1917 KO Fim3 strain, or to strain B1917 KO BvgS, confirming lack of expression of Fim2 or Fim3 (Fig. 1A&B). These results were also confirmed by whole cell ELISA, where the cells were coated onto ELISA plates and detection performed using the anti-Fim2 and anti-Fim3 antibodies (results not shown). FimD-specific mAbs were not available and therefore FimD expression was not confirmed for the strains in this study.

3.2. The fimbriae-encoding AdHu5 vaccine candidates induce antigen-specific serum IgG responses in mice

Three recombinant replication-deficient AdHu5 vectors were constructed to express either Fim2 (181 aa, 19,203.5 Da), Fim3 (179 aa, 19,248.7 Da), or FimD (365 aa, 39,313.5 Da) using Gateway-compatible entry plasmids and the corresponding three adenovirus vectors (AdHu5 Fim2, AdHu5 Fim3, AdHu5 FimD) were generated. All three vaccines passed the quality control assessment as described previously [24] (data not shown) and progressed to immunogenicity assessment in mice.

A first immunogenicity study comprised seven groups of mice. Mice were either naïve, or received one of the following vaccines: wP, AdHu5 Fim2, AdHu5 Fim3, AdHu5 FimD or a combination of AdHu5 Fim2 and AdHu5 Fim3, or a combination of the three adenoviruses (Table 2). In a separate experiment, another group of mice was vaccinated with aP. Serum samples were collected at 6 weeks post a single vaccine dose, and the presence of Fim-specific antibodies was analyzed by whole cell ELISA against two *B. pertussis* strains: UK22, which expresses Fim2 only and possibly FimD, and B1917, which expresses Fim3 only and possibly FimD. Serum IgG binding to UK22 (Fim2, FimD) and B1917 (Fim3, FimD) following immunization with the AdHu5 Fim2 or AdHu5 Fim3 vaccines occurred in a serotype-specific manner: mice immunized with AdHu5 Fim2 elicited a higher IgG titre to UK22 than mice immunized with AdHu5 Fim3, and conversely (Fig. 2). The response elicited by AdHu5 FimD against both strains was low or absent ($p = 0.0525$ for UK22 and $p = 0.0221$ for B1917).

AdHu5 Fim2 induced a similar IgG titre to aP against strain UK22, and only a slightly lower response than wP, which is remarkable since unlike wP, AdHu5 Fim2 is only presenting one antigen. The combinations of AdHu5 vaccines induced IgG titres similar to AdHu5 Fim2 alone against strain UK22, indicating that the Fim2 component may be responsible for most of the IgG bound.

As expected, AdHu5 Fim3 induced a higher IgG titre against strain B1917 than AdHu5 Fim2. This vaccine also induced a significantly higher response than aP ($p < 0.0001$), and an equivalent response to that induced by wP (Fig. 2). The antibody titres induced by the combinations of AdHu5 vaccines against B1917 were significantly lower than titers elicited by AdHu5 Fim3 alone.

Total IgG binding onto *B. pertussis* strains was also investigated by flow cytometry (Fig. 3). IgG binding following the AdHu5 vaccines to *B. pertussis* UK22, B1917, B1917 KO Fim3 and B1917 KO BvgS is shown in Fig. 3A, B and C. The IgG binding occurred in a serotype-specific manner for the AdHu5 Fim2 and AdHu5 Fim3 vaccines. Sera from mice that received the AdHu5 FimD vaccine showed similar IgG binding MFI values to the wild type UK22 and B1917 strains and a significantly reduced binding to the Fim3 KO and BvgS KO strains.

Total IgG binding induced by wP and aP vaccines or individual and combined AdHu5 vaccines can be compared for each strain in Fig. 3 D-G.

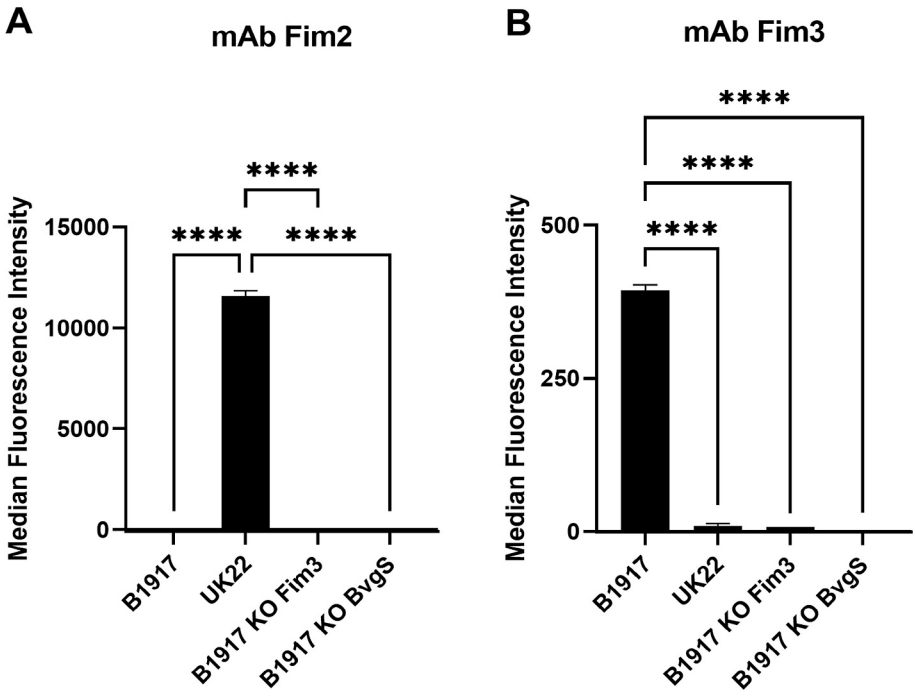

Fig. 1. Characterization of Fimbriae variants. Quantification of IgG binding onto UK22, B1917, B1917 KO Fim3 and B1917 KO BvgS, using monoclonal antibody directed against either Fim2 (A) or Fim3 (B). The fluorescence intensity was determined from a technical duplicate. Means and standard deviation errors are represented. Distribution of the data was assessed before performing a one-way ANOVA followed by Turkey's multiple comparisons test. Significant p values are indicated (****$p \leq 0.0001$).

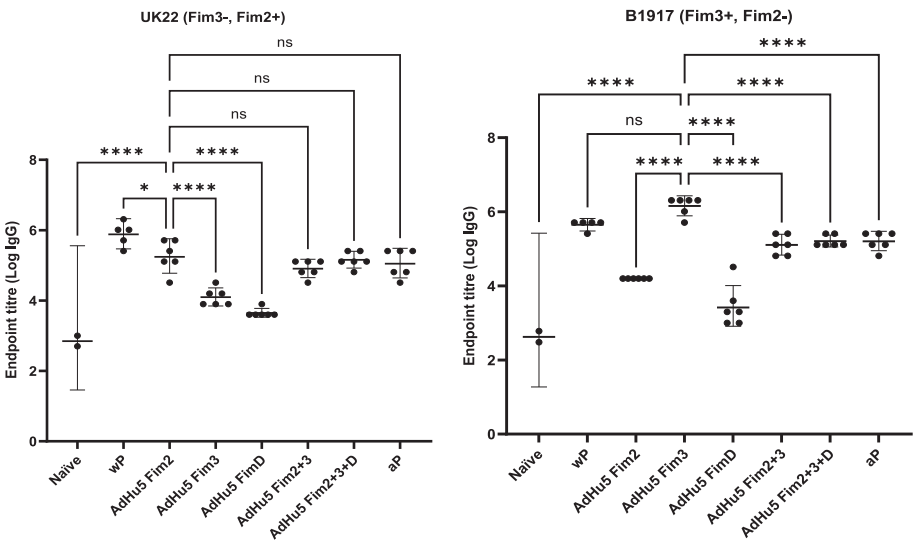

Fig. 2. Quantification by ELISA of IgG binding onto *B. pertussis* following vaccination. Sera from unvaccinated mice (naïve) or from mice immunized with either a single dose of whole cell pertussis vaccine (wP), five fimbriae adenovirus constructs (AdHu5), or two doses of acellular pertussis vaccine (aP) were tested on UK22 and B1917. Geometric means and 95 % confidence intervals are represented. Statistical analyses were performed by one-way ANOVA followed by Turkey's multiple comparisons test and significant p values are indicated (****p ≤ 0.0001, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

wP vaccine induced high IgG binding to all of the strains, including the KO strains, likely due to the induction of IgG specific for a large number of surface antigens. For strain UK22, vaccination with AdHu5 Fim2 induced significantly lower IgG binding than wP vaccination ($p < 0.0001$, Fig. 3D). In addition, compared to wP, low IgG binding onto UK22, the Fim2-expressing strain, is observed after vaccination with the AdHu5 Fim3 or AdHu5 FimD vaccines (Fig. 3D).

High IgG binding onto B1917 was observed with sera from mice vaccinated with AdHu5 Fim3 which was similar to that induced by aP or wP vaccines (Fig. 3 E). Lower IgG binding onto B1917 is observed using the AdHu5 FimD construct serum compared to AdHu5 Fim3 (Fig. 3E), suggesting a lower production of specific antibody, and/or a lower expression of FimD than Fim3 at the surface of the bacteria, which would be expected due to the lower stoichiometry of FimD to the major Fim subunits, and potentially poorer surface accessibility of FimD [22]. The IgG binding to B1917 obtained from groups immunized with AdHu5 Fim3 in combinations with AdHu5 Fim2 and AdHu5 FimD were slightly lower than AdHu5 Fim3 alone but no significant difference was observed (Fig. 3E). IgG binding to the Fim3-negative strains, B1917 KO Fim3 and B1917 KO BvgS were similar and low, except for sera from wP vaccinated mice, as expected based on their phenotypes (Fig. 3F and G).

Altogether, these results show that the Fim adenovirus vaccine candidates allow the generation of Fim-specific antibodies in mice after a single dose. Using a flow cytometry readout, we show that immunization with AdHu5 Fim3 elicited the highest IgG binding to B1917 amongst the AdHu5 vaccines. Interestingly, B1917 has less mAb Fim3 binding onto its bacterial surface than UK22 with mAb-Fim2 used at the same concentration (Fig. 1), suggesting that AdHu5 Fim3 mediated greater production of antibodies than AdHu5 Fim2. The IgG responses induced by the vaccine mixtures have no significant difference as compared with single vaccine, but there is a trend towards higher IgG binding onto UK22 with the triple Fim-combination compared to the single vaccines (Fig. 3D).

3.3. *The Fim3-specific antibodies produced after vaccination with AdHu5 Fim3, AdHu5 Fim2 + AdHu5 Fim3 and AdHu5 Fim2 + AdHu5 Fim3 + AdHu5 FimD mediate complement deposition*

The function of the antibodies was measured by the ADCD assay. Sera from mice vaccinated with wP, AdHu5 Fim3, AdHu5 Fim2 + AdHu5 Fim3 and AdHu5 Fim2+ AdHu5 Fim3+ AdHu5 FimD mediated C3c/iC3b and C5b-9 deposition onto B1917 (Fig. 4A and B). Greater C3c/iC3b deposition was elicited after adenoviral-vectored vaccines injection as compared to the wP vaccine, whereas wP antibodies induce a higher C5b-9 deposition onto B1917 than adenoviral-vectored vaccines (Fig. 4B). These results demonstrate that the AdHu5 Fim3 construct generates complement-fixing antibodies against a Fim3-expressing strain. C3b/iC3b and C5b-9 deposition on the Fim2-expressing strain UK22 was only observed in sera from mice immunized with the wP vaccine (Fig. 4C and D), suggesting that even if the AdHu5 Fim2 induced the production of Fim2-specific antibodies they are not able to deposit complement onto bacteria. The B1917 KO Fim3 strain was used as a control and as expected, complement deposition against this strain was only observed after wP vaccination (Fig. 5A&B).

3.4. *AdHu5 Fim3 induces protection against mouse lung infection following aerosol challenge with B. pertussis B1917*

Since AdHu5 Fim2 and AdHu5 FimD failed to induce functional IgG against strain B1917, the efficacy of AdHu5 Fim3 was assessed against pertussis infection with the B1917 strain. Groups of mice ($n = 10$) were immunized with AdHu5 Fim3 (Table 3). At day seven post aerosol challenge with B1917, CFUs recovered from lungs were determined. A two-log reduction in CFU in the lungs of mice immunized with a single dose of the AdHu5 Fim3 vaccine was observed compared to naïve mice ($p < 0.0001$, Fig. 6). A single immunization with a 5aP vaccine containing Fim3, and one-dose immunization with wP vaccine both induced partial protection, and in some mice full protection (compared with naïve mice, day seven, $p < 0.0001$, Fig. 6).

4. Discussion

In this study, we created three adenoviral-based vaccine candidates encoding for the fimbriae antigens Fim2, Fim3 and FimD. We demonstrated that the vectors elicited antigen-specific antibody responses in mice. Only immunization with AdHu5 Fim3 or AdHu5 Fim3-containing combinations (AdHu5 Fim2 + AdHu5 Fim3; AdHu5 Fim2 + AdHu5 Fim3 + AdHu5-FimD) were able to elicit similar antibody responses as the wP vaccine and the aP vaccine as measured by IgG binding on the surface on bacteria. Furthermore, only the Fim3-containing vaccine induced antibodies that mediated complement deposition and had the capacity to clear *B. pertussis* infection in mice. A study of nasal

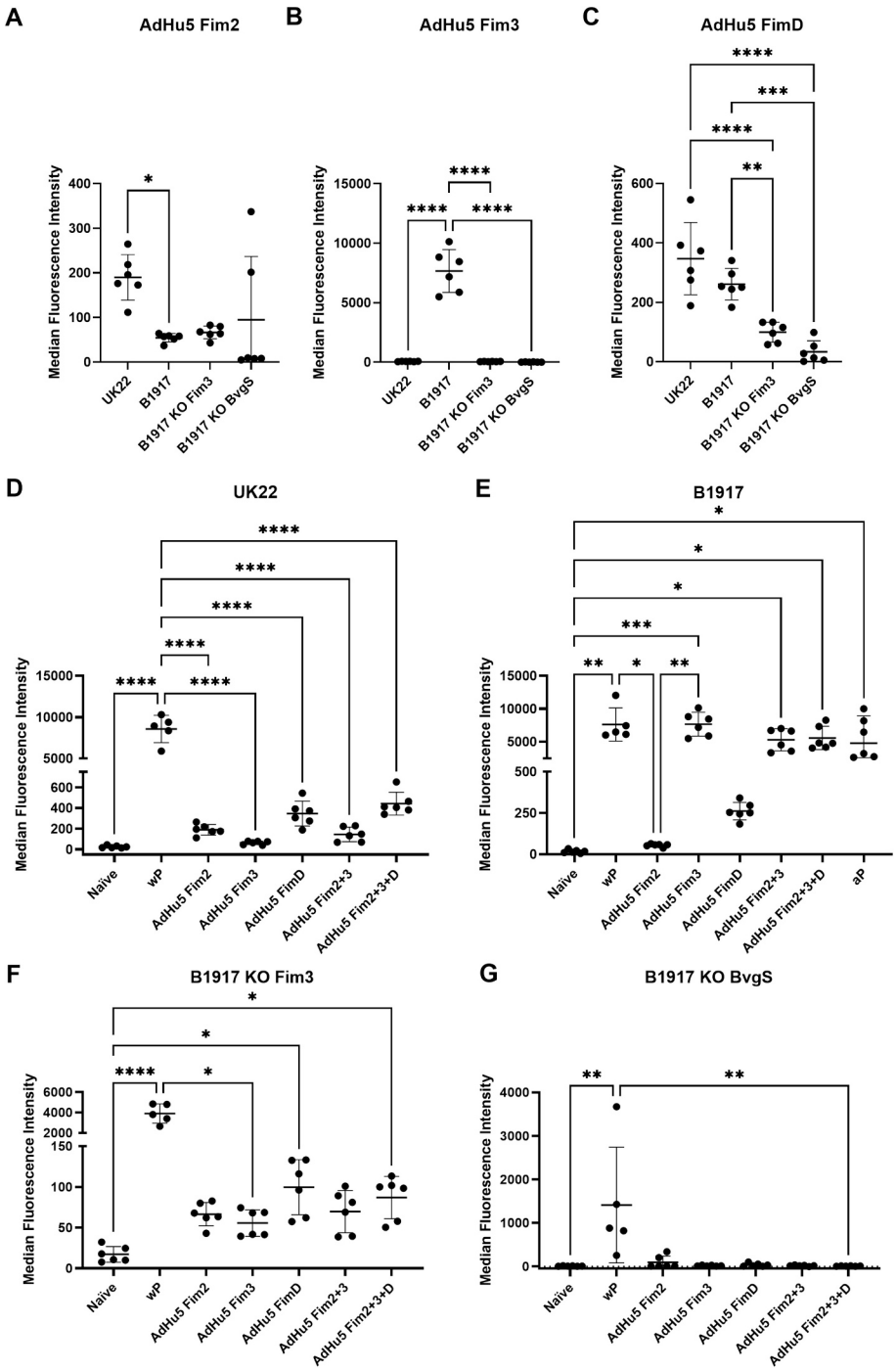

Fig. 3. Quantification by flow cytometry of IgG binding onto *B. pertussis* following vaccination. Sera from unvaccinated mice (naïve) or from mice immunized with either a single dose of whole cell pertussis vaccine (wP), or five fimbriae adenovirus constructs (AdHu5), or two doses acellular pertussis vaccine (aP) were tested for antibody binding to UK22, B1917, B1917 KO Fim3 and B1917 KO BvgS strains. Panels A-C show IgG binding to *B. pertussis* UK22, B1917, B1917 KO Fim3 and B1917 KO BvgS following the AdHu5 vaccines immunizations. Total IgG binding induced by wP and aP vaccines or individual and combined AdHu5 vaccines are compared for each strains in D-G. The fluorescence intensity was determined from a technical duplicate. Means and standard deviation errors are represented. Distribution of the data was assessed before a one-way ANOVA followed by Dunn's (panels A, E, F, G) or Turkey's (panels B, C, D) multiple comparison test. Significant p values are indicated (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

colonization following challenge of immunized mice with *B. pertussis* was also attempted but issues with contamination could not be overcome.

The *fim* genes of the Fim2 and Fim3-expressing strains (UK22 and B1917, respectively), used for the IgG surface binding, differed only slightly from the Tohama I gene sequence: for the Fim2 nucleotide sequence, there is 99.5 % identity in 636 residues overlap with UK22; the Fim3 of Tohama I and B1917 have a 99.8 % identity in 615 residues overlap. It is unlikely, therefore, that changes in the nucleotide sequence between the Tohama I (vaccine sequence) and the *B. pertussis* isolates used for analysis may have influenced the IgG fine specificity or complement-binding function induced by AdHu5 Fim2. Differences in conformation, post-translational modification (a single S—S bond formation) or polymerization of vector-expressed polypeptides during

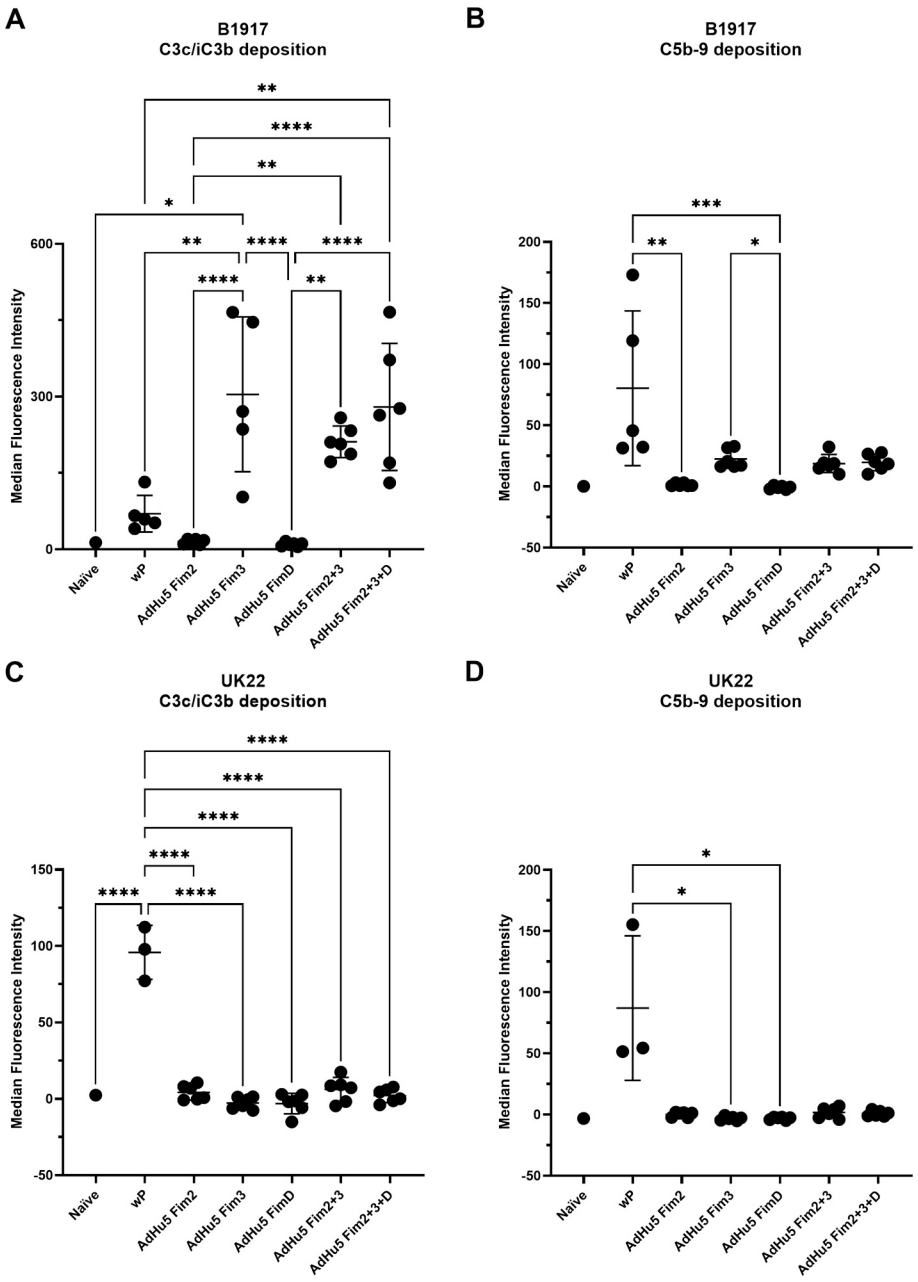

Fig. 4. Antibody-mediated complement deposition onto *B. pertussis*. Quantification of C3c/iC3b (A and C) or C5b-9 (B and D) deposition onto either UK22 or B1917 using sera from unvaccinated mice (naïve) or from mice immunized with either a single dose of whole cell pertussis vaccine (wP) or five fimbriae adenovirus constructs (AdHu). The fluorescence intensity was determined from a technical duplicate. Means and standard deviation errors are represented. Distribution of the data was assessed before performing a one-way ANOVA followed by Turkey's (panels A and B) or Dunn's (panels C and D) multiple comparison test. Significant p values are indicated (****$p \leq 0.0001$, ***$p \leq 0.001$, **$p \leq 0.01$, *$p \leq 0.05$).

translation in mice compared to its natural production in bacterial cells should also be considered, although comparable ELISA titres were obtained with the AdHu5 Fim2 and the 5aP vaccine, which contains Fim2 and Fim3 antigens. To date, recombinant protein fimbriae made in bacterial cells have failed to adopt conformations that form the polymerized fimbriae thought to be required for protection [35,36].

Single subunits of Fim2 and Fim3 are of similar molecular weight (19.2 kDa), yet have variant isoelectric points (Fim2, 5.8; Fim3, 8.8) which may have influenced epitope accessibility when expressed by the adenoviral vectors. They have 57 % amino acid concordance [15,16]. Both gene sequences used for the adenoviral-vectors were derived from Tohama I, a strain most commonly used, together with 10,536 strain, for the production of aP vaccines [37]. With conserved as well as hypervariable regions of aa sequence, the major fimbriae subunits have distinct epitope maps and induce serotype-specific antibodies [35,38]. Our results confirm the serotype-specificity of the anti-Fim antibodies induced by the AdHu5 vaccines. In comparison with the wP vaccine and 5aP which contain several antigen targets, the immunogenicity of AdHu5 Fim3, which targets a single antigen, was particularly high. Moreover, only one dose of the AdHu5 vaccine was given compared with two for 5aP. Antibodies induced by the wP vaccine against B1917 KO Fim3 and B1917 KO BvgS were significantly lower compared with strains expressing Fim2 or Fim3 but this is to be expected as the expression of many virulence factors is regulated by BvgS [39]. The response elicited by AdHu5 Fim2 + AdHu5 Fim3 and the mix of AdHu5 Fim2 + AdHu5 Fim3 + AdHu5 FimD were also comparable with those

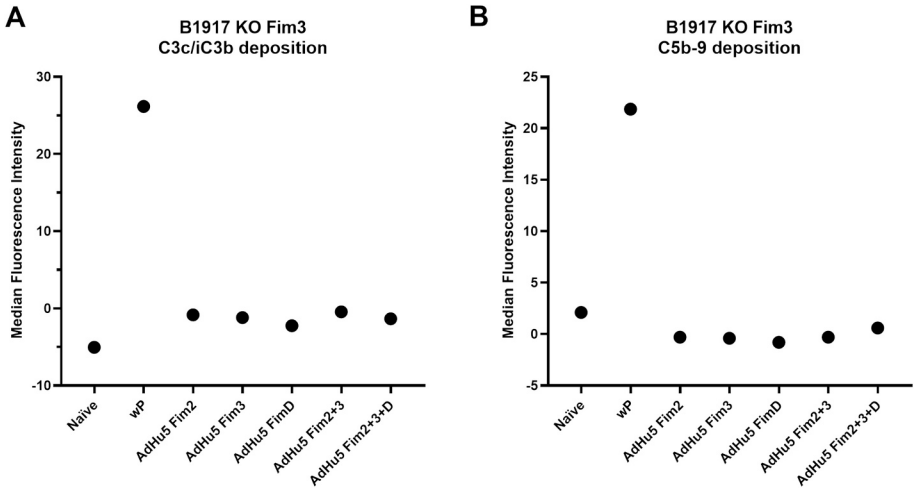

Fig. 5. Antibody-mediated complement deposition onto B1917 KO Fim3 variant. Quantification of C3c/iC3b (A) or C5b-9 (B) using pooled sera from unvaccinated mice (naïve) or from mice immunized with either a single dose of whole cell pertussis vaccine (wP) or five fimbriae adenovirus constructs (AdHu). The fluorescence intensity was determined from a technical duplicate and means are represented.

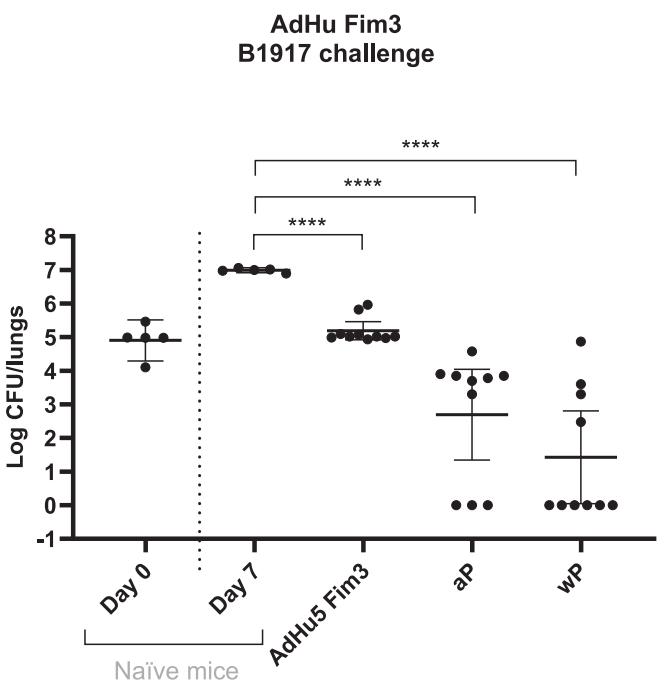

Fig. 6. Assessment of protective capacity of AdHu5 Fim3 vaccine against *B. pertussis* B1917 strain in mice. Mice ($n = 10$/group) were immunized intramuscularly once with AdHu5 Fim3 vaccine, aP and wP vaccine. Non-immunized mice served as negative control. Six weeks after the vaccination, mice were challenged via aerosol route with 1×10^9 bacteria/ml of B1917 strain. Lungs were removed one week after the challenge, homogenized and CFU counts were established five to seven days after plating the homogenized lungs on charcoal agar plates containing 10 % (*v*/v) defibrinated horse blood. Data in each graph were analyzed with a one-way ANOVA test followed by Dunn's multiple comparisons test to compare differences between vaccination groups and naïve mice on day seven (negative control group), ****; $p < 0.0001$.

elicited by the wP vaccine and 5aP. However, our results suggest that the responses induced by the mix were most likely driven by the AdHu5 Fim3 component only.

The literature regarding the minor subunit FimD, the so called "tip adhesion", in *B. pertussis* is scarce and there have been no reports or studies about its pathogenesis and role in the development of the disease since the 1990s, despite published results indicating that the FimD plays an important role in the colonization of the bacterium on the mouse respiratory epithelium [22]. Therefore, we investigated the potential of the FimD antigen when expressed in the adenovirus platform. Immunization with AdHu5 FimD induced specific IgG recognizing strains UK22, B1917 and B1917 KOFim3, with flow cytometry affirming the weaker response seen by whole cell ELISA. The response to B1917 was much lower compared with the response elicited by AdHu5 Fim3, as expected, and only slightly higher than the response elicited by AdHu5 Fim2 on UK22. Weak cross-reactivity between anti-FimD antibodies and Fim2 and Fim3 antigens is suggested by the finding that IgG binding of antibodies elicited by AdHu5 FimD on Fim2 and Fim3-expressing strains was higher than IgG binding on the strain not expressing Fim2 and Fim3 ($p \leq 0.01$). The lack of expression of Fim2 and Fim3 on B1917 KO Fim3 and B1917 KO BvgS could also have an impact on the FimD accessibility on the bacterial surface, as previously hypothesized [22], and this might lead to decreased IgG binding.

Prior to this study, a direct comparison of our AdHu5 vaccines with wP and aP vaccines in mice had not been done. For the adenoviral-vectored vaccines, a dose of 10^9 IU was used. Studies using other antigens have demonstrated that 10^8–5×10^8 IU is the dose where the plateau of the dose response starts when aiming for T-cell responses and when prime-boost approaches have been used [40,41]. The higher dose in this study was selected as this dose is more immunogenic when used as a single dose for induction of antibody responses [24,42]. Queenan et al. also proposed increasing the dose of Fim antigen in aP vaccines [43].

In addition, the blood sampling was performed six weeks after immunization with a single dose of the AdHu5 vaccines or the wP vaccine and three weeks after a booster dose of the aP vaccine. Studies using adenoviral-vectored vaccines in mice showed that antibody titres after immunization can increase up to 20 weeks [42].

Previous studies have shown that fimbrial expression is variable and that *B. pertussis* has the ability to switch fimbrial expression on and off [44–46]. In addition, fim2, fim3, and fimX genes are affected by transcriptional control, known as fimbrial phase variation. This acts as a defense mechanism by allowing the bacteria to switch between different fimbrae expression which prevents the host's immune system from recognizing and eliminating the pathogen [47,48]. The expression of fimbriae is also regulated by small mutations such as insertions or deletions within a stretch of a cytosine-rich promoter region, and these mutations contribute to phase variation [49]. For this reason, the development of additional adenoviral vectored vaccine against other virulence factors, that are surface-accessible or secreted, i.e., pertussis

toxin, is desirable. Also, the AdHu Fim3 candidate vaccine could be used as part of a multicomponent vaccine, for example, added onto aP.

Sequence conservation between Fim2 and 3 has been proposed to account for their similar packing arrangements in helical fimbria which can extend to >700 nm in length, containing about 275 repeating units, or single Fim subunits [50]. The protective response to a fimbrial vaccine has been thought to be dependent on the native conformation of fimbriae, in turn, regarded as essential for polymerization of the subunits [33,35]. This study has demonstrated that an adenoviral-expressed Fim3 can induce a functional immune response that can disrupt or prevent colonization. The surface-accessibility of Fim3 on *B. pertussis* may have contributed to the success of this approach.

5. Conclusions

Three different *B. pertussis* vaccine candidates based on fimbrial antigens were created using an adenoviral-vectored vaccine platform. Vaccines specific for fimbriae of serotypes 2 and 3 and combinations thereof elicited specific antibody responses in mice as detected by ELISA. Further, IgG binding determined by flow cytometry showed that a single dose of AdHu5 Fim3 vaccine induced an antibody response comparable with those elicited with wP vaccine, and higher or equivalent to two doses of aP vaccine. These antibodies were found to mediate complement deposition and partial clearance of *B. pertussis* infection from mouse lungs following aerosol challenge. Future studies could address some of the limitations of this study, such as determining whether these vaccines can induce a long lasting Th1 response, induction of T cell and secretory IgA responses, have the ability to prevent nasal colonization or transmission via different routes of administration or vaccination schedules (including boosting). However, this study demonstrated that the adenoviral-vectored vaccine technology is a promising vaccine platform for the development of novel *B. pertussis* vaccines that can induce protective immune responses.

CRediT authorship contribution statement

Alexa Dierig: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Martina Kristof:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **Christina Dold:** Writing – review & editing, Methodology, Conceptualization. **Elodie Lesne:** Writing – review & editing, Methodology, Investigation, Data curation. **Catpagavalli Asokanathan:** Writing – review & editing, Methodology, Investigation. **Luke Blackwell:** Writing – review & editing, Investigation. **Barbara Bolgiano:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization. **Andrew R. Gorringe:** Writing – review & editing, Methodology, Formal analysis. **Andrew J. Pollard:** Writing – review & editing, Methodology, Conceptualization. **Kevin Markey:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization. **Christine S. Rollier:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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