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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.

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 Al^{3+}) 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 CTU_{100} of PT and incubated for 90 min at 37 °C. 50 μl of serum/PT mixtures was added to 50 μl CHO-K1 cells (4×10^5 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.

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