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Increasing FIM2/3 antigen-content improves efficacy of *Bordetella pertussis* vaccines in mice *in vivo* without altering vaccine-induced human reactogenicity biomarkers *in vitro*

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ABSTRACT

Current acellular-pertussis (aP) vaccines appear inadequate for long-term pertussis control because of short-lived efficacy and the increasing prevalence of pertactin-negative isolates which may negatively impact vaccine efficacy. In this study, we added fimbriae (FIM)2 and FIM3 protein to licensed 2-, 3- or 5-component aP vaccines (Pentavac®, Boostrix®, Adacel®, respectively) to assess whether an aP vaccine with enhanced FIM content demonstrates enhanced efficacy. Vaccine-induced protection was assessed in an intranasal mouse challenge model. In addition, potential reactogenicity was measured by biomarkers in a human whole blood assay (WBA) *in vitro* and benchmarked the responses against licensed whole cell pertussis (wP) and aP vaccines including Easyfive®, Pentavac® and Pentacel®. The results show that commercial vaccines demonstrated reduced efficacy against pertactin-negative versus pertactin-positive strains. However, addition of higher amounts of FIM2/3 to aP vaccines reduced lung colonization and increased vaccine efficacy against a pertactin-negative strain in a dose-dependent manner. Improvements in efficacy were similar for FIM2 and FIM3-expressing strains. Increasing the amount of FIM2/3 proteins in aP formulations did not alter vaccine-induced biomarkers of potential reactogenicity including prostaglandin E_2, cytokines and chemokines in human newborn cord and adult peripheral blood tested *in vitro*. These results suggest that increasing the quantity of FIM proteins in current pertussis vaccine formulations may further enhance vaccine efficacy against *B. pertussis* infection without increasing the reactogenicity of the vaccine.

1. Introduction

Bordetella pertussis is the causative agent of whooping cough, a contagious respiratory infectious disease most harmful to infants. Once considered well-controlled by vaccines, many countries are experiencing an increase in pertussis cases in older children and adolescents despite high vaccine coverage, with transmission to vulnerable infants too young to be vaccinated [1]. One contributing component of the recent resurgence in whooping cough cases in developed countries is related to the faster waning immunity after vaccination with acellular pertussis (aP) vaccines compared with whole cell pertussis (wP) vaccines [2,3]. Other contributing factors include increased awareness and improved detection methods, and

Abbreviations: aP, acellular pertussis vaccines; CFU, colony-forming units; DTaP, diphtheria, tetanus, and aP; FHA, filamentous hemagglutinin; FIM2/3, fimbriae type 2 and type 3; IL, interleukin; PGE_2, prostaglandin E2; LPS, lipopolysaccharide; PTX, pertussis toxoid; PRN, pertactin; WBA, whole blood assay; wP, whole-cell pertussis vaccine.

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strain adaptation to vaccine pressure. In particular, a percentage of recent pertussis clinical isolates in the United States, Australia, Europe and Japan no longer express the vaccine antigen pertactin (PRN) [4].

Currently available aP vaccines consist of between one and five detoxified *B. pertussis* virulence factors. All aP vaccines contain pertussis toxoid (PTX), with multicomponent aP vaccines additionally including the adhesins filamentous hemagglutinin (FHA), PRN and fimbriae (FIM) type 2 and type 3 (FIM2 and FIM3). Potential strategies to overcome the current pertussis problem include maternal immunization, changes to schedules to optimize the timing and frequency of booster doses, returning to using wP vaccines in infancy, using different adjuvants or new components in vaccines, or improving components present in existing vaccines.

Fimbriae are surface filaments that serve a role in adhesion of the bacteria to host cells [5]. There are two major types, FIM2 and FIM3, which are serologically distinct. FIM2/3 are currently included in marketed vaccines as antigens in five-component aP formulations produced by Sanofi Pasteur MSD Ltd. This antigen consists of a mixture of both FIM serotypes, co-purified and added to the vaccine at 5 μg per pediatric dose. There are strong data supporting the inclusion of FIM2/3 in vaccine formulations, as *B. pertussis* mutants lacking FIM2/3 are deficient in colonization of the mouse trachea [6], and antibodies to FIM interfere with *B. pertussis* attachment to human alveolar epithelial cell line [5]. In the mouse intranasal challenge model, intraperitoneal administration of 10 μg/dose of fimbrial proteins provided protection against *B. pertussis* lung colonization [7], and addition of FIM2/3 to a three-component aP vaccine increased its efficacy [8]. In human clinical trials, the five-component aP vaccine was more effective than two or three-component vaccines, suggesting that FIM2/3 added value to aP vaccines [9,10]. However, the topic is still debatable, and some authors claim that there is no sufficient evidence to support higher effectiveness of aP vaccines with differing numbers of components [11]. There is evidence that five-component aP is effective against *B. pertussis* strains expressing FIM2 but not against strains expressing FIM3 [12]. FIM 2/3 is only approved for human use at a concentration of 5 μg/dose and is considered an important component of current five-component formulations [13,14] but in light with the resurgence of whooping coughing cases, there is a urgent need for vaccine improvement.

Because of their short-lived efficacy compared to wP vaccines, and the increasing prevalence of PRN-negative isolates which may impact efficacy, current aP vaccines appear inadequate for long–term pertussis control. There is a need for improved aP vaccines with efficacy against contemporary clinical isolates. Using an *in vivo* mouse model of pertussis infection, we investigated whether the addition of higher amounts of FIM2/3 impacted aP vaccines efficacy. In addition, to gain insight into potential reactogenicity, we characterized the human innate immune response induced by vaccine formulations containing different amount of FIM2 and FIM3 proteins and benchmarked the responses to both licensed wP and aP vaccines including Easyfive®, Pentavac® and Pentacel® *in vitro*. We demonstrate that an aP formulation containing increased FIM protein content enhanced protective immune responses against *B. pertussis* challenge *in vivo* with limited effects with respect to reactogenicity biomarker profile *in vitro*.

2. Materials and methods

2.1. Ethics statement

Balb/c mice were purchased from Envigo (previously Harlan Laboratories), Indianapolis, IN, USA. All mice were maintained in an AAALAC approved animal facility at Janssen Spring House, PA under controlled temperature and humidity and maintained on a 12 h light/dark cycle, with free access to food and water. All procedures were performed according to the guidelines established in the Guide for the Care and Use of Laboratory Animals (8th Edition) [15], and the protocols were approved by the Janssen Institutional Animal Care and Use Committee of Spring House, PA, USA. The studies reported here are compliant with the ARRIVE Guidelines for reporting animal research [16]. Non-identifiable cord blood samples were collected with approval from the Ethics Committee of The Brigham & Women's Hospital, Boston, MA (protocol number 2000-P-000117) and Beth Israel Deaconess Medical Center Boston, MA (protocol number 2011P-000118). Blood samples from adult volunteers were obtained after written informed consent with approval from the Ethics Committee of Boston Children's Hospital, Boston, MA (protocol number X07-05-0223).

2.2. Bacterial strains

The standard *B. pertussis* WHO-18323 strain was obtained from the American Type Culture Collection (ATCC) (#9797). The FIM2-expressing strains p584/99 and p111/97 were a generous gift from Professor Hans Hallander (Public Health Agency of Sweden). *B. pertussis* 19-81 and 20-29 were shared by Alan Evangelista (Drexel University) and previously characterized as PRN-negative [17]. *B. pertussis* I-195 (PRN-negative) is a recent clinical isolate of the FIM3 serotype and was shared by Dr. Tod Merkel (Center for Biologics Evaluation and Research, US Food and Drug Administration). Strains were streaked from frozen stocks and passaged two times on Bordet-Gengou agar at 35 °C before starting an overnight culture in Stainer-Scholte medium the day before challenge.

2.3. Vaccination and challenge models

The mouse intranasal challenge model was used as described previously [18,19]. In brief, female Balb/c mice were vaccinated at 4 and 7 weeks of age. Vaccines were given subcutaneously (s.c.) in fractions of a human dose, 1/10 or 1/25. For accurate dosing, vaccines at 1/10 or 1/25 human dose were diluted in saline for a dose volume of 0.1 ml. One wP vaccine (Easyfive®, Panacea Biotec) and four aP vaccines containing between 2 and 5 pertussis antigens were studied (Table 1): diphtheria, tetanus, and aP (DTaP2,

Table 1
Composition of studied vaccines.

Vaccine	Brand name	PTXμg	FHAμg	PRNμg	FIM2/3μg	DTIU	TTIU	Al^{3+}mg
DTaP2	Pentavac®	25	25	–	–	≥30	≥40	0.3
Tdap3	Boostrix®	8	8	2.5	–	≥2	≥20	0.5
DTaP3	Infanrix®	25	25	8	–	≥30	≥40	0.5
Tdap5	Adacel®	2.5	5	3	5	≥2	≥20	0.33
DTaP-IPV*	Pentacel®	20	20	3	5	15 Lf	5 Lf	0.33
wP	Easyfive®	–	–	–	–	20 Lf	7.5 Lf	0.25

PTX: pertussis toxoid; FHA: Filamentous hemagglutinin; PRN: pertactin; FIM2/3: fimbriae types 2 and 3; DT: Diphtheria toxin; TT: Tetanus toxin; IU: international units.
* DTaP-IPV also contains inactivated polioviruses [40 D-antigen units (DU) Type 1 (Mahoney), 8 DU Type 2 (MEF-1), 32 DU Type 3 (Saukett)], and 10 μg PRP of *H. influenzae* type b covalently bound to 24 μg of tetanus toxoid (PRP-T).

Pentavac®, Sanofi Pasteur MSD Ltd.), Tdap3 (Boostrix®, GlaxoSmithKline Biologicals S.A.), DTaP3 (Infanrix®, GlaxoSmithKline Biologicals S.A.) and Tdap5 (Adacel®, Sanofi Pasteur MSD Ltd.) were administered at either 1/10 or 1/25 human dose. When FIM was added to a regimen, it was injected s.c. as 0.1 ml in the flank. The FIM2/3 formulation was prepared by mixing dilutions of purified FIM2/3 (200 μg/ml, List Biological Laboratories, Inc.) in PBS with Alhydrogel to a final concentration of aluminum of 0.6 mg/ml at 4 °C. FIM2/3 was added in amounts of 0.5–5 μg/ injection, which at 1/10 human dose would correlate with 5 μg to 50 μg in a pediatric dose. At 9 weeks of age, mice were challenged intranasally with ~10^6 Colony-forming units (CFU) of different *B. pertussis* strains. Four to five mice per treatment group were sacrificed 2 h post challenge and 8–10 mice 5 days after challenge, which was determined to be the peak of infection. Lungs were harvested, homogenized and plated on Bordet-Gengou agar. The plates were incubated at 35 °C for 4–5 days, and CFU were counted and converted to CFU/lung. A limit of detection, based on plating undiluted samples, was determined to be 20 CFU/lung ($\log_{10}$ 1.3). Tail or terminal bleeds were used to obtain blood samples for antibody titer and agglutination testing.

2.4. Total IgG determination

Total IgG antibody levels to PTX, FHA and FIM2/3 were determined using the BioPlex Multiple Immunoassay (BioRad) system [20]. The mice were vaccinated at 4 weeks of age and boosted 3 weeks later. 2 weeks after the boost, total IgG was measured on sera from individual mice. 50 μl/well 4-fold serial dilutions of sera in PBS, 0.1% Tween 80, 3% BSA (starting at 1:50 dilution) were added to Bio-Plex Pro™ Flat Bottom Plates. Four thousand beads/well were added for each antigen. The plates were mixed and incubated at room temperature for 45 min in the dark on a plate shaker at 600 rpm and washed twice with PBS. Goat anti-mouse R-Phycoerythrin (RPE)-conjugated IgG secondary antibody (Invitrogen, catalog #P852) at 1:250 dilution in PBS was added in a volume of 50 μl. The plates were incubated at room temperature for 30 min under continuous shaking and then washed twice with PBS. Beads were re-suspended in 125 μl of PBS and analyzed on the Bio-Plex 200 in combination with Bio-Plex Manager software. The WHO reference serum NIBSC 97/642 was included to express IgG levels in standard International Units (IU)/ml.

2.5. Agglutination assay

Functionality of FIM2/3-induced antibodies was evaluated by testing their ability to agglutinate suspensions of FIM2 and FIM3-expressing *B. pertussis* strains. Serum samples from vaccinated mice were pre-incubated with 25% kaolin prior to testing in order to reduce auto-agglutination background [21]. Sera from each treatment group (N = 5–15) were pooled for analysis. Bacteria grown overnight at 35 °C in Stainer-Scholte broth were washed in saline, adjusted to OD_{600} 1.0 and mixed with an equal volume of pooled test serum. After overnight incubation at 35 °C, the plates were scored visually, with a positive agglutination test indicated when agglutination prevented pellet formation at the bottom of the well. The agglutination titer was defined as the reciprocal of the dilution that caused complete agglutination of a FIM2 (*B. pertussis* p584/99) or FIM3 (*B. pertussis* I-195) bacterial suspension.

2.6. Human blood sample processing and in vitro stimulation

Peripheral blood was collected from healthy adult volunteers, while human newborn cord blood was collected immediately after Cesarean section delivery of the placenta. Births to known HIV-positive mothers were excluded. Human experimentation guidelines of the U.S. Department of Health and Human Services, The Brigham & Women's Hospital, Beth Israel Deaconess Medical Center Boston, and Boston Children's Hospital were observed, following protocols approved by the local institutional review boards. Human blood was anti-coagulated with 20 units/ml pyrogen-free sodium heparin (American Pharmaceutical Partners, Inc.; Schaumberg, IL). All blood products were kept at room temperature and processed within 4 h from collection. For human whole blood assays, neonatal cord blood or adult whole blood (WB) was first diluted with sterile RPMI 1640 medium (Invitrogen; Carlsbad, CA) and 175 μl of the diluted WB was added to each well of a 96 well U-bottom plate (Becton Dickinson; Franklin Lakes, NJ, USA) containing 75 μl of RPMI, freshly prepared FIM proteins, or licensed vaccines at 10× the final concentration. The same formulations were used for both *in vivo* and *in vitro* assessment, so the FIM2/3 formulation was prepared by mixing dilutions of purified FIM2/3 (200 μg/ml, List Biological Laboratories, Inc.) in PBS, which at 1/10 human dose would correlate with 5 μg/250 μl. The candidate vaccine components including sterile FIM buffer (10 mM Tris HCl, pH 8, 150 mM NaCl, 0.1% sucrose, 0.05% Tween 20), FIM2 and FIM3 protein. FIM protein antigens (endotoxin < 1 EU/ml) were adsorbed onto alum in FIM buffer at 4 °C for 2 h. Proteins were prepared in RPMI from stocks and they were verified free of endotoxin (<1 EU/ml) according to the manufacturer, as performed by endotoxin detection limulous amoebocyte lysate (LAL) assay. Licensed vaccines evaluated included wP (Easyfive® Panacea Biotec), DTaP-IPV (Pentacel®, Sanofi Pasteur) and DTaP2 (Pentavac®, Sanofi Pasteur) (Table 1) were obtained from the corresponding manufacturers. Suspensions containing 250 μl/well were gently mixed by pipetting and incubated for 6 h at 37 °C in a humidified incubator at 5% CO_2. The final dilution of the WB was 1:1 vol/vol. After culture, plates were centrifuged at 500*g* and supernatant was carefully removed by pipetting without disturbing the cell pellet and stored at −80 °C.

2.7. ELISA and multi-analyte assay

Supernatants derived from WB stimulation were assayed by ELISA for TNF (BD Biosciences; San Jose, CA, USA) and IL-1β (eBiosciences; San Diego, CA). Additionally, assay supernatants were analyzed by customized 14-plex magnetic bead multiplex cytokine/chemokine assay (EMD Millipore; Billerica, MA, USA) and analyzed on the Luminex 100/200 System employing xPOTENT software (Luminex; Austin, TX) and EMD Millipore Milliplex Analyst (version 3.5.5.0). The minimum threshold for each analyte was set at the minimum detectable concentration for a given assay, defined as three standard deviations above the mean background. Supernatants were assayed by ELISA by competitive monoclonal enzyme immunoassay (EIA) for PGE_2 (Cayman Chemical, Ann Arbor, MI, USA). PGE_2 concentrations were determined using the analysis tool at www.myassays.com.

2.8. Data analysis, statistics and graphics

CFU counts per lung were converted to $\log_{10}$ CFU and analyzed with Tobit regression to account for observations below the detection limit of the assay. When comparing vaccine groups or challenge strains, the Tobit model contained either vaccine or challenge strain as explanatory factors. When testing for a dose dependent linear improvement in vaccine efficacy when adding FIM2/3, the Tobit model contained dose as a continuous covariate. Log_{10} CFU data were graphed as mean +/- standard deviation (SD). FHA- and PTX-specific IgG levels were analyzed using Tobit regression and FIM-specific IgG levels were analyzed using Wilcoxon Rank Sum test to determine significant differences between groups. P-values were adjusted for multiple testing using either

the Tukey method in case of all pairwise groups comparisons, or the Dunnett method in case of comparisons to a fixed reference group. P-values < 0.05 were considered statistically significant. All statistical analyses were performed in R 3.3.1 (R Core Team). *In vitro* data were analyzed using Prism for Mac OS X versions 5.0c and 7.0 (GraphPad Software Inc., San Diego, CA) and Microsoft Excel for Mac 2011 version 14.6.0 (Microsoft Corporation, Redmond, WA). Bar graphs and line graphs represent means +/- standard error mean (SEM), radar plots represent means and scatter plots show individual data points with means indicated by horizontal lines. Wilcoxon matched pairs signed rank test was applied at each concentration to individual treatments. Two-way repeated measure ANOVA test and Bonferroni post-test were applied to overall comparisons of treatments with different concentrations. p values < 0.05 were considered significant and indicated as follows: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

3. Results

3.1. Currently licensed aP vaccines show less efficacy against PRN-negative challenge strains

Two-, three, and five-component licensed acellular pertussis vaccines and one wP vaccine were evaluated for vaccine efficacy at 1/10 human dose using PRN-positive and PRN-negative strains. Fig. 1 shows that at day 5 post-infection, the three-component aP vaccine (Tdap3) was more efficacious against WHO 18,323 PRN-positive *B. pertussis* strain than against 19-81 and I-195 PRN-negative strains ($p = 0.0269$ and $p = 0.0022$, respectively). Similar results were observed with a five-component aP vaccine (Tdap5). A significant reduction in lung CFU counts was observed in mice that were challenged with WHO 18,323 PRN-positive *B. pertussis* strain when compared to 19-81 and I-195 PRN-negative strains ($p = 0.0001$ for both strains).

Although both vaccines Tdap3 and Tdap5 showed similar trends toward lower efficacy against the PRN-negative *B. pertussis* 20-29 strain when compared to PRN-positive strain WHO 18323, the differences in CFU did not reach statistical significance.

DTaP2 and DTaP3, vaccines formulated with higher concentrations of PT and FHA and the wP vaccine, were more efficacious in reducing lung CFU counts when compared to Tdap3 and Tdap5 for all strains tested; however, these vaccines did not show differential efficacy when comparing PRN-positive versus PRN-negative strains (Fig. 1).

3.2. Addition of FIM2/3 overcomes lower efficacy against PRN-negative strains

As Tdap5 demonstrated the least protection against the PRN- strains, we tested if a high concentration of FIM2/3 would increase protection. Vaccination with Tdap5 at 1/10 of human dose (HD) containing 0.5 μg of FIM-2/3 with additional FIM2/3 (5 μg (10× higher), demonstrated increased protection at Day 5 post-infection against the PRN-positive and 2 out of 3 negative *B. pertussis* strains when compared to Tdap5 alone (strain WHO 18323, $p = 0.0139$; strain 20–29, $p = 0.0497$; strain I-195, $p < 0.0001$, Fig. 2). The addition of 5 μg of FIM2/3 to Tdap5 did not improve vaccine efficacy against the PRN-negative *B. pertussis* strain 19–81 ($p = 0.1631$).

3.3. Addition of FIM2/3 to two-component aP increased vaccine efficacy against a PRN-negative strain in a dose dependent manner

Using *B. pertussis* I-195, a recent PRN-negative clinical isolate, we tested if additional FIM2/3 would improve efficacy of a two-component vaccine (DTaP2) that includes higher concentrations of PTX and FHA as compared to Tdap3 and Tdap5 (Table 1). Mice were vaccinated with DTaP2 administered at 1/25 (Fig. 3a) or 1/10 (Fig. 3b) of human dose and with increased concentrations

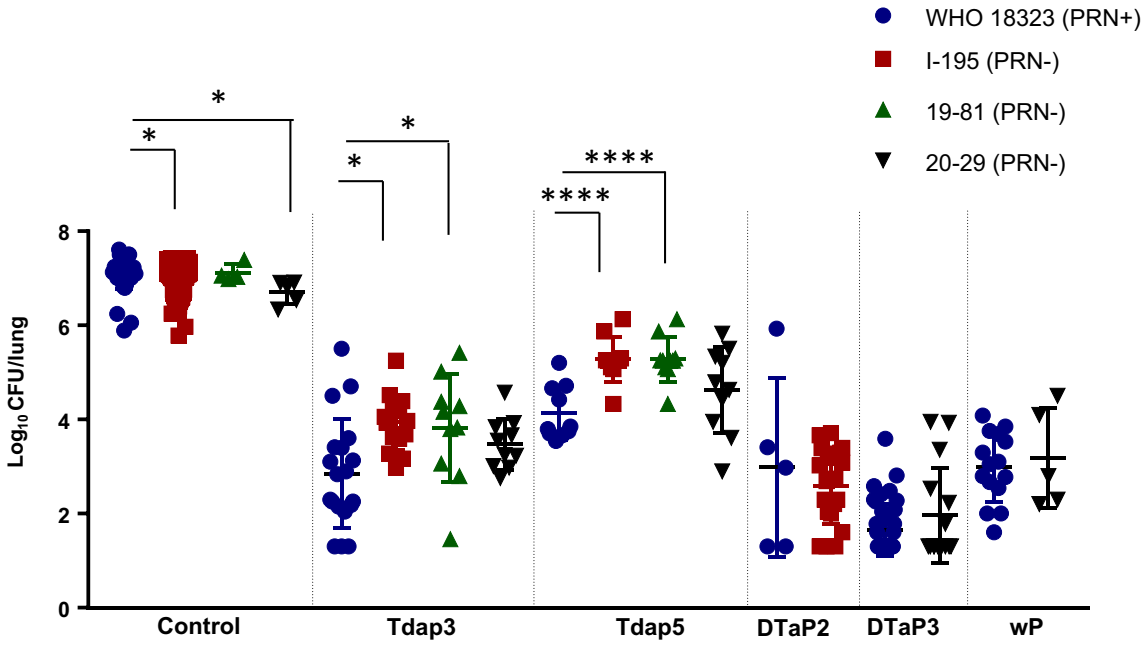

Fig. 1. Acellular pertussis vaccines are less efficacious against PRN-negative *B. pertussis* strains. Mice were vaccinated s.c. with commercially available vaccines, Tdap3, Tdap5, DTap2, DTaP3 and wP (1/10 human dose) followed by challenge with PRN-positive and PRN-negative *B. pertussis* strains. CFU counts were determined 5 days post-infection. Graph shows mean and SD of $\log_{10}$ lung CFU. WHO 18323, PRN+ (blue); I-195, PRN- (red); 19–81, PRN- (green); 20–29, PRN- (black). $^{*}p \leq 0.05$, $^{****}p \leq 0.0001$.

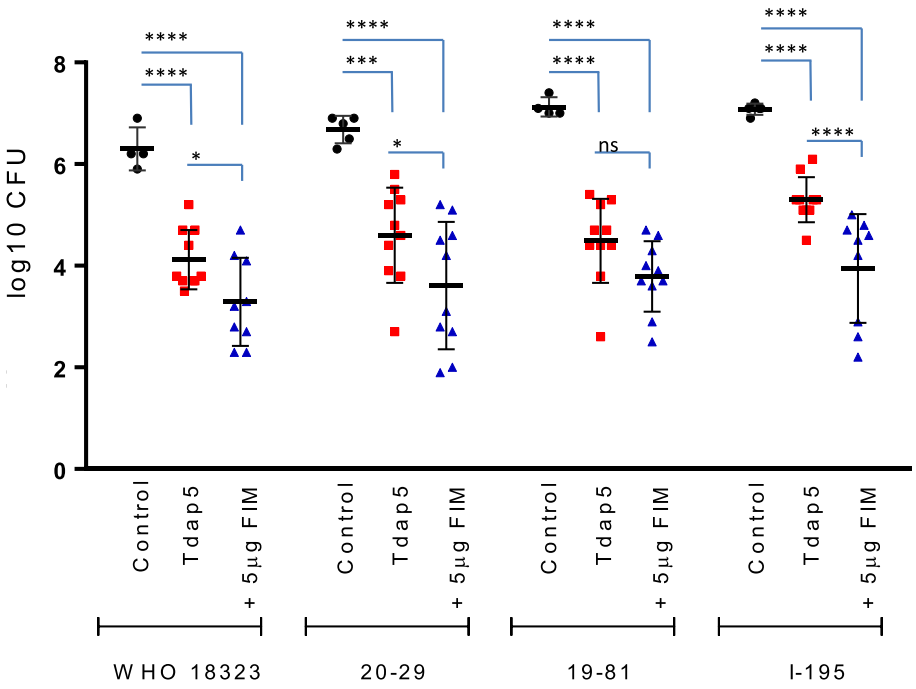

Fig. 2. Vaccination with additional FIM2/3 improves Tdap5 vaccine efficacy against PRN-negative *B. pertussis* strains. Mice were vaccinated with Tdap5 or Tdap5 administered with additional 5 μg of FIM2/3 followed by intranasal challenge with PRN-positive (WHO18323) or PRN-negative (20–29, 19–81, I-195) *B. pertussis* strains. Lung CFU were determined 5 days post-infection. Graph shows mean and SD of $\log_{10}$ CFU. $^{*}p \leq 0.05$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

of FIM2/3 (0.5 to 5 μg). Addition of FIM2/3 to DTaP2 administered at 1/25 of human dose improved vaccine efficacy in a dose-dependent manner at 5 days post-infection (Fig. 3a). Similar results were observed when the vaccine was administered at 1/10 HD in combination with different concentrations of FIM2/3 (Fig. 3b); however, with this regimen, a dose dependent improvement in vaccine efficacy was evident as early as 2 h p.i. (Fig. 3b). Interestingly, addition of 5 μg of FIM2/3 to DTaP2 at 1/10 human dose seems to impact adherence, assessed by measuring CFU/lung 2 h post infection, which was approximately 1 $\log_{10}$ lower than the CFU from mice vaccinated with DTaP2 alone ($p < 0.0001$) (Fig. 3b). Overall, for both vaccine dilutions (1/10 and 1/25 human dose), the addition of increasing concentrations of FIM2/3 resulted in a dose-dependent increase in vaccine efficacy 5 days post challenge, which reached statistical significance at FIM2/3 concentrations of 2 μg and above (Fig. 3a and b).

3.4. Addition of FIM2/3 increased vaccine efficacy after challenge with either FIM2- or FIM3-expressing strains

We investigated whether the FIM serotype of the *B. pertussis* strains used in the challenge model impacts the improved efficacy observed with addition of increased concentrations of FIM2/3. Mice were vaccinated at 4 and 7 weeks of age with a two-component aP vaccine (DTaP2) at 1/25 human dose with or without the addition of FIM2/3 at 2 or 5 μg/dose. The FIM2 expressing-strain, *B. pertussis* p111/97, and the FIM3 expressing-strain, *B. pertussis* I-195, were used for the intranasal challenge. The addition of 2 or 5 μg of FIM2/3 to DTaP2 significantly improved vaccine efficacy against FIM2-expressing *B. pertussis* strain (2 or 5 μg FIM2/3, $p < 0.0001$, Fig. 4). Similarly, DTaP2 vaccine efficacy against *B. pertussis* strain-expressing FIM3 is significantly improved by the addition of 2 or 5 μg FIM2/3 (2 μg FIM2/3, $p = 0.0232$; 5 μg FIM2/3, $p = 0.0004$). In summary, addition of FIM2/3 increased DTaP2 efficacy for both FIM2 and FIM3-expressing *B. pertussis* strains.

3.5. Antibody responses after addition of FIM2/3 to a two-component aP vaccine

IgG antibody levels to PTX, FHA and FIM2/3 were measured in mice vaccinated at 4 weeks and boosted at 7 weeks with DTaP2 at 1/25 human dose with or without the addition of FIM2/3 in a range of 0.5–5 μg/dose. Total IgG was measured from serum collected at 2 h post-infection. As shown in Table 2, addition of different concentrations of FIM2/3 induced a significant increase in antibody responses to FIM2/3 when compared to DTaP2 alone ($p = 0.004$ for 0.5, 1.0, 1.5 and 2.5 μg/dose of FIM2/3 and $p < 0.0001$ for 2.0 and 5.0 μg/dose of FIM2/3 compared to DTaP2 alone); however, dose-dependent antibody responses against FIM2/3 was not observed. Antibody levels against PTX and FHA were not significantly different in the presence of additional FIM2/3 when compared to DTaP2 alone (Table 2).

3.6. FIM2/3 antibodies are functional

The ability of sera from vaccinated mice to agglutinate either a FIM2- or a FIM3-expressing strain was used as a measure of antibody functionality. Table 2 shows the agglutination titers in pooled sera from mice vaccinated with 1/25 human dose of DTaP2 with addition of increasing doses of FIM2/3. All FIM2/3 concentrations tested elicited a strong agglutination response compared to DTaP2 alone.

3.7. FIM proteins adjuvanted with alum induces modest amounts of cytokine production in human blood

To characterize the potential reactogenicity and immunogenicity of formulations containing FIM2 and FIM3 proteins for pertussis vaccine development, we benchmarked pertussis vaccine formulations to licensed whole cell and acellular pertussis vaccines (Table 1) using a human *in vitro* whole blood assay. FIM antigen formulations were prepared in three different dilutions, to 1:10,

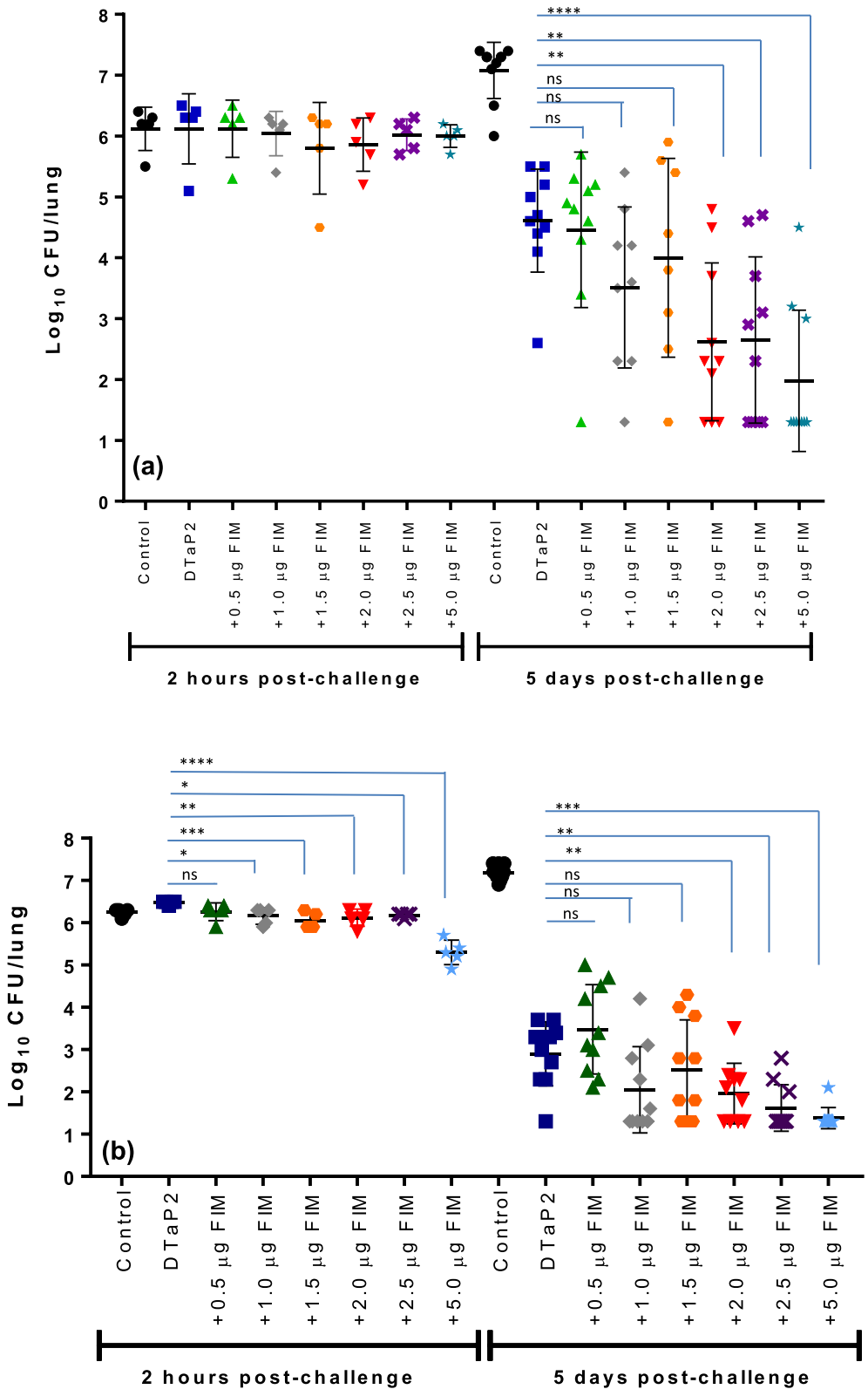

Fig. 3. (a) FIM2/3 improves DTaP2 vaccine efficacy in a dose dependent manner. Mice were vaccinated with 1/25 human dose of DTaP2 or DTaP2 including increasing concentrations of FIM2/3 followed by intranasal challenge with PRN-negative *B. pertussis* strain (I-195). Mean and SD of $\log_{10}$ CFU were determined 2 h and 5 days post-infection. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$. (b) FIM2/3 improves DTaP2 vaccine efficacy in a dose dependent manner. Mice were vaccinated with 1/10 human dose of DTaP2 or DTaP2 including different concentrations of FIM2/3 followed by intranasal challenge with PRN-negative *B. pertussis* strain (I-195). Mean and SD of $\log_{10}$ CFU were determined 2 h and 5 days post-infection. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

1:100 and 1:1000 vol/vol of original concentration (Supplemental Fig. 1). Newborn cord blood and adult peripheral blood were collected, and within 4 h, stimulated *in vitro* at 37 °C, 5% CO_2 for 6 further hours and supernatants were collected for ELISA and multiplex assays as outlined in the workflow diagram (Supplemental Fig. 1). Analyses using multiplex assays to measure the produc-

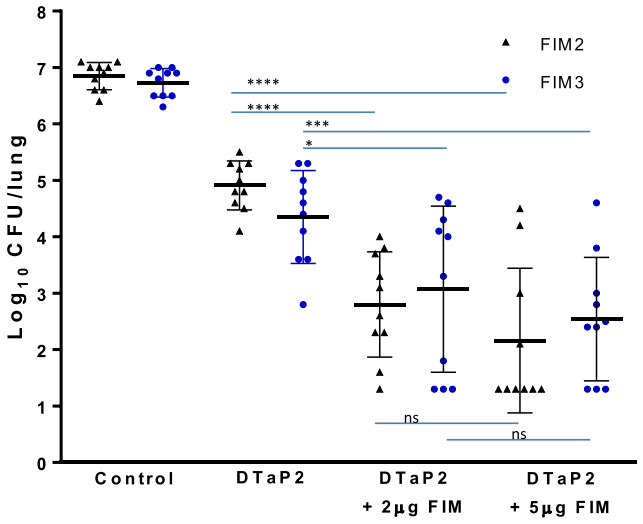

Fig. 4. Additional FIM2/3 (2 μg or 5 μg/dose) improves DTaP2 vaccine efficacy for both FIM2 and FIM3-expressing *B. pertussis* strains. Mice were vaccinated with DTaP2 (1/25 HD) or DTaP2 (1/25 HD) including 2 or 5 μg of FIM2/3 followed by challenge with FIM2- or FIM3-expressing *B. pertussis* strains. Graph shows mean and SD of $\log_{10}$ lung CFU 5 days post-challenge.

tion of a total of 14 cytokines and chemokines demonstrated that FIM protein alone induced modest production over the RPMI vehicle control. For example, for TNF production in newborn blood, as compared to FIM protein alone (~16 fold; from 4.92 to 78 pg/ml) or FIM absorbed onto alum (~138 fold; from 4.92 to 678 pg/ml) induced modest production over the RPMI control (Fig. 5A). A similar trend was evident for adult blood (Fig. 5B). Interestingly, in both human newborn and adult whole blood treated with the same concentration of FIM or FIM absorbed onto alum, a modest skewing towards a production of Th17-polarization cytokines (IL-1, IL-6, GM-CSF) and chemoattractive (CCL2, CCL3, CXCL8) chemokines was observed (Fig. 5A, B).

3.8. aP vaccine formulations with enhanced FIM content do not demonstrate increased reactogenicity biomarker profiles

We next determined whether FIM proteins could alter reactogenicity biomarker profiles by supplementing FIM2 and FIM3 proteins into licensed acellular vaccine DTaP-IPV (Pentacel®, Sanofi Pasteur) that contains small amounts of FIM proteins (5 μg). As expected, we found that the wP vaccine (Easyfive®, Panacea) induced significantly more IL-1β than the aP vaccines DTaP-IPV (Pentacel®, Sanofi Pasteur, 5 μg FIM) and DTaP2 (Pentavac®, Sanofi Pasteur, which does not contain any FIM proteins) at 1:1000 and 1:100 dilutions (Supplement Fig. 1). The difference tapered off at 1:10 dilution of the vaccine formulation (Supplement Fig. 1). Overlapping radar plots demonstrate that adding more FIM proteins to DTaP-IPV (Pentacel®, Sanofi Pasteur) did not change the overall cytokine and chemokine profile (Fig. 5C). Next, to gain insight into potential reactogenicity in early life, newborn (Fig. 5D) whole blood was assessed for vaccine-induced production of PGE_2, a molecule whose *in vitro* production has been correlated with reactogenicity *in vivo* [22–25]. Newborn responses were also benchmarked against conventional licensed wP (Easyfive®, Panacea, 1:10 vol/vol). Here we focused on the correlation of both PGE_2 and IL-1β production, as co-production of both in human monocytic assays may predict rabbit pyrogenicity (i.e., fever) *in vivo* [24]. Overall, a common trend was observed. The wP vaccine (Easyfive®, Panacea) induced the highest production of both PGE_2 and IL-1β. Conversely, aP vaccines (Pentacel®) with or without extra FIM induced a lower PGE_2/IL-1β profile than wP vaccine (Easyfive®, Panacea) similar to the low reactogenicity pediatric vaccines PCV13 and HBV (Fig. 5D, and not shown) [22,23,25].

4. Discussion

The mouse model of lower respiratory tract infection has demonstrated value in the evaluation of both wP and aP vaccine formulations and has been used for several decades. In the past, this model, which measures the clearance of bacteria from the lungs of mice, detected the decreased clinical efficacy of a substandard wP vaccine and also demonstrated that increasing the number of components in aP vaccines correlated with increased efficacy [8,26,27]. In our study, we employed the mouse model of lower respiratory tract infection to evaluate current and experimental vaccines, particularly regarding efficacy against PRN-negative strains. Recent reviews of human infections reveal that PRN-negative *B. pertussis* may have a selective advantage in settings where aP vaccines are used routinely [28]. Previous studies have demonstrated that in aP vaccinated mice PRN-negative strains have a selective advantage over a PRN-positive strains in colonization of the upper respiratory tract [29]. Accordingly, we observed that vaccine efficacy was lower when PRN-negative strains, as compared to when PRN-positive strains, were used for intranasal challenge in the mouse model. We also demonstrate that the efficacy of a 3-component aP vaccine (TdaP3) with higher amounts of antigens was higher than a 5-component vaccine (TdaP5). In addition, we used the mouse model of lower respiratory tract infection to investigate whether addition of FIM2/3 in a range of 0.5–5 μg/dose to a 2-component aP vaccine (DTaP2, Pentavac®) increased vaccine efficacy compared to DTaP2. While the current FIM2/3 dose in pediatric pertussis containing vaccines is 5 μg, we

Table 2
Levels of aP2 antigen-specific induced antibodies and their functionality assessment.

	IgG antibody levelsGMT (95% CI)			Agglutination titer	
	PTX	FHA	FIM2/3	FIM2	FIM3
DTaP2	263 (136–509)	466 (286–760)	0.02 (0.003–0.165)	<1:10	<1:10
+0.5 μg FIM	119 (16–790)	551 (120–2534)	180 (76–428)	120	320
+1.0 μg FIM	740 (252–2171)	695 (149–3333)	234 (94–584)	160	960
+1.5 μg FIM	400 (210–762)	440 (195–995)	185 (58–593)	240	640
+2.0 μg FIM	309 (199–478)	406 (236–698)	300 (174–516)	160	960
+2.5 μg FIM	274 (114–661)	597 (156–2283)	222 (53–938)	320	640
+5.0 μg FIM	221 (110–445)	650 (389–1088)	373 (250–556)	640	640

Pertussis antigen specific IgG levels and the FIM agglutination titers determined in serum samples collected from mice at 9 weeks of age, after vaccination at 4 and 7 weeks of age with aP2 Pentavac® at 1/25 human dose and after addition of different amounts of FIM.
IgG antibody levels expressed in IU/ml. FIM agglutination titers determined against a FIM2 and FIM3 expressing strain.
n = 15 for aP2, +2.0 μg FIM, +5.0 μg FIM groups.
n = 5 for +0.5 μg FIM, +1.0 μg FIM, +1.5 μg FIM, +2.5 μg FIM groups.

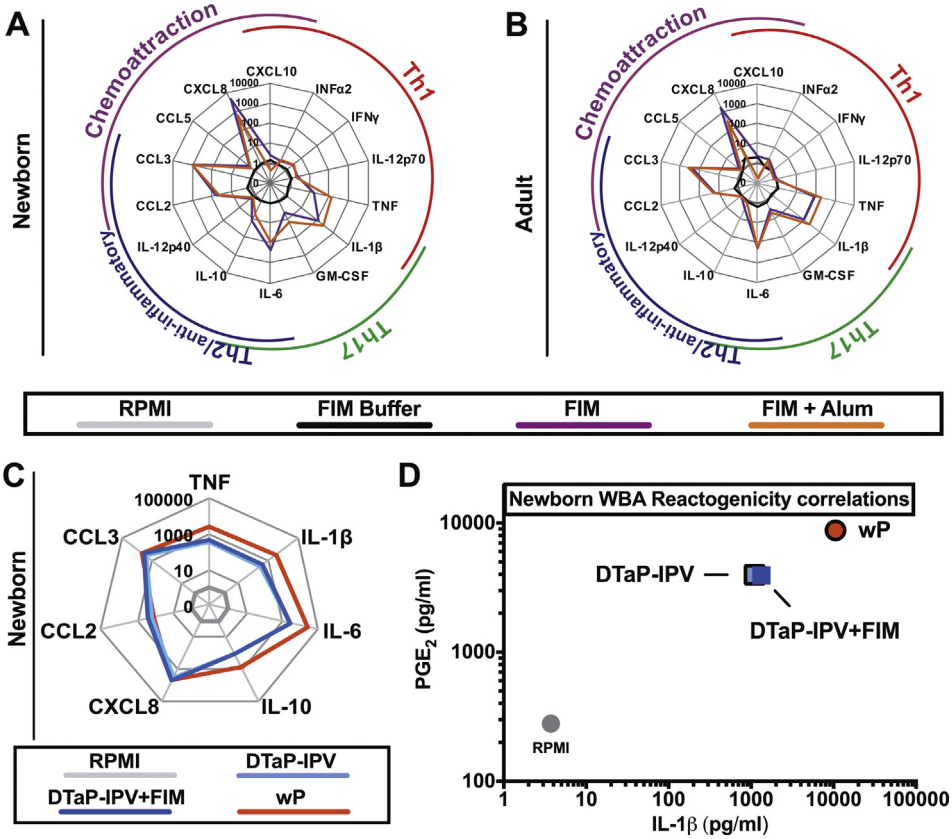

Fig. 5. A FIM-enriched aP vaccine formulation induces lower concentrations of reactogenicity biomarkers than a wP vaccine. (A) newborn and (B) adult whole blood, with data depicted as fold-change over RPMI control, stimulated with vehicle (control) or FIM2/3 proteins (final dilution 1:10). (C) Newborn blood stimulated with indicated formulations of DTaP-IPV (Pentacel®, Sanofi Pasteur), DTaP-IPV + FIM2/3 or wP (Easyfive®, Panacea) (all at 1:100 vol/vol). Mean ± SEM, n = 4. Radar plots show the production of 14 cytokines/chemokines analyzed by multiplex assay. Data depicted as fold-change over RPMI control. (D) Depiction of vaccine-induced biomarkers of potential reactogenicity in the human newborn WBA: PGE2 as a function of IL-1β.

added FIM2/3 in amounts representative of up to 50 μg/pediatric doses, basing this estimate on vaccinating the mice with 1/10 human dose containing 5 μg additional FIM2/3.

Because anti-FIM antibodies persist and show correlation with vaccine efficacy, inclusion of FIM2/3 adds value to aP vaccines [30]. Even though the FIM type expressed on *B. pertussis* strains has shifted over time from FIM2 to FIM3, both types are likely expressed on the bacterium during infection, supporting the rationale to include both FIM2 and FIM3 in current vaccines. Herein we demonstrated that levels of FIM 2/3, representing doses higher than those currently approved in pediatric vaccines, increased vaccine efficacy in the mouse challenge model. This effect was observed using FIM2- and FIM3-expressing *B. pertussis* strains. The increased vaccine efficacy was also observed when using a PRN-negative isolate, which is specifically relevant in the context of current epidemiology, where a significant increase in the presence of PRN-negative strains has been reported [4]. In a household study, Storsaeter et al. showed a significant correlation between anti-PRN and anti-FIM 2/3 antibody levels and clinical protection. As currently more pertussis strains are PRN negative, antibodies to FIM are gaining importance for protection [31].

During the latter 20th century, effective but reactogenic wP vaccines were replaced by aP vaccines formulated with purified bacterial antigens. Adding extra FIM2/3 should not come at the expense of increased reactogenicity of the aP vaccines. Therefore, we characterized the human innate immune response induced by vaccine formulations containing different amount of FIM2 and FIM3 proteins and benchmarking the responses to both licensed wP and aP and demonstrated that an aP formulation containing increased FIM protein content had limited effects with respect to reactogenicity biomarker profile *in vitro*. This is in line with a clinical trial using aP vaccines with different amounts of FIM [33] in which formulations containing up to 10 μg of FIM were immunogenic and well tolerated in humans [32–34].

Adding 5 μg of FIM to DTaP2 at 1/10 human dose influenced the initial bacterial adherence, assessed by measuring CFU/lung 2 h post infection. The CFU found in the lungs of mice treated with at DTaP2 1/10 human dose + FIM 5 μg was approximately 1 $\log_{10}$ lower than the CFU from mice vaccinated with DTaP2 alone. This reduction in adherence was not observed when the vaccine was used at the lower 1/25 human dose. In addition to the high FIM dose, it is likely that the higher amount of FHA at the 1/10 human dose, which also plays a role in adherence, was important in reducing the establishment of colonization [6,14].

Antibody responses, FIM2/3 IgG levels and agglutination, observed in our study were generally robust, but not traditionally titratable. Other Ig classes or subclasses may be differentially produced (e.g., IgA, IgG1, IgG2b, IgG3 etc.), resulting in increased functional activity. In humans, levels of FIM2/3 of 0.8, 5 and 10 μg in vaccines have demonstrated dose-dependent IgG responses and agglutination titers [32]. Antibody effector mechanisms, such as antibody mediated opsonophagocytosis and/or inhibition of colonization, may demonstrate a dose-dependent response to FIM2/3 content, and are topics for follow-up investigations. The increased efficacy could also be the result of an improved IgA or IgG subclasses response, or perhaps enhanced Th1/Th17 cellular response to the infection. Effector Th1 and Th17 cells were implicated in the clearance of *B. pertussis* by recruitment and activation of neu-

trophils and macrophages in mice [35] and baboons [36]. Ross et al., 2013 showed that aP vaccines induced pertussis specific $CD4^+$ T cells that secrete IL-17A and found an essential role for IL17A, but not for IL-4 or IFN-γ, in protective immunity induced by aP in mice [35].

As PRN, PT and FHA are present in most commercial vaccines, data is available on the proliferation and cytokine induction for these antigens and less for FIM antigen. An aP5 (Pentacel®, manufactured by Sanofi Pasteur) vaccine induced T cells with a heterogenous cytokine profile, both IFN-γ and IL-5 production was detected in PBMC upon antigen specific-stimulation [37]. Preferential induction of Th1 cytokines, as evidenced by a significant increase in IFN-γ production in response to the PT and FIM antigens was found and a significant increase in IL-2 production in response to the PT, FHA, and PRN antigens [37]. Therefore, the higher vaccine efficacy observed when mice were vaccinated with increased amounts of FIM2/3 may lead to a more robust Th1/Th17 responses.

In summary, using the mouse intranasal challenge model we have shown that aP vaccine efficacy increases when adding higher amounts of FIM2/3, up to 50 μg per pediatric dose (5 μg per mouse). This finding is of potential importance, considering the current rise in pertussis disease and the urgent need for improved vaccines. Further examination of the immunological response to FIM2/3, testing of duration of immunity and efficacy testing in other animal models, including newborn and infant models as well as the recently developed baboon model of pertussis disease [36,38], are warranted, as high levels of FIM2/3 may improve current aP vaccines. Of note, increasing the amount of FIM proteins in an aP vaccine formulation did not affect the level of potential reactogenicity biomarkers including PGE_2, cytokines and chemokines induced in human newborn and adult whole blood, suggesting a relatively low potential for reactogenicity [22,23,25]. Further efforts to establish safety and efficacy of such FIM-enhanced aP formulations are warranted.

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Conflict of interest statement

AMQ and JF are employees of Janssen Research & Development, Raritan, NJ, United States. KF, JS, GvdD and JP are employees of Janssen Vaccines & Prevention, Leiden, The Netherlands. SW and AF were employees of Janssen Vaccines & Prevention, Leiden, The Netherlands. Janssen was granted a US patent (US 8,916,173) increased FIM in aP vaccine compositions. All other authors report no potential conflicts.

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Author contributions

AMQ, WKC, DJD, GvdD, JP and OL designed the study. DJD, WKC, PF, SJ, SB conducted the *in vitro* experiments. AMQ, JF, produced antigens and conducted the *in vivo* experiments. Statistical analysis was done by JS. AMQ, KF, SW, AF, GvdD, WKC and DJD wrote the manuscript. JP and OL provided overall mentorship and assisted in writing the manuscript. All authors contributed helpful discussions and the careful approval of the final manuscript. All authors have given final approval for the version submitted for publication.

Appendix A. Supplementary material

Increasing the content of FIM2/3 proteins in a pertussis vaccine formulation does not alter human whole blood cytokine/chemokine profile in vitro. Human newborn cord and adult peripheral blood were collected in pyrogen-free heparin, diluted with RPMI 1:1 (vol:vol), then stimulated with DTaP-IPV (Pentacel®) or DTaP-IPV + additional FIM2/3 proteins and incubated at 37 °C, 5% CO2 for 6 hours. Supernatants were collected, and cytokines measured by ELISA. Data are depicted as mean ± SEM. Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2018.11.028.

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