



Addition of a TLR7 agonist to an acellular pertussis vaccine enhances Th1 and Th17 responses and protective immunity in a mouse model



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ABSTRACT

A resurgence of whooping cough (pertussis) has been observed in recent years in a number of developed countries, despite widespread vaccine coverage. Although the exact reasons of the recurrence of pertussis are not clear, there are a number of potential causes, like antigenic variation in the circulating strains of *Bordetella pertussis*, changes in surveillance and diagnostic tools, and potential differences in protection afforded by current acellular pertussis (aP) vaccines compared to more reactogenic whole cell (wP) vaccines, which they replaced. Studies in animal models have shown that induction of cellular as well as humoral immune responses are key to conferring effective and long lasting protection against *B. pertussis*. wP vaccines induce robust Th1/Th17 responses, which are associated with good protection against lung infection. In contrast, aP vaccines induce mixed Th2/Th17 responses. One research option is to modify current aP vaccines with the intention of inducing protective T cell responses, without compromising on their low reactogenicity profile. Here we found that formulation of an aP vaccine with a novel adjuvant based on a Toll-like receptor 7 agonist (TLR7a) adsorbed to aluminum hydroxide (alum) enhanced *B. pertussis*-specific Th1 and Th17 responses and serum IgG2a/b antibodies, which had greater functional capacity than those induced by aP formulated with alum alone. Furthermore, addition of a TLR7a enhanced the protective efficacy of the aP vaccine against *B. pertussis* aerosol challenge; protection was comparable to that of a wP vaccine. These findings suggest that alum-TLR7a is a promising adjuvant for clinical development of next generation pertussis vaccines.

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1. Introduction

The recent resurgence of pertussis is reopening many questions on a disease against which effective vaccines are available and routinely used. Although the basis of this is still not fully clear, resurgence of pertussis is prominent in countries using acellular pertussis (aP) vaccines. It is becoming more evident that aP vaccines do not induce long lasting protection [1] and studies in animal models have suggested that aP vaccination fails to prevent nasal colonization and transmission of *B. pertussis* [2]. Studies in a baboon challenge model showed that immunization with an alum-formulated aP vaccine protected animals from disease, but did not prevent nasal colonization or bacterial transmission to naïve animals; nasal colonization in these animals lasted at least as long as that in unvaccinated controls [2]. In contrast, baboons

immunized with wP vaccine cleared the bacteria faster, whereas previously infected animals were completely protected, with no nasal colonization detected after re-challenge. This suggests that while aP vaccines may protect individuals from disease, there may be little to no contribution to herd immunity. These recent learnings together with changes in the antigenicity of circulating *B. pertussis* strains, guide potential strategies for the development of new and improved pertussis vaccines.

A mouse respiratory challenge model [3] has proved very useful for studies on the mechanisms of protective immunity to *B. pertussis*. Studies using this model have demonstrated that *B. pertussis*-specific T cells, as well as antibodies, mediate natural and vaccine-induced protective immunity [4]. Experiments in knockout mice showed that IFN- γ and IL-17, secreted by Th1 and Th17 cells respectively, contribute to protection, and vaccines that induce both of these T cell subtypes are most effective [5]. While natural infection and wP vaccination induce a mixed Th1/Th17 response in mice [5,6], a more Th2-polarized response is induced by immunization with conventional aP vaccines [5]. Importantly,

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the immune responses induced by infection or vaccination in baboons are similar to those in mice, further validating the mouse model; infection induces long-lived Th1/Th17 responses, wP vaccination induces more Th17-dominated responses, whereas aP vaccines induce Th2-polarised responses [2,7].

All current aP vaccines utilize chemically detoxified pertussis toxin (PT), one of the major virulence factors of *B. pertussis*, and subject to immune-driven antigenic variation [8]. However, chemical treatment lowers to some extent immunogenicity of PT. In contrast, genetically detoxified PT mutants – including PT-9K/129G, that lacks enzymatic activity – retain the functional and immunological properties of wild-type PT [9]. PT-9K/129G was shown to be safe and immunogenic as a component of a DTaP vaccine evaluated in two large Phase III efficacy trial in infants [10,11]. Furthermore, PT-9K/129G induced higher anti-PT antibody titres and longer lasting protection when compared with a vaccine containing chemically detoxified PT. PT-9K/129G may therefore have advantages over chemically detoxified PT for inclusion in new generation vaccines.

Besides modification or addition of vaccine antigens, current aP vaccines might also be improved by the inclusion of new generation adjuvants. Toll like receptors (TLRs) detect the presence of conserved molecules expressed by different classes of pathogens and their ligation results in activation of the innate immune system. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and recognize pathogen-derived molecules, such as components of bacterial and fungal cell walls. TLR7, TLR8 and TLR9 are present in intracellular vesicles and recognize microbial DNA and RNA species. The type of TLR-signaling that is triggered determines the nature and magnitude of the innate immune response as well as the ensuing adaptive response that are induced. Ligation of intracellular TLRs is especially effective at promoting IL-12 production from innate immune cells, which promotes development of Th1 responses that are crucial for protection against viruses, tumors and intracellular bacteria. Consequently, TLR ligands have considerable potential as adjuvants for infectious disease vaccines and the TLR4 ligand MPL has already been licensed for use in humans [12].

A number of studies have explored the use of TLR7 agonists (TLR7a) as vaccine adjuvants and have employed different strategies, including conjugating TLR7a to pathogen antigens or adsorption to alum, to optimize their immune stimulating effects, while minimizing toxicity [13–15]. Adsorption of the TLR7a to alum reduces its systemic circulation and excessive cytokine release into serum. Therefore, alum adsorption of TLR7a offers an advantage of creating a universal, low reactogenic Th1-promoting adjuvant and could represent the ideal solution for the design of an improved aP vaccine. Here we show that adjuvantation of an aP vaccine with alum-TLR7a – a novel adjuvant made of alum adsorbed SMIP7.10, a small molecule selected from a benzonaphthyridines series of TLR7a – significantly enhanced its immunogenicity, promoting Th1 and Th17 responses, IgG2a/b antibodies and protection against *B. pertussis* infection in a mouse model.

2. Materials and methods

2.1. Antigens and adjuvants

Aluminum hydroxide (alum), Tetanus toxoid (TT) and diphtheria toxoid (DT) from GSK Vaccines (Marburg, Germany); the clinical-grade antigens genetically detoxified pertussis toxin (PT-9K/129G), filamentous haemagglutinin (FHA) and pertactin (PRN) produced by Lonza Group (Basel, Switzerland), and wP vaccine (88/522; non WHO Reference Material) from NIBSC were used.

Alum-TLR7a is a proprietary GSK adjuvant in development, made by adsorption of the TLR7 agonist SMIP7.10 to alum [16].

TdaP vaccine formulations were prepared by adsorbing antigens (at the respective concentrations given in figure legends) to alum (2 mg/ml, corresponding to 0.69 mg/ml Al³⁺) alone or together with the TLR7a at 4 °C overnight under mild agitation.

Antigen identity, integrity and alum-adsorption was evaluated by Western blot analysis and endotoxin content of all formulations respected the 5–20 EU/ml recommended range.

2.2. Mouse immunization

BALB/c mice (female, 6-weeks-old) (Charles River Laboratories International Inc., Wilmington, MA) received intramuscular (i.m.) immunizations twice, 4 weeks apart, with 100 µl formulations (50 µL/leg). Sera were collected 2 weeks after each immunization. Alternatively, C57BL/6 mice (female, 6–8-weeks-old) were vaccinated once or twice (4 weeks apart) intraperitoneally (i.p.) with 100 µl vaccine formulations and challenged 2 weeks later as described below. 4 mice/group were sacrificed on day of challenge to measure antigen-specific T-cell responses in spleen cells and antibody titres in serum. All animal studies were performed in compliance with local and European laws, approved by the respective institute's Animal Welfare Body, and authorized by the Italian Ministry of Health or Ireland's Health Products Regulatory Authority.

2.3. Serological assays

B. pertussis-specific IgG titres in individual mouse sera were determined by bead-based (Luminex immunoassay) or plate-based ELISA after each immunization, while subclasses and functional titres were measured after the second immunization.

2.3.1. Luminex immunoassay

Total IgG titres against all vaccine antigens were analysed by Luminex penta-plex immunoassay as described elsewhere [16]. Titres are expressed as Relative Luminex Units per ml (RLU/ml), resulting from conversion of the registered median fluorescence intensities (MFI) through hyper-immune reference antisera. IgG subclasses are reported as MFI.

2.3.2. Antibody titres by ELISA

FHA-specific antibodies were quantified by ELISA using plate-bound FHA (1 µg/ml), biotin-conjugated anti-mouse IgG1 or IgG2a and peroxidase-conjugated streptavidin (BD Pharmingen, Franklin Lakes, NJ). Antibody levels are expressed as the mean end point titre ± SEM, determined by extrapolation of the linear part of the titration curve to 2 SE above the background value obtained with nonimmune serum.

2.3.3. PT neutralization assay

CHO-K1 cell line (Chinese hamster ovary cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD). PT neutralization assay was optimized as described elsewhere [16].

Two-fold serially diluted sera were mixed with 4 CTU₁₀₀ of PT and incubated for 90 min at 37 °C. 50 µl of serum/PT mixtures was added to 50 µl CHO-K1 cells (4 × 10⁵ cells/ml) and incubated for 16 h at 37 °C, followed by evaluation of morphological alterations (clustered phenotype) by light microscopy. Endpoint titres are the reciprocal of the highest dilution able to inhibit cell clustering. Naïve sera were used as negative controls.

2.3.4. FHA binding inhibition assay

A549 cells (Human lung epithelium) were obtained from ATCC and FHA binding inhibition assay was performed as described elsewhere [16].

Cells were seeded on 96-well plates (2.5×10^4 /well) and cultured for one day. Total IgG at 1 mg/ml were two-fold serially diluted in F12-K medium and pre-incubated with 2.5 μ g/ml AlexaFluor488-labeled FHA for 1 h at 37 °C, then added to cells and incubated 30 min further. After extensive washing to remove unbound protein, the cell-associated fluorescence was measured at excitation/emission 485/535 nm by Tecan Infinite F200PRO microplate reader.

2.4. *B. pertussis* respiratory challenge

C57BL/6 mice were aerosol challenged with a virulent strain of *B. pertussis* (Bp338) as described before [17]. Bacterial burden at different time points post infection was evaluated by performing CFU counts on serially diluted lung homogenates from individual mice.

2.5. Antigen-specific T cell responses

To measure the T-cell cytokine production, spleen cells (2×10^6 /mL) from immunized mice were cultured with purified FHA, PRN, hiPT (heat inactivated at 90 °C for 30 min) or with

medium alone as control at 37 °C and 5% CO₂. Supernatants were removed after 72 h and IL-5, IL-17 and IFN- γ concentrations determined by ELISA.

2.6. Statistical analysis

Graphpad Prism 7 software was used to perform the statistical analysis of the data. One- or two-way ANOVA was used to test for statistical significance of differences between more than two experimental groups. Area under the curve, which is an inverse measure of vaccine potency, was calculated for CFU curves using GraphPad Prism 7 software.

3. Results

3.1. Alum-TLR7a promotes higher levels of functional antibodies against *B. pertussis* antigens

To evaluate the adjuvant effect of alum-TLR7a on an aP vaccine formulation, mice were vaccinated twice with aP+alum, aP+alum-TLR7a (10 μ g) and aP+alum-TLR7a (50 μ g) or with PBS as control. Antibody titres were assessed following each immunization, while antibody functionality was assessed after the booster immunization. Analysis of antibody responses revealed that formulation of the aP vaccine with alum-TLR7a resulted in significantly increased

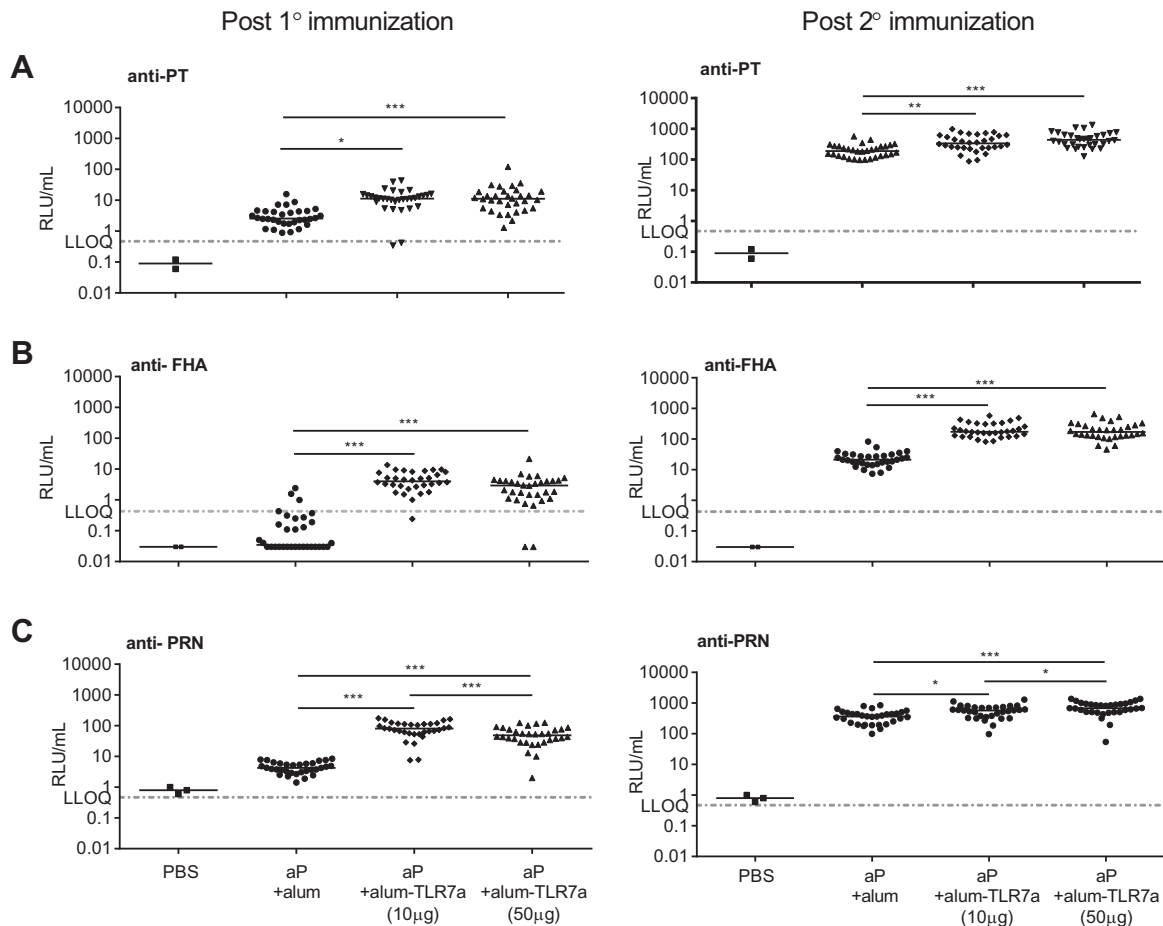


Fig. 1. Alum-TLR7a adjuvant enhances *B. pertussis*-specific IgG production induced by aP vaccination. BALB/c mice (32/group) were immunized i.m. at 0 and 4 weeks with 100 μ l (50 μ L /leg) of Tdap formulations containing: 4 IU TT, 0.4 IU DT, 0.8 μ g PT-9K/129G, 0.8 μ g FHA, 1.6 μ g PRN, adsorbed to alum and where indicated TLR7a (10 μ g or 50 μ g), corresponding to 1/5th human dose. Two weeks after each immunization sera were collected and total IgG specific for PT, FHA and PRN measured by Luminex assay. Graphs show (A) anti-PT (B) anti-FHA and (C) anti-PRN titres. LLOQ – lower limit of quantification. Graphs show values for individual mice and the median value (bar). n = 32 mice, *** p < 0.001, ** p < 0.01, * p < 0.05 by One-way ANOVA and Bonferroni's multiple comparisons test.

IgG responses against all three *B. pertussis* antigens, PT, FHA and PRN (Fig. 1A–C). After a single immunization, mice immunized with aP+alum-TLR7a had significantly higher IgG titres compared with mice immunized with aP+alum. Both concentrations of TLR7a in the adjuvant lead to similar enhancement of IgG titres. Serum IgG titres were enhanced following booster vaccination with all vaccines tested. However, TLR7a-containing vaccines were superior to the aP vaccine with alum and also promoted significant IgG2a and IgG2b titres against all antigens and slightly greater IgG1 responses than the aP+alum vaccine (Fig. 2A).

Having shown that alum-TLR7a enhances IgG against all aP vaccine antigens as well IgG2a/b class switching, we investigated whether the antibodies also had enhanced functional properties, including ability to neutralize PT and to block adhesion of FHA to epithelial cells. A commercial vaccine based on chemically inactivated pertussis toxoid was used as control. Genetically inactivated PT-9K/129G induced significantly higher PT-neutralizing titres than chemically detoxified PT, even though PT-9K/129G was used at a lower dosage. The alum-TLR7a adjuvant further enhanced this neutralizing activity when compared with the antibodies induced with aP formulated with alum-only (Fig. 2B). Furthermore, IgG from mice immunized with aP+alum-TLR7a had a significantly greater ability to inhibit FHA binding to the human lung A549 cell line (Fig. 2C).

3.2. Alum-TLR7a adjuvant promotes induction of Th1 and Th17 responses and enhances protective immunity in mouse aerosol challenge model

Having demonstrated that alum-TLR7a adjuvant enhanced antibody responses, especially IgG2a/b against aP antigens in BALB/c mice, we examined immunogenicity in a second mouse strain, and also evaluated T cell responses and protective efficacy of the experimental aP vaccine in the *B. pertussis* mouse aerosol challenge model. C57BL/6 mice were immunized twice (0 and 4 weeks) with 1/5 human dose of aP vaccine formulated with alum or alum-

TLR7a. Control mice were immunized with a wP vaccine or PBS. Each vaccine preparation induced potent anti-FHA IgG in serum (Fig. 3A). The responses were weakest in mice immunized with aP+alum. Likewise, all vaccines induced FHA-specific IgG1, with rather modest responses detected in the wP-immunized mice. In contrast, anti-FHA IgG2a was very strong in mice immunized with wP or aP+TLR7a-alum, but was undetectable in mice immunized with aP+alum.

Assessment of antigen-specific T cell responses in the spleen revealed that mice immunized with wP or aP+alum-TLR7a had robust Th1 and weak Th2-type responses; significant IFN- γ production was detectable in supernatants of spleen cells re-stimulated *in vitro* with heat-inactivated PT, FHA or PRN (Fig. 3B). In contrast, high concentrations of IL-5 but low IFN- γ were detected in *B. pertussis* antigen re-stimulated spleen cells from aP+alum-immunized mice. *B. pertussis*-specific IL-17 was more variable, but induced to similar extent by all formulations. Taken together the alum-adjuvanted aP vaccine induced a Th2/Th17 response, while the other formulations induced a Th1/Th17 profile.

Mice were challenged by exposure to an aerosol of *B. pertussis* 2 weeks after the second immunization. Control mice immunized with PBS developed a persistent infection, characteristic of that previously observed in naive mice [5]. Mice immunized with wP or aP+alum-TLR7a had cleared the infection by day 3 post challenge (Fig. 3C). In contrast, aP+alum-immunized mice still had detectable though low numbers of bacteria in the lungs on days 3 and 14 post challenge.

Given that vaccination of mice with the 1/5 human dose resulted in rapid clearance of bacteria from the lungs, a lower dose (1/40 human dose) of the vaccines was employed in an attempt to further discriminate between the different aP-adjuvant combinations. Immunization of mice with aP+alum-TLR7a formulation was found to confer the highest level of protection against respiratory challenge with *B. pertussis* (Fig. 4A). The protection observed was equivalent to that induced with a wP vaccine and surpassed

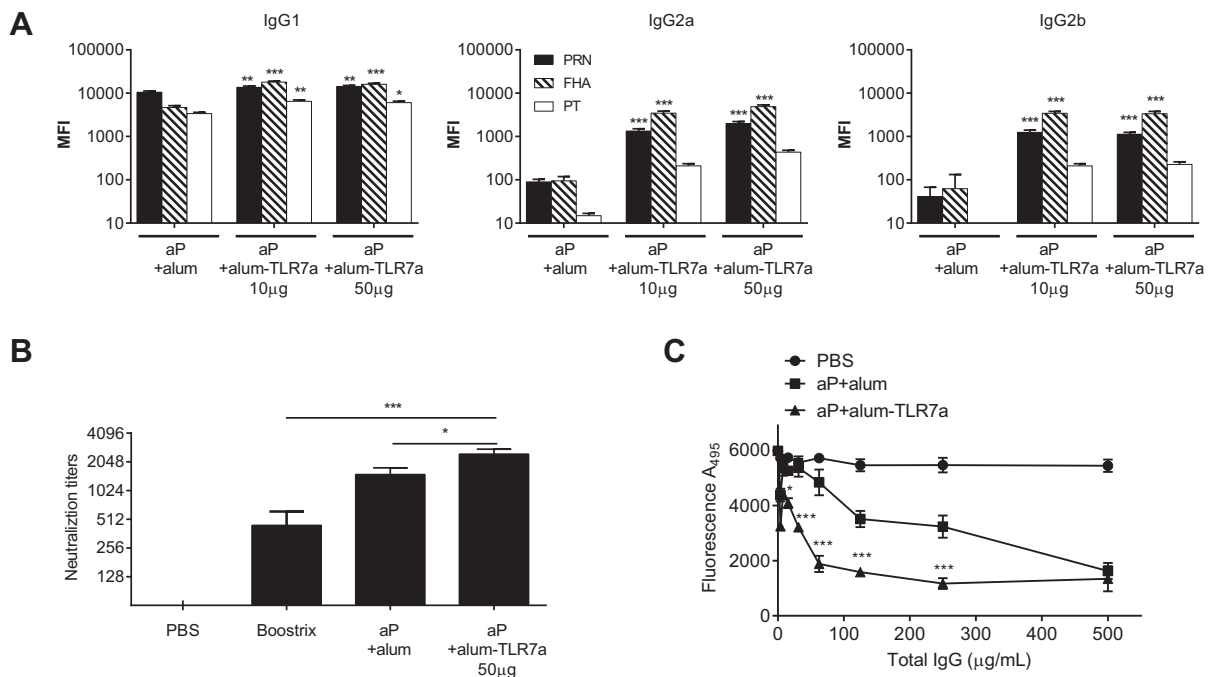


Fig. 2. Alum-TLR7a adjuvant promotes IgG2a and IgG2b class switched antibodies with a greater functional capacity. BALB/c mice were immunized as specified for Fig. 1. Commercial Tdap vaccine (Boostrix, GSK) was used as a control, 100 µl containing: 4 IU TT, 0.4 IU DT, 1.6 µg PT(chem), 1.6 µg FHA, 0.5 µg PRN. Sera were collected two weeks after the booster immunization and antibody isotypes and their functional capacity analyzed. (A) IgG1, IgG2a and IgG2b specific for PTg, FHA and PRN titres were analysed by Luminex assay (B), PT neutralizing titre. (C) Inhibition of FHA binding by total IgG. Results are mean ± SEM, ***p < 0.001, **p < 0.01 *p < 0.05 by two way ANOVA with Tukey's post test (A and C) or by one way ANOVA with Tukey's post test (B). Statistical differences are relative to aP+alum vaccinated mice unless otherwise indicated.

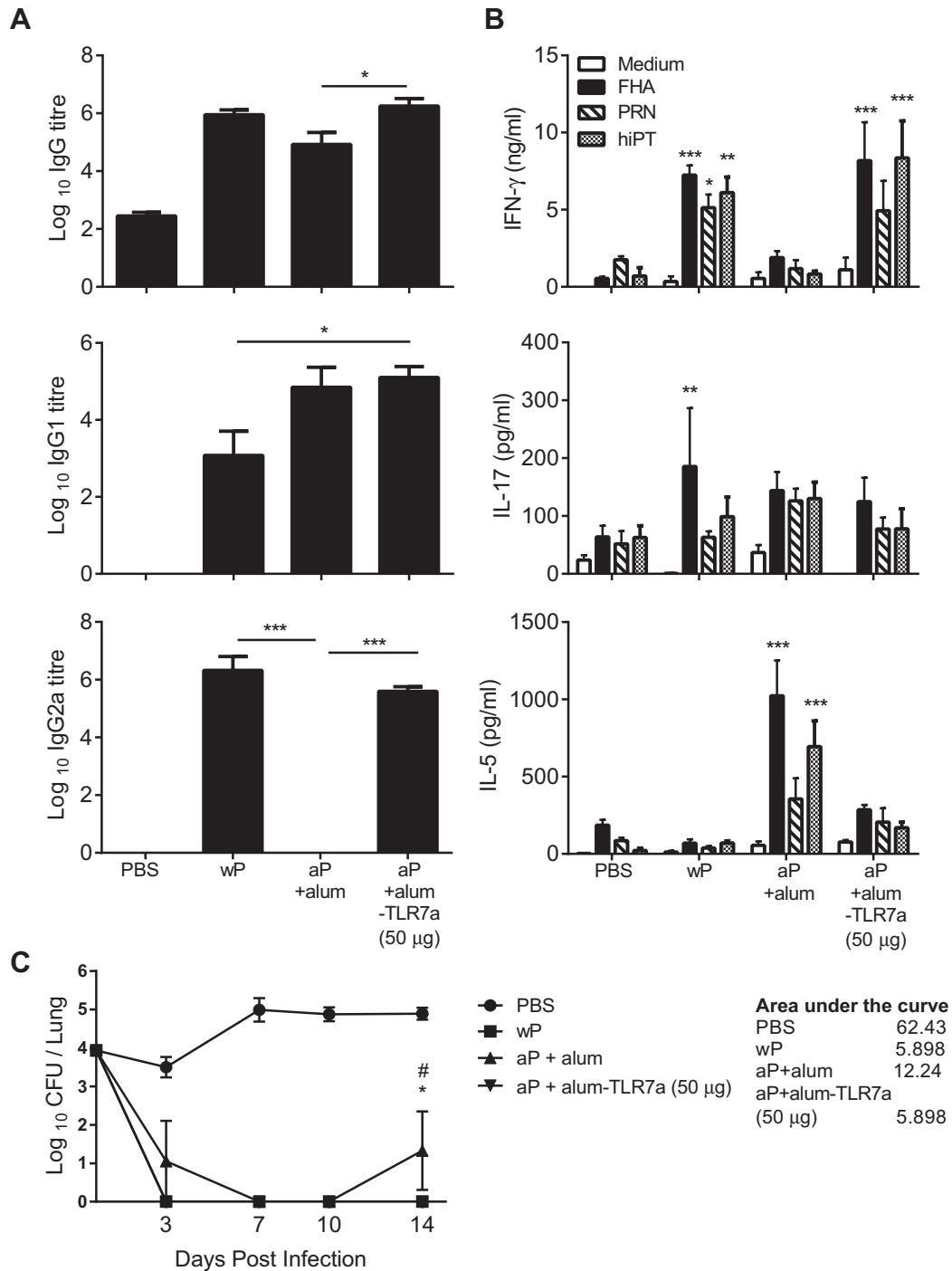


Fig. 3. TLR7a promotes antigen-specific Th1 responses in mice either alone or in combination with alum. C57BL/6 mice were immunized i.p. at 0 and 4 weeks with formulations as specified for Fig. 1 or with 1/5 human dose wP vaccine and challenged 2 weeks later. (A) FHA-specific antibodies present in the sera of immunized mice on the day of challenge were analysed by ELISA. ***p < 0.001, *p < 0.05 by One way ANOVA with Bonferroni's post test (B) Spleen cells (2 × 10⁶/mL) from vaccinated uninfected mice were cultured in the presence of FHA (0.5 µg/mL), PRN (1 µg/mL) and hiPT (1 µg/mL). After 72 h the concentration of IL-17, IFN-γ and IL-5 in the supernatants was analysed by ELISA. *p < 0.05, **p < 0.01, ***p < 0.001 compared to unstimulated cells by Two way ANOVA with Bonferroni's post test. (C) Mice were infected with *B. pertussis* (Bp338) and the bacterial burden in the lungs assessed by performing CFU counts on lung homogenates at the time points indicated. *p < 0.05 aP vs wP, # p < 0.5 aP vs aP+alum+TLR7a vaccinated mice by Two way ANOVA with Tukey's post test. Results are mean ± SEM (n = 4 mice).

that generated by aP+alum. The bacterial counts in the lungs of mice immunized with aP+alum-TLR7a were significantly lower 3 and 10 days after challenge when compared with aP+alum-immunized mice. Based on the areas under the bacterial clearance curve, aP+alum-TLR7a was more potent than aP+alum or wP.

FHA-specific IgG1 antibodies were induced in response to all 3 vaccines, and the highest titres were detected in mice immunized with aP vaccines. Anti-FHA IgG2a antibodies were induced in mice immunized with wP or aP+alum-TLR7a, but not with aP-alum (Fig.4B).

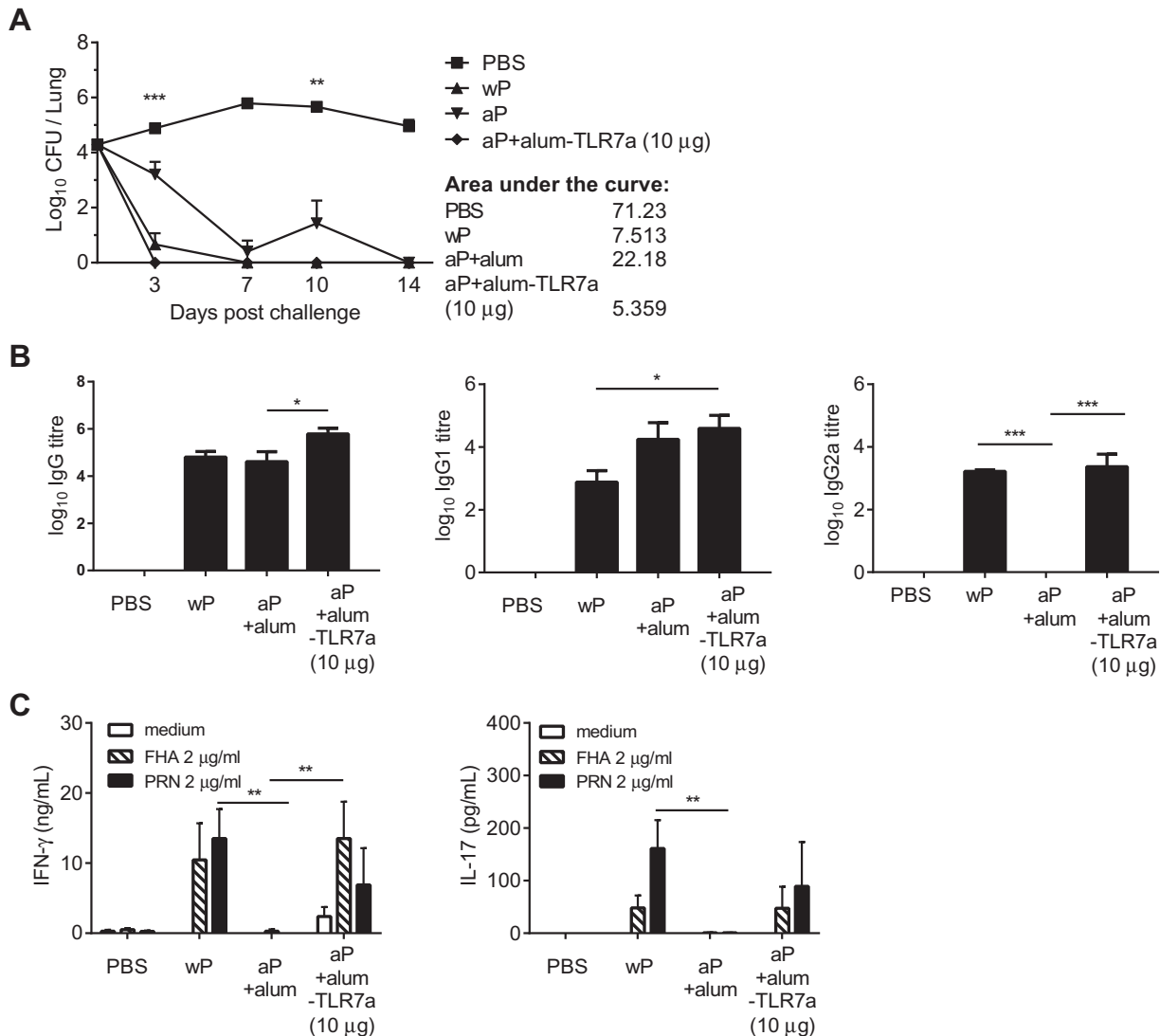


Fig. 4. TLR7a adjuvanted aP vaccine confers similar level of protection as a wP vaccine. C57BL/6 mice were immunized i.p. at 0 and 4 weeks with 100 µl of TdaP formulations containing: 0.13 IU TT, 0.06 IU DT, 0.2 µg PT-9K/129G, 0.2 µg FHA, 0.06 µg PRN, adsorbed to Alum or alum-TLR7a (10 µg or 50 µg), corresponding to 1/40th human dose, or with 1/40 human dose wP vaccine and challenged 2 weeks later. (A) Mice were infected with *B. pertussis* (Bp338) and the bacterial burden in the lungs assessed by performing CFU counts on lung homogenates at the time points indicated. *** $p < 0.001$ ** $p < 0.01$ aP+alum vs aP+alum-TLR7a, by Two way ANOVA with Bonferroni's post test (B). (B) FHA-specific antibodies present in the sera of immunized mice on the day of challenge were analysed by ELISA. *** $p < 0.001$ * $p < 0.05$ by One way ANOVA with Tukey's post test, (C) Spleen cells (2×10^6 /mL) from vaccinated uninfected mice were cultured in the presence of FHA (2 µg/mL) and PRN (2 µg/mL). After 72 h the concentration of IL-17 and IFN-γ in the supernatants was analysed by ELISA. ** $p < 0.01$ by Two way ANOVA with Bonferroni's post test. Results are mean \pm SEM ($n = 4$ mice).

T cell responses were weaker following immunization with the low dose of the vaccines. However, similar to what was observed with the higher dosed vaccines, immunization with aP+alum-TLR7a or wP, but not aP+alum promoted FHA- and PRN-specific IFN-γ or IL-17 production (Fig. 4C). Antigen-specific IL-5 production was weak (aP+alum) or undetectable (aP+alum-TLR7a or wP; data not shown).

Given the clearly superior antibody titres induced by aP+alum-TLR7a after a primary immunization (Fig. 1), the protective efficacy of the experimental vaccine was evaluated by challenging mice 3 weeks following a single immunization. Again, aP+alum-TLR7a conferred the highest level of protection, which was significantly greater than that induced by aP+alum (Fig. 5). In addition, no protection was observed in mice vaccinated with the adjuvant alone (TLR7a). Taken together the data demonstrate that addition of a TLR7a to an aP vaccine enhances its capacity to induce antigen-specific Th1 and Th17 cells, IgG2a antibody responses and protection against respiratory challenge with *B. pertussis*.

4. Discussion

The results of this study demonstrate that addition of a TLR7a to an alum-adjuvanted aP vaccine converts it from a Th2-inducing vaccine to a more Th1/Th17-inducing vaccine with higher protective capacity, equivalent to or greater than that of a wP vaccine. Most wP vaccines have high efficacy in children but have been linked to side effects and were replaced in developed countries with aP vaccines formulated with alum. Although significantly less reactogenic than wP vaccines, there is evidence to suggest that the aP vaccines might not induce as long lasting protection against *B. pertussis* – despite demonstrating similar efficacy at earlier time-points. Furthermore, recent findings from a baboon model suggest that aP vaccines prevent disease, but do not prevent nasal colonization or transmission of *B. pertussis* [2].

Studies in mice have shown that wP vaccines protect through induction of a mixed Th1/Th17 response, while the aP vaccine promotes Th2/Th17 polarized responses [5,7], with Th2 responses

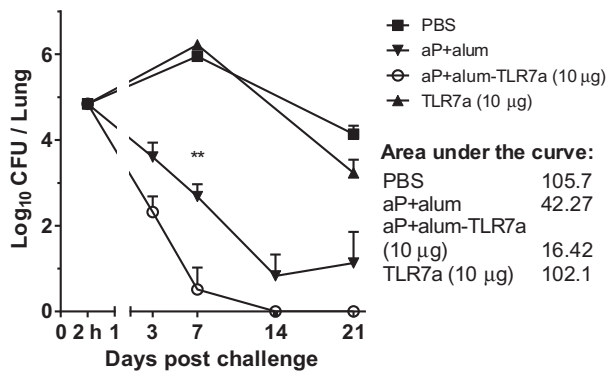


Fig. 5. A single immunization with aP+alum-TLR7a confers greater protection against *B. pertussis* aerosol challenge than immunization with an alum adjuvanted aP vaccine. C57BL/6 mice were immunized i.p. only once at 3 weeks prior to challenge with 100 µl of Tdap formulations as specified in Fig. 1. The mice were then infected with *B. pertussis* (Bp338) and the bacterial burden in the lungs assessed by performing CFU counts on lung homogenates at the time points indicated. ** $p < 0.01$ aP vs aP+Alum-TLR7a vaccinated mice by Two way ANOVA with Bonferroni's post test. Results are mean \pm SEM ($n = 4$ mice).

being dispensable for protection. Developed countries are not likely to revert to wP vaccines due to their higher reactogenicity. Therefore, one approach to solving the pertussis resurgence is to modify the aP vaccine by addition of a Th1/Th17-inducing adjuvant and eventually by including novel pertussis antigens. In this study we have focused on a modified aP vaccine containing a genetically detoxified PT mutant and a novel TLR agonist-containing adjuvant. TLR agonists are known to induce the production of innate IL-12 that polarizes naïve T cells to develop into Th1 cells and IL-1, IL-6 and IL-23 that polarize the development and expansion of Th17 cells [18]. We found that immunization of mice with an aP vaccine formulated with alum-TLR7a promoted the induction of Th1 and Th17 cells, similar to that induced by a wP vaccine. Furthermore, alum-TLR7a-adjuvanted aP vaccine selectively promoted antigen-specific IgG2a and IgG2b subclasses, which is consistent with a more pronounced Th1 type response. When compared with IgG from mice immunized with aP+alum, IgG from aP+alum-TLR7a-immunized mice had greater functional capacity, including significantly higher PT-neutralizing titres and increased inhibition of FHA binding to lung epithelial cells. It is likely that enhanced IFN- γ production in aP+alum-TLR7a-vaccinated mice contributed to enhanced opsonizing and complement-fixing antibodies, as IFN- γ is known to promote antibody affinity maturation [19] and isotype switching to IgG2a/b. The enhanced clearance of bacteria following *B. pertussis* challenge of mice immunized with aP+alum-TLR7a is likely to be mediated by a combination of different immune responses, including cytokine secretion by Th1 and Th17 cells, leading to enhanced recruitment and activation of phagocytic cells, and higher titres of IgG2a/b antibodies titres, that are known to promote opsonization and complement-mediated killing of bacterial cells. Similar to our findings, addition of a TLR7 agonist to an influenza vaccine formulation promoted protective IFN- γ and IgG2a responses in mice [20]. Importantly, TLR7-adjuvanted influenza vaccine produced potent immune responses with low reactogenicity. A TLR7 agonist has also been shown to promote protective Th1 and inhibit detrimental Th2 responses when formulated in a whole cell *Leishmania major* vaccine [21]. Similarly, an alum-TLR7a adjuvant was shown to enhance immune responses to glycoconjugate vaccines with significant increase in the frequency of antigen-specific Th1 and decrease in Th2 cells [22]. Adsorption of TLR7a to alum or its conjugation to the vaccine antigen will likely improve tolerability by reducing dissipation of the small molecule TLRa from the site of injection. Furthermore,

much lower concentrations of the TLR7a would be needed to have local immune activating effects at the site of injection.

In the current study the aP+alum-TLR7a vaccine was found to be superior to the standard alum-adjuvanted vaccine at promoting protective Th1-polarized response even at a highly reduced antigen dose. Furthermore, significantly enhanced protection against *B. pertussis* challenge was observed following a single immunization with aP+alum-TLR7a. When taken together with previous studies, our data suggests that alum-TLR7a is a promising adjuvant for vaccines requiring cellular immunity, where Th1 and Th17 responses are required for optimal protection.

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Sponsorship and conflict of interest

This study was sponsored by Novartis Vaccines, now acquired by the GSK group of companies. GSK has pending patent applications relating to TLR adjuvanted Tdap vaccines (WO2013/132041) where inventors include BCB and DTO.

RL, BG, BCB, UDO, DTO, MP and AS were employees of Novartis Vaccines and Diagnostics Srl at the time of the study. Following acquisition of Novartis Vaccines by the GSK group of the companies in March 2015, RL, BG, BCB, UDO, DTO, MP and AS are now employees of the GSK group of the companies.

Author contribution

BCB, UDO, DTO, MP, AS and KHGM were involved in the conception and design of the study. AM, ACA, BG and RL acquired and analyzed the data. All authors were involved in drafting the manuscript or revising it critically for important intellectual content. All authors had full access to the data and approved the manuscript before it was submitted by the corresponding author.

References

- [1] Klein NP, Bartlett J, Rowhani-Rahbar A, Fireman B, Baxter R. Waning protection after fifth dose of acellular pertussis vaccine in children. *N Engl J Med* 2012;367:1012–9.
- [2] Warfel JM, Zimmerman LI, Merkel TJ. Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proc Natl Acad Sci U S A* 2014;111:787–92.
- [3] Mills KH, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. *Infect Immun* 1998;66:594–602.
- [4] Mahon BP, Brady MT, Mills KH. Protection against *Bordetella pertussis* in mice in the absence of detectable circulating antibody: implications for long-term immunity in children. *J Infect Dis* 2000;181:2087–91.
- [5] Ross PJ, Sutton CE, Higgins S, Allen AC, Walsh K, Misiak A, Lavelle EC, McLoughlin RM, Mills KH. Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. *PLoS Pathog* 2013;9:e1003264.
- [6] Higgs R, Higgins SC, Ross PJ, Mills KH. Immunity to the respiratory pathogen *Bordetella pertussis*. *Mucosal Immunol* 2012;5:485–500.
- [7] Warfel JM, Merkel TJ. *Bordetella pertussis* infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. *Mucosal Immunol* 2013;6:787–96.
- [8] Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, Willems RJ. Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infect Immun* 1998;66:670–5.
- [9] Pizzi M, Covacci A, Bartoloni A, Perugini M, Nencioni L, De Magistris MT, Villa L, Nucci D, Manetti R, Bugnoli M, et al. Mutants of pertussis toxin suitable for vaccine development. *Science* 1989;246:497–500.
- [10] Greco D, Salmasso S, Mastrantonio P, Giuliano M, Tozzi AE, Anemona A, Ciofi degli Atti ML, Giammanco A, Panei P, Blackwelder WC, Klein DL, Wassilak SG. A

- controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. *N Engl J Med* 1996;334:341–8.
- [11] Salmaso S, Mastrantonio P, Wassilak SG, Giuliano M, Anemona A, Giammanco A, Tozzi AE, Ciofi degli Atti ML, Greco D. Persistence of protection through 33 months of age provided by immunization in infancy with two three-component acellular pertussis vaccines stage II Working Group. *Vaccine* 1998;16:1270–5.
- [12] Garçon N, Van Mechelen M. Recent clinical experience with vaccines using MPL- and QS-21-containing adjuvant systems. *Expert Rev Vaccines* 2011;10:471–86.
- [13] Donadei A, Balocchi C, Mancini F, Proietti D, Gallorini S, O'Hagan DT, D'Oro U, Berti F, Baudner BC, Adamo R. The adjuvant effect of TLR7 agonist conjugated to a meningococcal serogroup C glycoconjugate vaccine. *Eur J Pharm Biopharm* 2016;107:110–9.
- [14] Liu R, Wang J, Yang Y, Khan I, Zhu N. Rabies virus lipopeptide conjugated to a TLR7 agonist improves the magnitude and quality of the Th1-biased humoral immune response in mice. *Virology* 2016;497:102–10.
- [15] Wu TY, Singh M, Miller AT, De Gregorio E, Doro F, D'Oro U, Skibinski DA, Mbow ML, Bufali S, Herman AE, Cortez A, Li Y, Nayak BP, Tritto E, Filippi CM, Otten GR, Brito LA, Monaci E, Li C, Aprea S, Valentini S, Calabromicron S, Laera D, Brunelli B, Caproni E, Malyala P, Panchal RG, Warren TK, Bavari S, O'Hagan DT, Cooke MP, Valiante NM. Rational design of small molecules as vaccine adjuvants. *Sci Transl Med* 2014;6:263ra160.
- [16] Agnolon V, Bruno C, Leuzzi R, Galletti B, D'Oro U, Pizza M, Seubert A, O'Hagan DT, Baudner BC. The potential of adjuvants to improve immune responses against Tdap vaccines: a preclinical evaluation of MF59 and monophosphoryl lipid A. *Int J Pharm* 2015;492:169–76.
- [17] Misiak A, Wilk MM, Raverdeau M, Mills KH. IL-17-Producing innate and pathogen-specific tissue resident memory $\gamma\delta$ T cells expand in the lungs of *Bordetella pertussis*-infected mice. *J Immunol* 2017;198:363–74.
- [18] Walsh KP, Mills KH. Dendritic cells and other innate determinants of T helper cell polarisation. *Trends Immunol* 2013;34:521–30.
- [19] Holland GP, Holland N, Steward MW. Interferon-gamma potentiates antibody affinity in mice with a genetically controlled defect in affinity maturation. *Clin Exp Immunol* 1990;82:221–6.
- [20] Goff PH, Hayashi T, Martínez-Gil L, Corr M, Crain B, Yao S, Cottam HB, Chan M, Ramos I, Eggink D, Heshmati M, Krammer F, Messer K, Pu M, Fernandez-Sesma A, Palese P, Carson DA. Synthetic toll-like receptor 4 (TLR4) and TLR7 ligands as influenza virus vaccine adjuvants induce rapid, sustained, and broadly protective responses. *J Virol* 2015;89:3221–35.
- [21] Zhang WW, Matlashewski G. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against *Leishmania major* in BALB/c mice. *Infect Immun* 2008;76:3777–83.
- [22] Buonsanti C, Balocchi C, Harfouche C, Corrente F, Galli Stampino L, Mancini F, Tontini M, Malyala P, Bufali S, Baudner B, De Gregorio E, Valiante NM, O'Hagan DT, Rappuoli R, D'Oro U. Novel adjuvant Alum-TLR7 significantly potentiates immune response to glycoconjugate vaccines. *Sci Rep* 2016;6:29063.