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A FORMULATION SCIENCE PERSPECTIVE APPLIED TO COMBINATION VACCINES

Particles-based multiplex immunoassays for improved antigens characterization and rational design of adjuvants for tailored immunity to acellular pertussis vaccines

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Guarda lontano,

e quando pensi di aver guardato lontano,

guarda ancora più lontano.

- Robert Baden Powell

SUMMARY

Vaccines are complex multi-component products, in which an interdependent relationship among constituents exists: the concomitant inclusion of antigens, adjuvants, and excipients is essential to guarantee the efficacy of the final product. To stimulate the immune system and raise immunogenicity against highly purified vaccine components most likely adjuvants are required, thus becoming more and more crucial elements of modern vaccine formulations. Physico-chemical and functional attributes of all components and of the whole vaccine formulation have to be controlled before injection. However, often characterization challenges are experienced with the currently available analytical techniques, also because of interferences with adjuvants.

In this thesis adjuvants were evaluated with the aim to improve quality and immunogenicity of combination vaccines, in which the inclusion of multiple antigenic components contributes to enhanced complexity of the final product.

In detail, three virulence factors of the Gram negative bacterium *Bordetella pertussis* (pertussis toxin PT, filamentous heamagglutinin FHA, and pertactin 69K) were chosen as model antigens. Currently licensed acellular pertussis (aP) vaccines are combined with diphtheria and tetanus toxoids in the DTaP/TdaP vaccine, which is considered a cornerstone of combination vaccines. This vaccine is typically adjuvanted with aluminum salts, known to induce mainly a humoral immune response which has been associated with recent pertussis outbreaks reported in many countries. This suggests that existing vaccines may not be ideal to provide protection against the disease. Thus, a pharmaceutical sciences approach focusing on adjuvants was applied to investigate the potential improvement of acellular pertussis vaccines through promotion of the analytical characterization and rational polarization of the quality of immune responses.

Improved *in vitro* characterization assays for combination vaccines adjuvanted with aluminum hydroxide

Vaccines *in vitro* characterization is required to identify optimal formulation conditions to ensure physical, chemical, and biological integrity of antigens and adjuvants. Analytical methods currently in use are mostly suitable for the characterization of unformulated antigens – requiring the complete desorption from aluminum-based adjuvants - and are not always able to reveal individual antigens in vaccine combinations. Here, the Luminex technology is proposed as proof of concept for the development of an improved analytical method for vaccine characterization, based on the use of specific antibodies bound to magnetic microspheres presenting unique digital signatures for simultaneous recognition of respective antigens in the entire formulation. TdaP combination vaccines were chosen as a model to develop an assay able to quantify acellular pertussis antigens and their levels of adsorption to adjuvant at the same time. This assay was directly applicable on the commercial vaccine product, avoiding any laborious procedures for separation of the antigens from the adjuvant. Accurate and reproducible quantification of aP antigens in TdaP vaccine has been achieved in a range between 0.78 and 50 ng/mL, providing information on antigen identity, quantity, and degree of adsorption to aluminum hydroxide. Importantly, the Luminex characterization method has the potential to be further evaluated as correlate of *in vitro* potency assays - ideally allowing reducing *in vivo* animal studies.

***In vivo* evaluation of novel adjuvants for improved aP-containing combination vaccines**

The successful approach of combining diphtheria, tetanus and pertussis antigens into a single vaccine has become the cornerstone of pediatric and adults immunization programs. Yet, even if vaccination coverage is high, a resurgence of pertussis has been recently reported in many countries suggesting that current vaccines may not provide adequate long-lasting protection.

In order to develop pertussis-containing vaccines able to induce a more durable and better tailored immune response different approaches have been proposed, including the use of novel adjuvants. Currently licensed pertussis vaccines contain

aluminum salts, which are the most common adjuvants for human use. By inducing mainly humoral immune responses aluminum salts might not be ideal for providing protection against pathogens which require a more cellular immune response, such as *Bordetella pertussis*.

Therefore, alternative adjuvants that induce more balanced T-helper profiles or even Th1-prone responses might be more adequate. In this project, we tested three different adjuvants: MF59 emulsion adjuvant – that induces a mixed Th1/2 response - as well as the combination of a Toll-Like Receptor 4 agonist (TLR4a) or a Toll-Like Receptor 7 agonist (TLR7a) with aluminum hydroxide to induce even further Th1 polarization. The adjuvants were evaluated for their ability to improve immune responses against a TdaP vaccine containing three *B. pertussis* antigens: genetically detoxified pertussis toxin (PT-9K/129G), filamentous hemagglutinin (FHA) and pertactin (69K).

The quicker onset of serum antibody titers and the changed quality of the antibody responses induced by the adjuvants evaluated here fully support the potential replacement of aluminum salts with alternative adjuvants to enhance pertussis immunogenicity in aP-containing combination vaccines.

Conclusions

Overall, the collected results suggested the possibility to modulate quality and efficacy of a complex vaccine product by carefully working on the adjuvant component. Improving the analytical characterization of vaccines while addressing a rational polarization of immune responses are proposed as effective strategies to be pursued for the development of next generation combination vaccines.

RIASSUNTO

I vaccini sono prodotti complessi costituiti da diversi elementi tra i quali esiste un rapporto d'interdipendenza: la presenza concomitante di antigeni, adiuvanti, ed eccipienti è essenziale per garantire l'efficacia del prodotto finale. Gli adiuvanti sono spesso necessari per stimolare il sistema immunitario e aumentare l'immunogenicità di un vaccino composto di antigeni molto purificati: diventano perciò sempre più importanti nelle moderne formulazioni di vaccini. Le proprietà fisico-chimiche di tutti gli elementi e dell'intera formulazione e devono essere controllate prima dell'iniezione. Tuttavia, le tecniche analitiche ora disponibili spesso interferiscono con gli adiuvanti, e problemi di caratterizzazione sono incontrati.

In questa tesi gli adiuvanti sono stati sfruttati con l'obiettivo di indagare la possibilità di migliorare la qualità e l'immunogenicità dei vaccini combinati, nei quali l'inclusione di più elementi antigenici contribuisce alla maggiore complessità del prodotto finale.

In particolare, tre fattori di virulenza del batterio Gram negativo *Bordetella pertussis* (tossina della pertosse PT, emoagglutinina filamentosa FHA, e pertactina 69K) sono stati scelti come antigeni modello. I vaccini acellulari di pertosse (aP) attualmente autorizzati sono combinati con le tossine inattivate di difterite e tetano nel vaccino DTaP/TdaP, che è considerato la pietra miliare dei vaccini combinati. Questo vaccino è tipicamente adiuvato con sali di alluminio, noti per indurre prevalentemente una risposta immunitaria umorale che è stata associata con le recenti epidemie di pertosse segnalate in molti paesi. Ciò suggerisce che il vaccino esistente non sia ideale per fornire una protezione contro la malattia. Pertanto, un approccio basato sulle scienze farmaceutiche e focalizzato sugli adiuvanti è stato applicato per studiare il potenziale miglioramento dei vaccini acellulari di pertosse, attraverso lo sviluppo della caratterizzazione analitica e la razionale polarizzazione della qualità della risposta immunitaria.

Sviluppo di saggi di caratterizzazione *in vitro* per vaccini combinati adiuvantati con idrossido di alluminio

La caratterizzazione analitica in vitro è necessaria per individuare le condizioni di formulazione ottimali che garantiscano l'integrità fisica, chimica e biologica di antigeni e adiuvanti. I metodi analitici ora in uso sono soprattutto adatti per la caratterizzazione di antigeni non formulati - richiedendo quindi il desorbimento completo dalla superfice degli adiuvanti a base di alluminio - e non sempre sono in grado di distinguere tra i singoli antigeni contenuti nei vaccini combinati. In questa tesi la tecnologia Luminex è proposta come *proof of concept* per lo sviluppo di un miglior metodo analitico per la caratterizzazione di vaccini. Questo metodo è basato sull'utilizzo di anticorpi specifici legati a microsfere magnetiche che sono univocamente riconoscibili tramite una firma digitale permettendo quindi l'identificazione simultanea dei rispettivi antigeni formulati nel vaccino.

I vaccini combinati TdaP sono stati scelti come modello per sviluppare un saggio in grado di quantificare simultaneamente gli antigeni acellulari della pertosse (aP) e il loro grado di adsorbimento all'adiuvante. Questo saggio è direttamente applicabile al vaccino commerciale, evitando qualsiasi procedura laboriosa per la separazione degli antigeni dall'adiuvante. Una quantificazione accurata e riproducibile degli antigeni aP nel vaccino TdaP è stata ottenuta in un intervallo di concentrazioni compreso tra 0,78 e 50 ng/mL, fornendo informazioni sull'identità degli antigeni, la loro quantità e il grado di assorbimento alla superficie dell'idrossido di alluminio. Il metodo di caratterizzazione sviluppato sulla tecnologia Luminex ha la potenzialità di essere valutato come test di *in vitro potency* - permettendo idealmente la riduzione degli studi effettuati sugli animali.

Valutazione *in vivo* di nuovi adiuvanti per migliorare i vaccini combinati contenenti aP

L'approccio di combinare gli antigeni di difterite, tetano e pertosse in un unico vaccino è diventato il fondamento dei programmi d'immunizzazione pediatrici e per adulti. Nonostante l'elevata copertura vaccinale, una riacutizzazione dei casi di pertosse è stata recentemente registrata in molti paesi, suggerendo che i vaccini attuali non possono fornire un'adeguata protezione di lunga durata.

Diversi approcci sono stati proposti al fine di sviluppare vaccini in grado di indurre una risposta immunitaria più durevole e più adeguata contro la pertosse, tra cui l'uso di nuovi adiuvanti. I vaccini contro la pertosse ora autorizzati contengono sali di alluminio, che sono gli adiuvanti più comuni per uso umano. Inducendo principalmente risposte immunitarie umorali, i sali di alluminio non sono ideali per fornire protezione contro gli agenti patogeni che richiedono una risposta immunitaria cellulare, come ad esempio *Bordetella pertussis*.

Pertanto, altri adiuvanti che stimolino l'induzione di cellule Th1 potrebbero essere più adeguati. In questo progetto, abbiamo provato tre diversi adiuvanti: l'emulsione MF59 - che induce una risposta mista Th1/2 - oppure la combinazione di un agonista del Toll-Like Receptor 4 (TLR4a) o del Toll-Like Receptor 7 (TLR7a) con idrossido di alluminio – che polarizzano la risposta verso il profilo Th1. Gli adiuvanti sono stati valutati per la loro capacità di migliorare le risposte immunitarie contro il vaccino TdaP contenente tre antigeni di *B. pertussis*: tossina della pertosse geneticamente detossificata (PT-9K/129G), emoagglutinina filamentosa (FHA) e pertactina (69K).

La rapida induzione di titoli anticorpali e la polarizzazione della qualità della risposta immunitaria indotte dagli adiuvanti qui esaminati sostengono pienamente la potenzialità di sostituire i sali di alluminio con adiuvanti alternativi che migliorino l'immunogenicità di vaccini combinati contenenti aP.

Conclusioni

Complessivamente, i risultati raccolti suggeriscono la possibilità di modulare la qualità e l'efficacia di un vaccino complesso lavorando sulla componente adiuvante. Migliorare la caratterizzazione analitica dei vaccini e indirizzare una polarizzazione razionale della risposta immunitaria sono proposte come strategie efficaci da perseguire per lo sviluppo dei vaccini combinati della prossima generazione.

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PREFACE

Vaccines, together with antibiotics, are among the most successful outcomes of modern medicine. Regardless of this, people reveal a declining confidence and compliance when facing vaccines, probably because these products are not considered useful in preventing diseases which are mostly unknown and not recognized as life-threatening. People are no longer aware of diseases that vaccines protect from as they have never experienced them. As a consequence, vaccination coverage decreases, resulting in large outbreaks of highly contagious diseases, as recently seen e.g. for measles [1]. Additionally the complexity of vaccine products contributes to concerns about vaccine ingredients and unexpected side-effects.
Vaccine research is a complex field, covering many areas ranging from the discovery and evaluation of new antigenic candidates to the continuous improvement and validation of products already available in the market.

During my PhD I have investigated the possibility to improve combination vaccines through the application of pharmaceutical sciences approaches directed and focusing on adjuvants. I have explored the potential of complete characterization and rational design of adjuvants within complex vaccine formulations, with the purpose of improving development and awareness of future vaccines - emphasizing the importance of adjuvants.

CHAPTER I.
GENERAL INTRODUCTION AND OBJECTIVE OF THE THESIS

VACCINES

At the end of the nineteenth century Louis Pasteur identified microorganisms as agents responsible for infectious diseases. Moreover, by accidentally leaving a bacteria culture without nutrients, he observed that it was possible to artificially obtain microorganisms showing attenuated virulence and able to confer immunity against the disease. Pasteur referred to attenuated bacteria as "vaccines" in honor of Edward Jenner's discovery - dated back at the end of the eighteenth century - about the possibility to protect people against smallpox through inoculation with cowpox, a less dangerous disease which he denoted as *Variolae Vaccinae* (from the Latin word *vacca*, cow).

More than a hundred years after Pasteur's observation, vaccines are considered a fundamental milestone for healthcare providing life-long protection against numerous infections. At first the leading principle was the basic one proposed by the French microbiologist, starting with the identification of the microorganism, inactivation through heat or exsiccation and subsequent injection. A big step

forward was made during the 1920s by Gaston Léon Ramon who developed the first two vaccines – against *Corinebacterium diphtheriae* and *Clostridium tetanii* - constituted not by the whole bacterium but only by a part of it. Diphtheria and tetanus toxins, identified as the causes of the respective diseases, were inactivated with formaldehyde following a procedure similar to the one used in modern vaccine manufacture [2].

A brief historical overview across vaccines discovery and development allow to categorize vaccines in three main groups: live-attenuated whole microorganisms, heat or chemically inactivated microorganisms, and microorganisms' subunits [3]. This classification is still valid in modern vaccinations but technology improvements and the continuous raising of safety concerns drive vaccine development to limit the use of whole pathogens in favor to the identification and isolation of well-characterized components responsible of conferring immunity to the disease. Indeed, reversion of toxicity and high levels of reactogenicity with subsequent adverse-effects are challenges that might be faced when working with whole inactivated microorganisms. Significant improvements in terms of safety were introduced by the advent of the recombinant DNA technology which allowed the production of recombinant antigens [4].

COMBINATION VACCINES

Continuous vaccine development (Figure 1 [5]) is pushing towards the constant progress in the development of combination vaccines, which allow providing immunity against more than one infectious disease. By incorporating separate antigens or individual vaccines into a single product, combination vaccines represent an important means of simplifying vaccination strategies. Their development started over sixty years ago with the combination of diphtheria toxoid (DT), tetanus toxoid (TT) and whole-inactivated pertussis bacteria (wP) into a single DTwP vaccine that has become the cornerstone of pediatric and adult immunization programs [6]. Over the years the wP component was replaced with less reactogenic acellular pertussis (aP) antigens, and further routine vaccines were

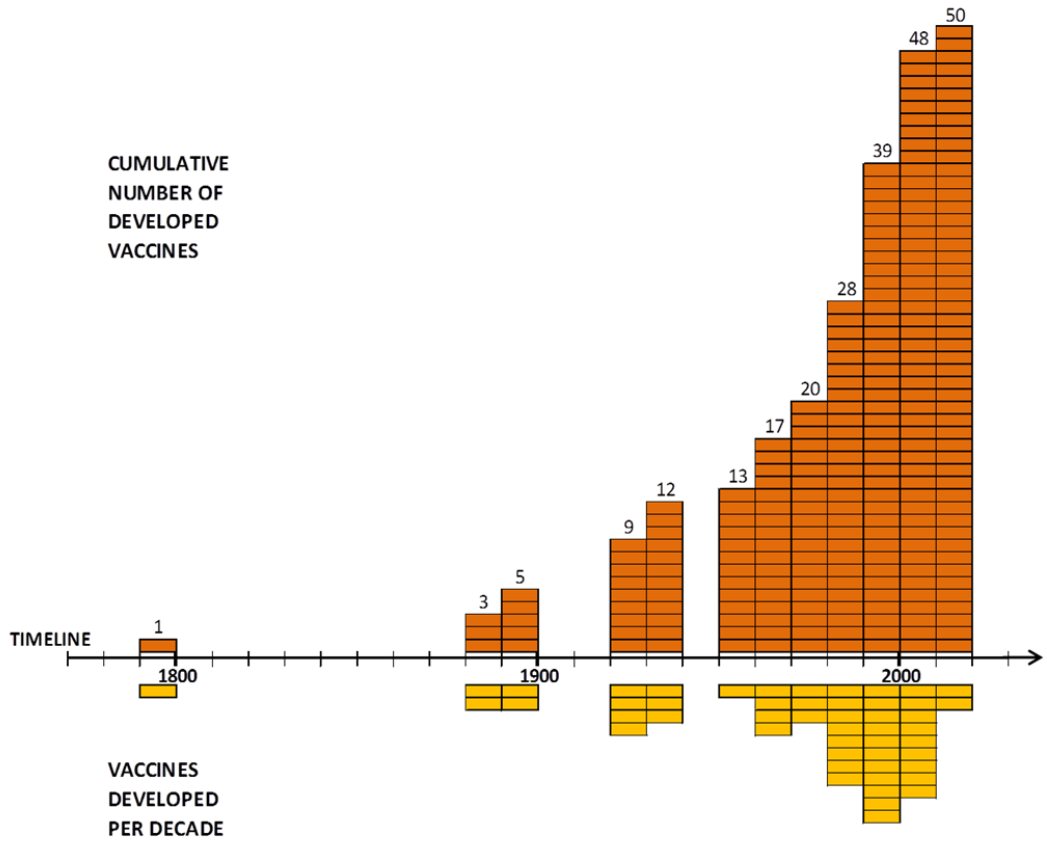

FIGURE 1. Timeline of vaccine development showing the number of vaccines introduced in the market every decade (yellow) since the discovery of the first vaccine at the end of the eighteenth century. In orange the progressive cumulative number of vaccine products is recorded.
1790s: smallpox.
1880s: rabies, typhoid.
1890s: cholera, plague.
1920s: diphtheria, tetanus, pertussis, tuberculosis.
1930s: yellow fever, influenza, typhus.
1950s: polio (injected, inactivated).
1960s: polio (oral, live), measles, mumps, rubella.
1970s: anthrax, meningococcal disease (polysaccharide), pneumococcal disease (polysaccharide).
1980s: adenovirus infection, rabies (cell culture), tick-borne encephalitis, hepatitis B (plasma derived), Haemophilus influenza type B infection (polysaccharide), hepatitis B (yeast or baculovirus, surface antigen), Haemophilus influenza type B infection (conjugate), typhoid (other variants).
1990s: cholera (killed whole cell), Japanese encephalitis (inactivated), cholera (recombinant toxin B), cholera (live attenuated), typhoid (capsular polysaccharide), varicella zoster, acellular pertussis, hepatitis A (inactivated), lyme disease, rotavirus infection, meningococcal disease (conjugate group C).
2000s: pneumococcal disease (7 valent, conjugate), cold-adapted influenza, meningococcal disease (4 valent, conjugate), varicella zoster (live), rotavirus infection (attenuated), human papilloma virus (4 valent, recombinant), cholera (whole cell), Japanese encephalitis (Vero cell colture), human papilloma virus (2 valent, recombinant).
2010s: (current decade): pneumococcal disease (13 valent, conjugate), meningococcal disease (recombinant group B).
Data collected from [5].

subsequently added to the DTaP combination following the growing number of childhood diseases for which vaccination was recommended.

The latest advance in DTaP combination vaccine development is defined by Infanrix™ -hexa from GSK, which protects against six different diseases by carrying diphtheria, tetanus, and acellular pertussis antigens (DTaP) together with inactivated polio vaccine (IPV), *Haemophilus influenzae* vaccine (Hib), and hepatitis B vaccine (HepB) [7]. Other significant combination vaccines are the 23-valent pneumococcal polysaccharide vaccine (Pneumovax 23™ from Merck) [8] or the 4-valent meningococcal conjugate vaccine (Menveo™ from Novartis Vaccines) [9], which respectively protect solely against pneumococcal and meningococcal infections. It is likely that new combination vaccines will reach the market in the coming years, combining DTaP-based with pneumococcal or meningococcal vaccines. Promisingly, these products are already being co-administered at the same vaccination visit, and no major negative effects have been reported [10].

Combination vaccines confer several advantages, starting with the simplification of the immunization schedule and subsequent reduction of the number of injections needed to confer protection against multiple diseases. Benefits can be identified on various levels of society, with fever shots and improved compliance for recipients, and lower costs, less medical visits needed, better coverage and the possibility to more easily add new vaccines into the immunization program for national healthcare systems [7].

Combining multiple vaccines into a single product potentially introduce some challenges since fine-tuning respective immunization schedules which foresee a different number of doses on different time points is not immediate (Figure 2). Moreover adverse interactions among combined antigens have to be avoided while maintaining the final product as safe and immunogenic as the individual vaccine components. Improvements in this context may involve the inclusion of adjuvants, which can potentially bring advantages, such as increased antigen stability and reduced interference among vaccine components, as well as antigen dose reduction and reduction of the number of scheduled immunizations [11].

Vaccine	Age										
	Birth	1 mo.	2 mo.	4 mo.	6 mo.	12 mo.	15 mo.	18 mo.	19-23 mo.	2-3 yr.	4-6 yr.
Diphtheria, Tetanus, Pertussis			DTaP	DTaP	DTaP		DTaP				DTaP
Measles, Mumps, Rubella						MMR					MMR
Hepatitis B	HepB	HepB			HepB						
Heamophilus influenzae type B			Hib	Hib	Hib	Hib					
Pneumococcal			PCV	PCV	PCV						
Rotavirus			RV	RV	RV						
Inactivated Poliovirus			IPV	IPV	IPV						IPV
Varicella						Var.					Var.
Hepatitis A						HepA (2 doses)					
Influenza					Flu (yearly)						

FIGURE 2. Recommended immunization schedule for children aged 0 through 6 years. Adapted from [7].

VACCINE ADJUVANTS

Adjuvants owe their name to the Latin word *adjuvare*, meaning "to help". Ramon, previously cited for his studies about diphtheria and tetanus toxins, was the first to describe adjuvants as "substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone" [12]. In whole live attenuated or inactivated vaccines the role of potentiation of the immune response is carried out by components naturally present in respective microorganisms, such as bacterial DNA or lipopolysaccharide, which are recognized as non-self by the host thus generating an immune response. The introduction of purified and recombinant antigens caused an evident increase in safety profile but, despite that, antigens alone resulted to be less immunogenic and not always protective. Therefore, vaccine development becomes a complex fine-tuning between safety and efficacy of the final product. In this perspective, adjuvants were introduced to balance the removal of natural immune-stimulatory components of the pathogens with the aim to enhance an immune response similar to the one triggered by the natural infection [11-13], while maintaining equilibrium between vaccine safety and efficacy.

The inclusion of adjuvants in vaccines provides a number of advantages which confer the potential to:

- Increase antigens immunogenicity;
- Lower the number of doses needed to confer protection – quicker response;
- Lower antigen doses with reduced impact on production costs;
- Modulate quality of the immune response to promote protection;
- Modulate quantity of the immune response by extending duration and potency of induced antibodies;
- Enable complex vaccine combinations overcoming competition among included antigens;
- Overcome limited immune response in endangered population such as the elderly, young children, and immunocompromised people.

For almost 80 years aluminum-based adjuvants - approved in the 1920s - were the only adjuvants licensed for human use, followed by MF59 approval for inclusion in an influenza vaccine in 1997 [13]. While the main feature of aluminum salts and MF59 is the delivery of antigens to immune cells, "second-generation adjuvants" have been developed combining a delivery system with an immune-potentiator molecule [11]. Among these AS04 [14] combine the lipopolysaccharide analog monophosphoryl lipid A (MPLA) with aluminum salts and was the first to be approved for inclusion in a vaccine against Hepatitis B virus (Fendrix® from GSK) in 2005 [15] and against human papilloma virus (Cervarix® from GSK) in 2007 [16]. In Table I a list of adjuvants licensed and evaluated in clinical studies is presented. However those are only a subset among the variety of adjuvants proposed and described in literature, indicating adjuvant development as a slow and difficult process, which undergo many challenges mainly from a safety and regulatory perspective [11, 17].

TABLE I. List of adjuvants licensed or under clinical evaluation for use in humans. Adapted from [17].

Adjuvant	Description	Status
Aluminum salts	Insoluble aluminum salts (e.g. hydroxides and phosphates); extensive safety record; antigens adsorbed to the surface.	Licensed in EU and US
MF59	Squalene oil-in-water emulsion; extensive safety record; antigens not associated with droplets.	Licensed in EU
AS03	Squalene oil-in-water emulsion added with the immune-potentiator α-tocopherol; associated with narcolepsy.	Licensed in EU and US
AS04	Combination of aluminum adjuvant with the TLR4 agonist monophosphoryl lipid A (MPLA), co-adsorbed.	Licensed in EU and US
AS01	Liposomes composed added with MPLA and QS21.	Clinical phase
CAF01	Cationic liposomes.	Clinical phase
Poly I:C	Synthetic double stranded oligonucleotide containing repeating units of inosine and cytosine (TLR3 agonist).	Clinical phase
IC31	Antimicrobial peptide bound to a TLR9 agonist.	Clinical phase
AS02	Co-mixture of AS03 emulsion adjuvant with MPLA and QS21.	Clinical phase
Imiquimod	TLR7/8 agonist small molecules immune potentiators (SMIP).	Clinical phase
CpG olugonucleotides	TLR9 agonists based on bacterial DNA.	Clinical phase
SE/SE-GLA	Squalene based emulsion added with the TLR4 agonist GLA.	Clinical phase
ISCOMS/ISCOMATRIX	Small (40 nm) lipid based adjuvants consisting of phospholipids, cholesterol and saponins.	Clinical phase

A growing number of research groups are directing their attention and investments at deepening the knowledge about adjuvants mechanism of action and studying adjuvants effect from an immunological point of view. Formulation science is focusing on the complexity induced by the addition of adjuvants to the vaccine formulation taking into consideration potential effects on product/antigen stability and efficacy [18]. A complex set of analytical characterization techniques is usually required for a complete characterization of formulated antigens and antigens-adjuvants interactions further complicate their application [19]. In the next section these challenges will be reviewed focusing on aluminum salts adjuvants, which are the mostly used adjuvants for human use [20].

ALUMINUM SALTS ADJUVANTS

Aluminum-salts based adjuvants have become a reference standard for the evaluation of new generation vaccines due to their long history of use. Formerly, aluminum salts were commonly used for proteins purification by taking advantage of their high absorbent capacity. The discovery of the adjuvant effect of this material dates back to 1926 when Alexander Glenny and his colleagues described the higher antigenic response of a diphtheria toxoid precipitated in presence of aluminum potassium sulfate in comparison to the soluble diphtheria toxoid [21]; a discovery that inspired Ramon's adjuvant definition. During the twentieth century aluminum adjuvants have been extensively studied and included in many human vaccines against infectious diseases showing good safety profile (e.g. vaccines against hepatitis B, *Haemophilus influenzae* and human papilloma virus). However, aluminum salts mechanisms of action as vaccine adjuvants is not yet fully understood, despite the role of many immunological components involved in the response have been recently elucidated [22]. Among the key concepts to exploit aluminum adjuvant potential there is the awareness for some antigens that adsorption is of key importance to guarantee vaccines stability and adjuvanticity [18, 23].

Two main types of aluminum salts have been identified as adjuvants having different chemical and physical compositions that strongly influence antigens adsorption profile on their surfaces [22, 24]:

- Aluminum hydroxide, chemically aluminum oxyhydroxide, has a crystalline structure composed of needle-shaped nanoparticles which tends to aggregate up to an average diameter of 10 μm. Aluminum hydroxide presents only hydroxyl groups on its surface and have a point of zero charge (PZC) of 11.4 thus being positive charged at the neutral pH used for vaccine formulations;
- Aluminum phosphate, chemically aluminum hydroxyphosphate, has an amorphous structure with a mean particles size of 50 nm which form irregular aggregates of several microns. Both hydroxyl and phosphate

groups cover aluminum phosphate surface with a ratio between the two that depends on manufacturing conditions. A PZC between 4.5 and 5.5 confer aluminum phosphate a negative charge at neutral pH.

Characterization of aluminum salts chemico-physical properties is of key importance for development of stable vaccines and induction of strong antigen-specific immune responses. The diverse properties of the two mainly employed aluminum salts adjuvants allow the rational choice of the one which most likely favors antigens adsorption, according to isoelectric points and chemical-physical characteristics. Indeed interactions between antigens and adjuvant surface are mainly affected by electrostatic forces, hydrophobic interactions and ligand exchange [25, 26]. Through antigen adsorption, aluminum salt adjuvant mainly act as a delivery system and promotes retention of antigens at the injection site [27-29] thus favoring uptake by immune cells [30, 31] while improving antigens physico-chemical stability and immunogenicity. Antigen-adjuvant interactions have to be optimized and characterized to ensure adsorption and favor the development of stable and efficacious vaccines.

A complex set of analytical techniques is needed to characterize antigens and antigen/adjuvant interactions. In particular, the adsorption of the antigen on the adjuvant surface is an important parameter to be monitored to control the adjuvant role of aluminum salts, along with total content and integrity of antigenic components [26, 32]. Many analytical tools commonly applied to monitor proteins (e.g. gel electrophoresis, Western blot, and liquid chromatography) cannot be applied directly to the vaccine product due to interferences with the adjuvant and other formulation excipients. Therefore, characterization of antigens in vaccines currently requires their complete recovery from aluminum adjuvanted formulations mainly through the use of high salt concentration and surfactants to dislocate antigens from the adjuvant surface. This desorption procedure could result in incomplete antigen recovery and could compromise the integrity of antigens [23, 33-35]. After centrifugation, antigens desorbed and released in the aqueous phase are recovered and analyzed, nonetheless desorption buffer components could interfere with the assays, too (e.g. surfactants impact HPLC methods, and histidine

buffers are not suitable for colorimetric assays) [19]. In addition, antigens are usually formulated in vaccines at doses significantly lower than the quantification limits of the analytical tools currently available, especially when considering booster dosages [36].

Thus the variety of analytical tools currently available for the characterization of soluble proteins is counteracted by adjuvant presence and antigen-adjuvant interactions which cause the analysis of antigens in formulated vaccines to be an enormous challenge [37]. In this perspective, the development of analytical assays which allow the characterization of antigen-adjuvant interactions and the investigation of their effect on antigens stability and immunogenicity is continuously needed. It has been already observed that antigens undergo structural changes due to interaction and adsorption to aluminum salts and the resulting effects on antigens physico-chemical stability due to changes in their environment have only recently started to be investigated [22, 32].

In the last years a variety of analytical techniques have been set up and improved for vaccine characterization without requiring preliminary phase separation procedures between antigens and aluminum salts adjuvant. The most commonly employed techniques are presented in Table II along with the type of information provided, the typical working range, and the specificity. The o-Phthalaldehyde (OPA) fluorescent protein assay is extensively used to determine antigen concentration while adsorbed to aluminum adjuvant and have a sensitivity (20 - 500 μg/ml) [38] similar to that of a chemo-luminescent method developed to quantify formulated antigens through determination of total nitrogen content [39]. A significant increase in terms of sensitivity is observed for assays which work on antibody recognition (DAFIA, flow-cytometry, SPR) [40-42]. The availability of antigen-specific and well-characterized monoclonal antibodies could allow gaining information on antigen identity and integrity through analysis of epitopes critical for vaccine immunogenicity, guaranteeing specificity to the assay. Spectroscopic methods or thermo-analytical techniques could be instead applied to investigate conformational and thermal stability of vaccine antigens while adsorbed on

aluminum adjuvants [43-48] and to screen different pH, formulation buffer components and stabilizers [49].

In order to avoid diminished potency and to ensure the development of high quality final products as required in modern vaccinology, a complex set of analytical techniques is needed and should be ideally applied since the early stages of research to characterize each antigen and antigen/adjuvant interaction. However, due to its

TABLE II. List and main features of the analytical methods currently available for characterization of vaccine antigens in presence of aluminum-based adjuvants.

Method	**Type of information provided**	**Working range**	**Specificity**	**References**
OPA (o-Phthalaldehyde assay)	Antigen quantity	20 - 500 µg/mL	-	[38]
CLND (Chemiluminescent Nitrogen Detection)	Antigen quantity	6.75 - 400 µg/mL	-	[39]
DAFIA (Direct Alum Formulation Immunoassay)	- Antigen-specific (antibody recognition) - Antigen quantity, identity, integrity - Direct analysis of immunological epitopes	0.16 - 10 µg/mL	+	[40]
Flow-cytometry	- Antigen specific (antibody recognition) - Antigen quantity, identity, integrity - Direct analysis of immunological epitopes - Content uniformity	6.2 - 200 µg/mL	+	[41]
SPR - Biacore (Surface Plasmon Resonance)	- Antigen specific (antibody recognition) - Direct analysis of immunological epitopes - Antigen conformational and thermal stability	20 µg/mL	+	[42]
Fluorescence spectroscopy (Intrinsic/Extrinsic)	Antigen conformational and thermal stability	50 - 200 µg/mL	-	[43, 44, 46, 49]
Near IR transmittance spectroscopy	Antigen quantity	0 - 1.75 mg/mL	-	[48]
FTIR (Fourier Transform IR spectroscopy)	Antigen conformational and thermal stability	0.5 - 1 mg/mL	-	[47]
DSC (Differential Scanning Calorimetry)	Antigen conformational and thermal stability	0.5 - 1 mg/mL	-	[44, 45]

complexity, current analytical panel results to be more suitable for proof of concept studies and research purposes. In this perspective there is an increasing need for an analytical method able to characterize formulated antigens with the sensitivity and the specificity typical for antibody-based assays. In particular the specificity should be considered as a necessary requirement to overcome the need of separate assays for each different vaccine component, which is typically required when working with multivalent formulations.

INTRODUCTION OF THE MODEL VACCINE: ACELLULAR PERTUSSIS-CONTAINING COMBINATION VACCINES

The successful approach of combining diphtheria, tetanus and pertussis antigens into a single vaccine has become a pillar in pediatric (DTaP) and adults (TdaP) immunization programs. Even if vaccination coverage is high, the highly contagious respiratory tract infection caused by the Gram negative bacterium *Bordetella pertussis* is still endemic worldwide and the number of cases has been increasing over the last years [50-54] suggesting that the current vaccine may not provide adequate long-lasting protection against the disease.

Being among combination vaccines per excellence and jumping into the news for worldwide resurgence were among the reasons why acellular pertussis vaccines were chosen for the objectives of this thesis and will be discussed in more detail in Chapter III.

In this PhD work, formulation science approaches directed on adjuvants were applied in order to improve current pertussis combination vaccines.

The aim was to put in the limelight vaccine characterization on one side and rational use of adjuvants to shape the desired immune response on the other, as key approaches for the development of improved acellular pertussis combination vaccines. Accordingly, the structure of the thesis reflects this vision and is therefore organized into two main chapters:

- Chapter II - *Improved in vitro characterization assays for aP combination vaccines* focusing on the development of improved *in vitro* characterization methods which are required to identify optimal formulation conditions and ensure physic-chemical integrity of antigens. Analytical methods currently in use commonly require the complete desorption of antigens from aluminum-based adjuvants, and are not always able to reveal individual antigens in vaccine combinations. The potential of Luminex technology was then exploited in an innovative way aiming to develop a multi-plex sandwich immunoassay for simultaneous characterization of multiple antigens in combination vaccines. The approach to directly analyze antigens without physical separation from the adjuvant has been explored with the potential to be widely applied to monitor integrity, quantity, and stability of antigenic components in aluminum salts formulations. The assay was developed for the simultaneous characterization of three acellular pertussis antigens when formulated with tetanus and diphtheria toxoids in current aluminum hydroxide adjuvanted TdaP vaccines.

- Chapter III - *In vivo evaluation of novel adjuvants for improved aP combination vaccines* pursue the approach to tailor immune responses to *B. pertussis* antigens contained in TdaP combination vaccines. This chapter takes its justification on the recent pertussis outbreaks reported in many countries, which suggest that the existing vaccine may not provide adequate long-lasting protection against the disease. The approach was designed

following the hypothesis that the humoral immune response induced by aluminum salts – currently included as adjuvants in the vaccine - might not be ideal for providing protection. In particular, MF59 emulsion adjuvant and the combination of Toll-Like Receptor agonists (TLRa) with aluminum hydroxide were rationally chosen as alternative adjuvants, characterized, and compared in a mouse model to assess their potential to enhance and fine-tune the immune response against TdaP antigens.

CHAPTER II. IMPROVED *IN VITRO* CHARACTERIZATION ASSAYS FOR aP COMBINATION VACCINES

The pharmaceutical industry has a little secret: even as it invents futuristic new drugs, its manufacturing techniques lag far behind those of potato-chips and laundry-soap makers.

Wall Street Journal - Sep 3, 2003

LUMINEX TECHNOLOGY

A complex set of analytical characterization techniques is usually needed to both qualitatively and quantitatively characterize antigens in vaccine formulations thus guaranteeing high quality final products. In this perspective, Luminex technology has been exploited with the potential to improve the analytical characterization of antigens in vaccine formulations. Through the set-up of solution-phase bead-array immunoassays the simultaneous analysis of identity, quantity, and stability of each formulated antigen can be potentially achieved, differently from traditional analytical methods which require a separate test for each immune response constituent. Luminex technology is indeed mainly known for the possibility to manage multiple analytes within a single assay run and has become of great impact in many biological evaluations.

The Luminex approach relies on polystyrene micro-particles, commonly called "beads", internally dyed with different intensities of a red and an infrared fluorophores. As a consequence it is possible to mix together differently dyed beads which can still be identifiable via their unique spectral signature: hence

Luminex peculiarities of allowing multiple measurements simultaneously, in the so called “multivalent approach”. Beads surface can be variously functionalized, e.g. carboxylated, allowing covalent coupling of analytes and bio-analytical reactions to take place over them. Inside the Luminex system, beads are lined up by the fluidics in a single file and pass through a detection chamber where a red laser excites their internal dyes allowing beads identification and a green laser quantifies the bio-molecular interaction occurring at the surface of the beads. Software analysis sort registered events by side scattering thus events larger or smaller than a single microsphere are excluded, as well as aggregated microspheres. The simultaneous analysis of multiple analytes offered by this technology appeared to be valuable in various fields ranging from clinical research studies for recognition and diagnosis of clinical markers [55, 56], to the vaccine field for the qualitative and quantitative profiling of immunological responses [57-59].

In this study Luminex technology was exploited in an innovative way for the simultaneous characterization of acellular pertussis (aP) antigens when formulated with tetanus toxoid (TT) and diphtheria toxoid (DT) in aluminum hydroxide (AlOH) adjuvanted TdaP vaccines. Luminex approach has been explored for the analysis of quality, quantity and degree of adsorption of antigenic components in AlOH-based formulations (Figure 3) avoiding physical separation procedures from the adjuvant. The purpose was to develop an analytical method having the accuracy, sensitivity and specificity typical of antibody-based immunoassays and able to fully characterize vaccine antigenic components within a single analysis.

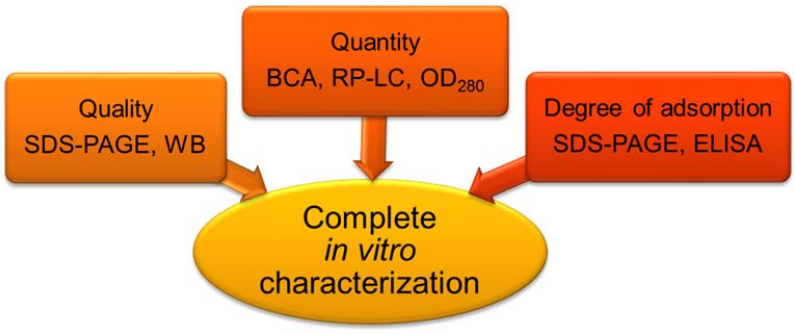

FIGURE 3. Characterization of vaccine antigens typically requires the application of various analytical tools to gain information on antigens quality, quantity and degree of adsorption to adjuvant surface. The approach based on Luminex technology has the potential to provide a complete characterization within a single analysis.

I*n vitro* vaccine characterization based on Luminex technology confers many advantages:

- Specificity of the assay – based on antigen-antibody recognition;
- Simultaneous analysis of multiple antigens, - "multivalent approach";
- Potential application on the entire vaccine formulation without separation between antigens and adjuvants.

The approach pursued in this PhD project foresees the application of Luminex for vaccine formulation characterization following two opposite layouts presented in Figure 4. The indirect assay approach relied on coupling of antigens on the surface of beads, and owed its name to the fact that a reduction on the fluorescence signal coming from beads through the green detection laser was indirectly proportional to an increase of the amount of antigens in vaccine. The direct approach rested on an opposite layout in which antibodies were coupled to the beads and required to directly recognize antigens in vaccine formulation.
The two approaches will be reviewed in detail in the following pages.

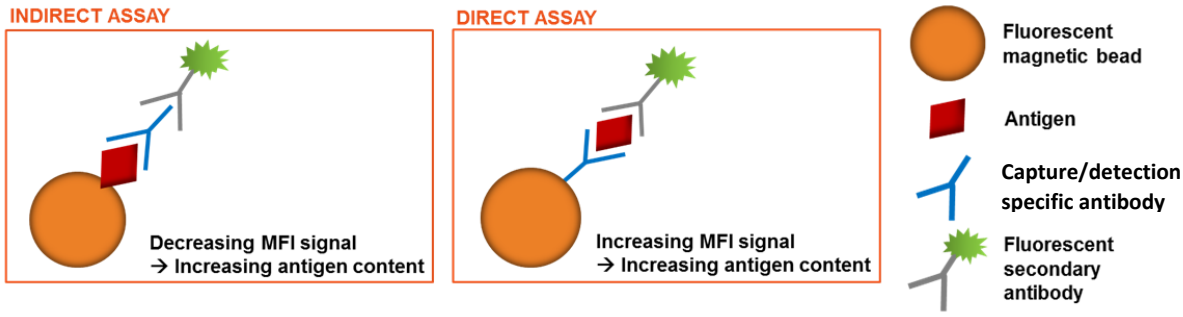

FIGURE 4. Schematic representation of the two opposite layouts investigated for i*n vitro* characterization of vaccine antigens using Luminex technology. Indirect assay: antigens are covalently coupled to the bead surface and the fluorescent (MFI) signal generated result indirectly proportional to the amount of formulated antigens. Direct assay: direct quantification of antigens in solution through a capture antibody linked to the bead surface, MFI signal result to be proportional to antigens amount.

The first approach taken into account to investigate the possibility of developing an analytical assay based on Luminex technology for vaccine formulations characterization relied on coupling of antigens to the surface of magnetic carboxylated beads. The underlying assumption (described in Figure 5) considered the definition of a calibration curve for antigens quantification through the addition of a fixed antibody dilution to known antigen concentrations. As a result only the complementary amount of antibodies would not be saturated by the free antigens thus being prone to be detected by the respective antigen-coupled beads. The quantification of the fluorescence signal associated to beads would then be directly proportional to the amount of not-saturated antibody and consequently indirectly proportional to the amount of antigens in the sample. The obtained fluorescent signal interpolated in the calibration curve provides a quantification of the antigen in the analyzed sample.

Materials and Methods

Antigens and Adjuvants

Aluminum hydroxide (AlOH) adjuvant was obtained from Novartis Vaccines (NVx, Marburg, Germany) manufacturing while the acellular pertussis antigens genetically detoxified pertussis toxoid (PT-9K/129G) [60], filamentous hemagglutinin (FHA), and pertactin (69K) were produced by Lonza Group (Basel, Switzerland).

Preparation of adjuvanted formulations

TdaP antigens were subsequently adsorbed onto 2 mg/mL AlOH suspension (correspondent to 0.45 mg/mL Al^{3+}) [24] at the following final concentrations: 10 Lf/mL TT, 4 Lf/mL DT, 8 µg/mL PT, 8 µg/mL FHA, and 16 µg/mL 69K. Formulations were then adjusted by the addition of 10 mM Histidine buffer and 9 g/l NaCl to the recommended physiological ranges of pH (6.5 ± 0.5) and isotonicity (osmolality, 300 ± 60 mOsm/kg).

FIGURE 5. Layout of the indirect assay: A. serial antigen dilution is incubated with B. a fixed antibody concentration; C. only not-saturated antibodies will be recognized by antigen-coupled beads. A calibration curve is obtained (more antigen in solution, less not-saturated antibodies, less fluorescent signal from beads surface) and is used for interpolation and quantification of antigens in formulation samples.

Characterization of antigens stability

Antigens integrity and stability when formulated with or without AlOH adjuvant in formulation buffer or in formulation buffer plus 0.01% (v/v) Tween20 was evaluated by Western Blot (WB) [61] and sandwich ELISA (according to internal NVx procedures).

Size and melting temperatures of antigens formulated without AlOH were determined with the particle size analyzer Zetasizer NanoZS (Malvern Instruments Ltd., Malvern, UK).

Antibodies

Monoclonal antibodies (mAb) anti-69K were obtained from Novartis Vaccines (NVx, Siena, Italy) manufacturing. Monoclonal antibodies (mAb) anti-PT or anti-FHA were produced by Areta International (Varese, Italy). Polyclonal

antibodies (pAb) anti-PT or anti-FHA were obtained through purification (Ab SpinTrap, GE Healthcare, Little Chalfont, Buckinghamshire, UK) of sera of mice immunized with monovalent antigens formulated with AlOH adjuvant as described above. IgG concentration was measured by UV spectrum at 280 nm. Phycoerythrin-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:400) was used as secondary detection reagent.

Luminex indirect assay procedure

Acellular pertussis antigens (PT, FHA, 69K, 20 µg each) were coupled to the carboxyl groups of 2.5 x 10^6 MagPlex microspheres (Luminex Corporation, Austin, TX) following manufacturer's instructions. Each antigen was coupled to a microsphere set, identifiable through its unique spectral signature.

Samples were diluted in formulation buffer (10 mM Histidine, 9 g/l NaCl) containing 0.01% (v/v) Tween20. Diluted antigens and antibodies were added (10 µl/well and 50 µl/well respectively) to black, opaque, 96-well flat-bottom plates (Bio-plex ProTM, Bio-Rad Laboratories, Inc., Hercules, CA). After a 30 minutes incubation step antigen-coupled beads were mixed at 1:5 dilutions in 10 mM phosphate buffer (PBS 1X) pH 7.2 containing 0.05% (v/v) Tween20 and were added to the samples (10 µl/well). After 30 minutes, phycoerythrin-conjugated anti-mouse secondary antibody (1:400) was used for detection in a 15 min incubation step. The resulting immune-complexes were resuspended in formulation buffer and analyzed on the Luminex LX-200 system.

After each incubation steps plates were washed with formulation buffer by an automatized magnetic washer HydroSpeed 96i (Tecan, Männerdorf, Switzerland). All reaction steps were carried out at room temperature, in the dark and under agitation motion using a horizontal shaker.

Data analysis

The Bio-Plex Manager 5.0 software (Bio-plex ProTM, Bio-Rad Laboratories, Inc., Hercules, CA) was used to determine the concentration of antigens as median fluorescence intensities (MFI) by using a not-adjuvanted aP formulation as

reference. MFI values were plotted against known concentration of the reference standard and a five-parameters nonlinear regression (5PL) [62] fitting was used to generate a curve and calculate the concentration of unknown samples through the equation: Y=d+((a-d)/(1+(X/c)^b)^g) where a, b, c, d, g were the parameters describing the curve, Y was the fluorescence reading and X the concentration to be determined.

Results and Discussion

A Luminex single-plex immunoassay was initially developed to characterize 69K antigen in mock formulations, i.e. model formulations in which all the conditions (formulation buffer, pH, and ionic strength) are preserved except the presence of adjuvant. The 69K antigen showed evidence of precipitation in its buffer what was even more evident in mock formulation conditions and working in vaccines concentration ranges (8-16 µg/ml). Various set of orthogonal techniques have been applied to study this precipitation phenomenon and improved 69K stability was achieved by the addition of 0.01% (v/v) Tween20 to the formulation buffer.

Western blot analysis

Western blot analysis was performed on 69K formulated with or without AlOH adjuvant in formulation buffer or in formulation buffer plus 0.01% (v/v) Tween20.

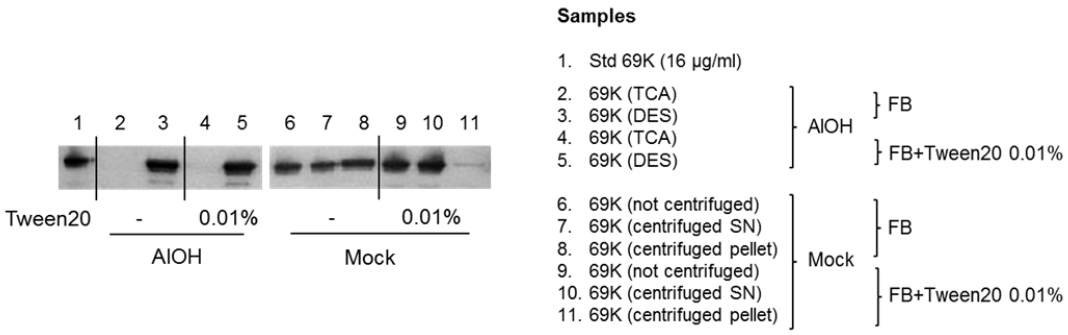

FIGURE 6. Western blot analysis showing Tween20 0.01% (v/v) effect on 69K antigen formulated at 16 µg/ml with or without (Mock) AlOH adjuvant. TCA: formulations supernatants (SN) treated with Trichloroacetic Acid to precipitate and reveal even low amounts of not-adsorbed antigens. DES: formulations treated with desorption buffer (KPi 300mM) to allow antigens separation from adjuvant surface. Mock formulations were analyzed directly or after centrifugation (both SN and resuspended pellet were loaded). The addition of Tween20 was shown to increase antigen stability.

TABLE III. Sandwich ELISA results showing Tween20 0.01% (v/v) stabilizing effect on 69K antigen formulated at 8 or 16 µg/ml with or without AlOH adjuvant. As expected, 69K is adsorbed to AlOH surface thus no antigen is quantified in AlOH supernatants. Accurate antigen quantification in mock formulation is obtained only in presence of Tween20.
N.d.: not defined. LOQ: limit of quantification of the method.

69K	Tween20 0.01%	µg/ml	
		Cexp	Cobs
AlOH SN	-	n.d.	< LOQ
	-	n.d.	< LOQ
	+	n.d.	< LOQ
	+	n.d.	< LOQ
Mock	-	8	1.10
	-	16	8.60
	+	8	8.80
	+	16	15.91

The presence of the surfactant was shown to not impact antigen adsorption profile onto AlOH surface. Moreover, Tween20 favored the recovery of 69K in vaccine supernatant after centrifugation, suggesting improved antigen stability (Figure 6).

Sandwich ELISA

In parallel to Western Blot analysis, sandwich ELISA were performed to investigate the effect of Tween20 on the stability of 69K antigen in AlOH or mock formulations. Again, antigen adsorption onto AlOH resulted not to be impacted by the presence of the surfactant in solution, which instead appeared essential for accurate antigen quantification in mock formulations at both the tested concentrations (Table III).

Dynamic Light Scattering

Dynamic Light Scattering (DLS) analysis revealed a remarkable increase in the count rate over-time when 69K antigen were analyzed at 16 µg/ml in its buffer or in mock formulations, suggesting antigen's tendency to increasingly aggregate. After 6 hours a drop in the count rate was registered, and protein precipitation occurred. This trend was reversed by the addition of Tween20 to the formulation buffer: when a stable count rate was recorded during the kinetic experiment, with

TABLE IV. Luminex indirect analysis of 69K antigen formulated at 8 or 16 µg/ml with or without AlOH adjuvant. Similarly to ELISA results, no antigen is quantified in AlOH supernatants, and accurate antigen quantification in mock formulation is obtained only in presence of Tween20.
N.d.: not defined. LOQ: limit of quantification of the method.

69K	Tween20 0.01%	µg/ml	
		Cexp	Cobs
AlOH SN	-	n.d.	0.1
	-	n.d.	< LOQ
	+	n.d.	< LOQ
	+	n.d.	< LOQ
Mock	-	8	0.87
	-	16	3.97
	+	8	7.34
	+	16	14.70

only a slight increase occurring after 6 hours. Moreover, a 4°C increase in the 69K melting temperature was registered when including Tween20 in the formulation, further proving the stabilizing effect of the surfactant (data not shown).

Gathering up the results provided by the various techniques applied, they all lead to the conclusion to include Tween20 surfactant in the formulation buffer as a useful tool to stabilize and better characterize antigens when formulated without AlOH. Tween20 is a surfactant commonly used in vaccine products (e.g. vaccines against Hepatitis A and B [63]) and was shown not to impact antigen adsorption profile onto AlOH adjuvant.

The Luminex immunoassay was then developed and optimized preparing both the calibration curve and the samples in formulation buffer containing 0.01% Tween20 (v/v). Analysis of TdaP vaccines formulated without AlOH adjuvant provided a precise 69K quantification at 8 and 16 µg/ml with observed concentrations (C_{obs}) in agreement with nominal amounts (C_{exp}) within a deviation of ± 10% (Table IV). In addition to the previously evaluated techniques, also Luminex approach suggested the need of Tween20 inclusion for accurate antigen quantification in mock formulations. Moreover, analysis of TdaP vaccines supernatants after vaccine centrifugation (10 min, 3500 x g) revealed that 69K adsorption rate onto AlOH

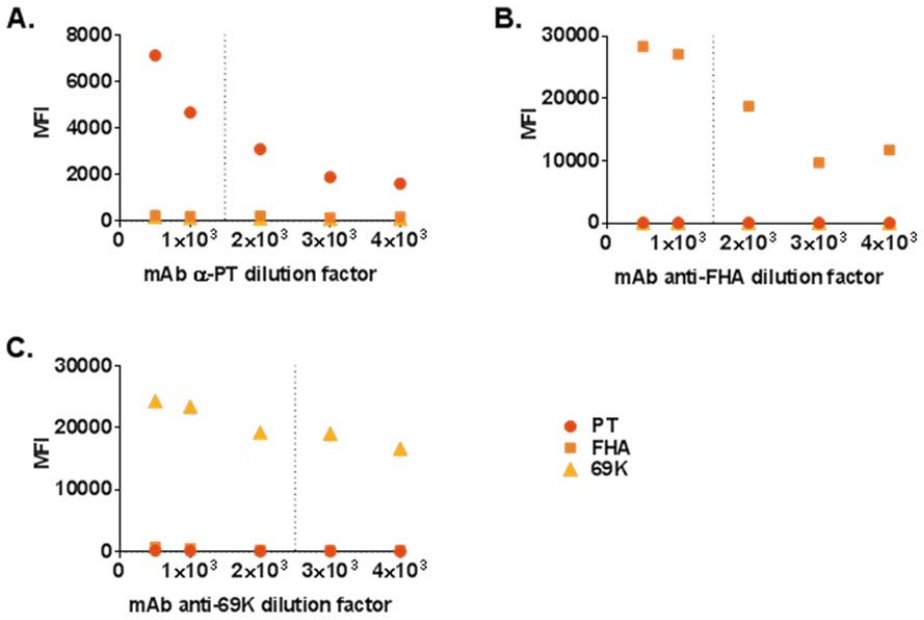

FIGURE 7. Evaluation of the cross-reactivity of the Luminex tri-plex assay. Different dilutions of mAb (A) anti-PT, (B) anti-FHA, (C) anti-69K were incubated with a mix of beads coupled with PT, FHA or 69K antigens. A detectable MFI signal was generated by each beads set in presence of the related antibody only, thus proving absence of cross-reactivity among analytes. Dotted lines represent mAbs working dilution, which corresponds to the concentration of detection antibody that yields 80% of the maximum obtainable signal (IC80).

adjuvant surface was almost complete at both the tested concentrations. Outcomes obtained from the developed Luminex immunoassay resulted to be consistent and fully comparable with those obtained by sandwich ELISA, which is considered a reference assay for aP antigens characterization in AlOH adjuvanted vaccines.

Luminex single-plex indirect assays for PT and FHA were later developed and optimized under the same conditions used for 69K antigen. Subsequently interference among the three bead sets was investigated to evaluate the feasibility to develop a tri-plex assay for the simultaneous detection of the three aP antigens. No differences in assay sensitivity and no cross-reactivity were observed among the single-plex assays thus confirming the feasibility to develop a unique tri-plex assay (Figure 7).

The ability of the tri-plex assay to characterize acellular pertussis antigens in terms of identity and quantity was evaluated on non-adjuvanted mock formulations, and once established TdaP vaccines formulated in presence of AlOH were analyzed.

The simultaneous characterization of the three aP antigens was performed on the whole TdaP vaccine in presence of AlOH with or without a preliminary treatment of the adjuvant with a saturating molecule to investigate if aspecific interactions of assay components with AlOH surface occurred. As expected, the analysis of non-saturated vaccine samples provided inaccurate quantification of antigens thus confirming the aspecific binding hypothesis. On the other hand, AlOH saturation with BSA or other saturating agents caused under-quantification of the antigens. The assumption, supported also by the observation in Figure 8, relied on a steric interference of the saturating molecule in the antigen-antibody recognition happening over the AlOH surface.

In conclusion, the indirect Luminex approach for the characterization of antigens was abandoned since in the presence of AlOH adjuvant antigen detection was not feasible. Hence the decision to move on with the direct approach was taken, relying on the direct antibody recognition, with the potential to work in a lower concentration range - thus being less subjected to adjuvant presence.

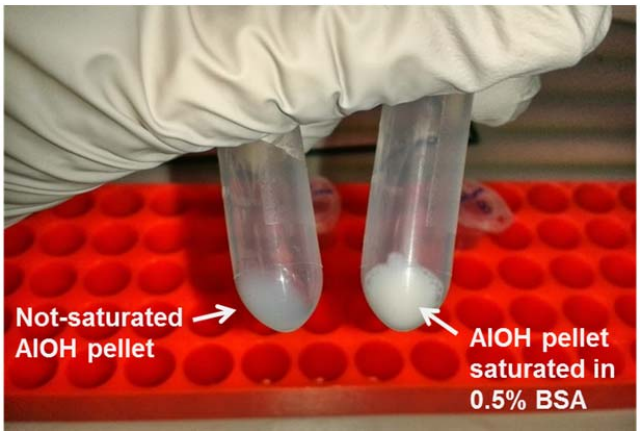

FIGURE 8. Centrifuged AlOH pellet not-saturated compared to pellet saturated in 0.5% BSA. The effect was interpreted as indication of a steric interference of the saturating molecule in the antigen-antibody recognition happening over AlOH surface.

DIRECT LUMINEX ASSAY FOR ANALYSIS OF FORMULATIONS

The direct Luminex approach for analysis of vaccine formulations consisted of coupling specific antibodies to magnetic carboxylated beads for the recognition of related antigens in vaccine formulation (Figure 9). The quantification of the fluorescence associated to each type of antibody-coupled bead led to the determination of the amount of related antigen in solution. The developed method was arranged like a sandwich ELISA but with two major differences:

- The multiplexing approach, by which multiple antigens could be analyzed simultaneously within the same sample by the use of uniquely identified fluorescent beads;
- The solution phase kinetic, which favors the detection of lower amounts of analytes compared to planar kinetic immunoassays.

The investigation of the Luminex technology as an improved analytical method for vaccine characterization through the application of the direct assay approach resulted in a manuscript entitled "Multiplex immunoassay for *in vitro* characterization of acellular pertussis antigens in combination vaccines" which was submitted to the Analytical Biochemistry Journal. The manuscript is reported in the following pages, describing in detail the procedures leading from assay development to acellular pertussis antigens characterization in AlOH adjuvanted TdaP vaccines.

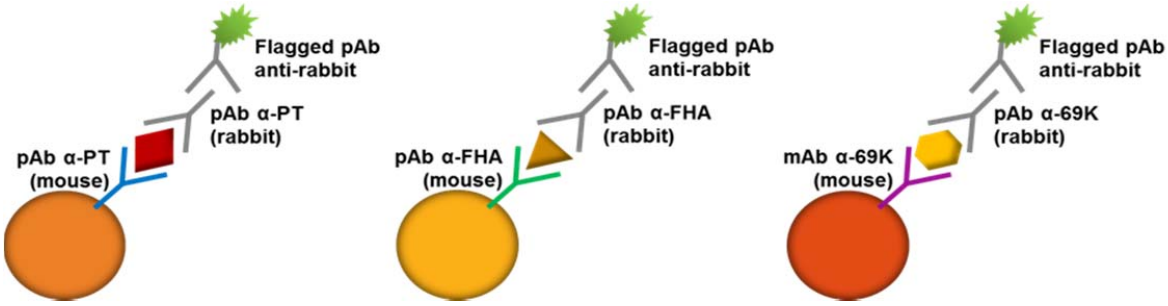

FIGURE 9. Layout of the direct Luminex assay developed for characterization of aP antigens in TdaP vaccines adjuvanted with AlOH. Capture antibodies are coupled to beads and directly recognize formulated antigens.

MULTIPLEX IMMUNOASSAY FOR *IN VITRO* CHARACTERIZATION OF ACELLULAR PERTUSSIS ANTIGENS IN COMBINATION VACCINES

Running title: "Luminex technology for vaccine characterization"

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Abstract

Vaccines *in vitro* characterization is required to identify optimal formulation conditions to ensure physical, chemical, and biological integrity of antigens and adjuvants. Analytical methods currently in use are mostly suitable for the characterization of unformulated antigens – thus requiring the complete desorption from aluminum-based adjuvants - and are not always able to reveal individual antigens in vaccine combinations. Here, Luminex technology is proposed as proof of concept for the development of improved analytical method for vaccine characterization, based on the use of specific antibodies bound to magnetic microspheres presenting unique digital signatures for simultaneous recognition of respective antigens in the entire formulation. TdaP (tetanus, diphtheria and pertussis antigens adjuvanted with aluminum hydroxide) combination vaccines were chosen as model to develop an assay able to quantify acellular pertussis (aP) antigens and their levels of adsorption to adjuvant at the same time. This assay was directly applicable on the whole vaccine, avoiding any laborious procedure for separation of the antigens from the adjuvant. Accurate and reproducible quantification of aP antigens in TdaP vaccine has been achieved in a range between 0.78 and 50 ng/mL, providing information on antigen identity, quantity, and degree of adsorption to aluminum hydroxide. Luminex characterization method has the potential to be evaluated as correlate of *in vitro* potency assays - ideally allowing reducing *in vivo* animal studies.

1. Introduction

Vaccination is an effective and successful medical intervention and vaccines have profoundly improved public health globally. Historically, progress in science and modern technologies have allowed vaccinology to move from the use of whole pathogen-based products to safer purified recombinant antigens, and adjuvants are often required to induce proper immune responses and provide adequate protection. Also, increasing interest has been shown in developing vaccines able to confer immunity against more than one infection by means of a single injection. These products are known as combination vaccines and consist of two or more immunogens physically combined into a single preparation. The value of combination vaccines is supported by the successful approach of combining diphtheria, tetanus and pertussis antigens into a single product, which has become the cornerstone of pediatric and adult immunization programs [6, 7]. Interestingly, pertussis vaccines also exemplify how the replacement of whole-cell antigens (wP) with the acellular antigens (aP) can significantly reduce reactogenicity and improve safety, paving the way to even more complex combinations (e.g., with inactivated polio, Haemophilus influenza and hepatitis B vaccine). In fact, it is expected that the need for larger combinations will further increase in future vaccine development, which may facilitate the introduction of new vaccines in current immunization schedules and simplify vaccination strategies by reducing the number of injections.

It is evident that, as vaccines move toward advanced adjuvanted combination strategies, an increase in product complexity is observed, which poses significant challenges for vaccine characterization. In fact, the development of high quality final products, as required by modern vaccinology, needs a complex set of analytical techniques to characterize each antigen and antigen/adjuvant interaction, which should be ideally applied since the early stages of research. For instance, when working with insoluble aluminum salt (e.g., aluminum hydroxide, AlOH; aluminum phosphate), antigens adsorption has to be monitored as an important parameter related to the immune-potentiator role of these adjuvants [26, 32]. However, several biochemical and biophysical methods applied to monitor antigens (e.g., gel electrophoresis, circular dichroism, liquid chromatography, colorimetric

assays) are yet mostly suitable for the characterization of unformulated antigens, due to interference with the adjuvant and/or the excipients. For example the turbidity caused by aluminum suspension inhibits direct antigen quantification by colorimetric assays (e.g., bicinchonic acid or Lowry [64]). Therefore, the need for antigens characterization in aluminum-adjuvanted vaccines is currently resolved by adjuvant dissolution [65] or by laborious and time-consuming desorption procedures, which are not always efficient and, more importantly, could alter antigens structure [23, 34, 35]. In this respect, the use of immunoassays, as the enzyme-linked immune-sorbent assay (ELISA), has represented a possible alternative for antigen quantification. Despite being reproducible and specific, ELISA methods require separate settings and analysis for each antigen included in a combination vaccine, and may require [66] or not [67] antigen desorption from aluminum salts prior quantitation, depending on the conditions. More recently antibody-based immunoassay [40] and flow cytometry [41] were also used to directly quantify antigens adsorbed onto aluminum adjuvant. However, there is an obvious need for methods able to provide information about quantity, identity and integrity of multiple formulated antigens along with the accuracy, sensitivity and specificity of immunoassays [19].

In the present study we evaluate the potential of the Luminex technology for simultaneous characterization of multiple antigens in a combination vaccine, as regard to identity, quantity and degree of adsorption to aluminum hydroxide adjuvant. Luminex is a solution-phase bead-array immunoassay, used for the analysis of multiple analytes within a single assay run (multi-plex analyses). This technology utilizes diverse sets of magnetic microsphere, each containing a different color-code (digital signature) and coated with a specific reagent, thus allowing the capture and detection of specific analytes from a sample. The analyzer can then identify each microsphere particle through the digital signature, and quantify the signal related to the captured analytes (e.g., as for classical sandwich immunoassays). This method is widely applied in clinical diagnosis and proteomics investigations [55, 56], as well as in the vaccine field for monitoring antibody titers [57-59]. As model, we here exploit the multi-plex Luminex sandwich immunoassay

for characterization of three aP antigens (genetically detoxified pertussis toxin, filamentous hemagglutinin, pertactin), when formulated with tetanus toxoid (TT) and diphtheria toxoid (DT) in AlOH adjuvanted TdaP vaccines. In fact, Luminex could combine ideally the specificity and sensitivity of immunoassays with the multi-plex capability required for the characterization of combination vaccines and the possibility to avoid laborious desorption procedure from AlOH, due to the use of magnetic solid phase and to high sensitivity.

2. Materials and Methods

2.1 Antigens and Adjuvants

TT, DT, and AlOH adjuvant were obtained from Novartis Vaccines (NVx, Marburg, Germany) manufacturing while the acellular pertussis antigens were produced by Lonza Group (Basel, Switzerland): genetically detoxified pertussis toxoid (PT, PT-9K/129G) [60], filamentous hemagglutinin (FHA) and pertactin (69K).

2.2 Preparation of adjuvanted formulations

TdaP antigens were subsequently adsorbed onto 2 mg/mL AlOH suspension (correspondent to 0.45 mg/mL Al^{3+}) [24] at the following final concentrations: 10 Lf/mL TT, 4 Lf/mL DT, 8 µg/mL PT, 8 µg/mL FHA, and 16 µg/mL 69K. Formulations were then adjusted by the addition of 10 mM Histidine buffer and 9 g/l NaCl to the recommended physiological ranges of pH (6.5 ± 0.5) and isotonicity (osmolality, 300 ± 60 mOsm/kg).

2.3 Antibodies

As capture reagents to be linked to Luminex beads polyclonal antibodies (pAb) anti-PT, polyclonal antibodies anti-FHA and a monoclonal antibody (mAb) anti-69K were used. Polyclonal antibodies anti-PT and anti-FHA were obtained through purification (Ab SpinTrap, GE Healthcare, Little Chalfont, Buckinghamshire, UK) of sera of mice immunized with monovalent antigens formulated with AlOH adjuvant, as described above. IgG concentration was measured by UV spectrometry at 280 nm. Anti-69K mAb clone E8D10D3 was obtained from Novartis Vaccines (NVx, Siena, Italy) manufacturing. As primary detection reagents polyclonal sera

anti-PT, anti-FHA and anti-69K were used. Sera were obtained by immunization of rabbits with monovalent antigens formulated with AlOH adjuvant. Animal studies were performed in compliance with the Italian law, approved by the local Animal Ethics Committee, and authorized by the Italian Ministry of Health.
Phycoerythrin-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:400) was then used as secondary detection reagent.

2.4 Assay procedure

A Luminex tri-plex immunoassay was developed to characterize acellular pertussis antigens in TdaP vaccines adjuvanted with AlOH. In detail, 40 µg of pAb anti-PT or anti-FHA and 60 µg of mAb anti-69K were coupled to the carboxyl groups of 2.5 x 10^6 MagPlex microspheres (Luminex Corporation, Austin, TX), through activation via EDC chemistry following manufacturer's instructions. Each antibody was coupled to a microsphere set, identifiable through its unique spectral signature.
Samples were diluted in 40 mM phosphate buffer (4X PBS) pH 7.2 containing 0.01% v/v Tween20 and added (50 µl/well) to black, opaque, 96-well flat-bottom plates (Bio-plex Pro™, Bio-Rad Laboratories, Inc., Hercules, CA). Antibody-coupled beads were mixed at 1:5 dilution in 1X PBS + 0.05% v/v Tween20 and were added to the samples (10 µl/well). Samples and beads were incubated for one hour. Afterwards, the primary antibodies were diluted (rabbit pAb anti-PT 1:10000, anti-FHA 1:6000, anti-69K 1:6000), mixed, and added to wells (50 µl/well). Phycoerythrin-conjugated anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 1:400) was then used for detection in a 15 min incubation step. The resulting immune-complexes were resuspended in 1X PBS pH 7.2 and analyzed on the Luminex LX-200 system. All reaction steps were carried out at room temperature, in the dark and under agitation motion using a horizontal shaker; after each incubation step, plates were washed in 1X PBS by an automatized magnetic washer HydroSpeed 96i (Tecan, Männerdorf, Switzerland).

2.5 Data analysis

The Bio-Plex Manager 5.0 software (Bio-plex Pro^{TM}, Bio-Rad Laboratories, Inc., Hercules, CA) was used to determine the concentration of antigens from median fluorescence intensities (MFI) by using a not-adjuvanted aP formulation as reference. MFI values were plotted against known concentration of the reference standard and a five-parameters nonlinear regression (5PL) [62] fitting was used to generate the curve and calculate the concentration of unknown samples through the equation: Y=d+((a-d)/(1+(X/c)^b)^g) where a, b, c, d, g were the parameters describing the curve, Y was the fluorescence reading and X the concentration to be determined.

2.6 Assay qualification

Repeatability of the assay was assessed by determination of intra-assay variation of eleven tri-plex assays performed in different days under the same operating conditions. Results were expressed as mean percentage coefficient of variation (%CV). Indication on assay sensitivity was obtained through the analysis of individual blank wells (n = 80) consisting of sample diluent. The detection limit (DL) and the quantification limit (QL) were calculated for each analyte and expressed respectively as DL = 3.3σ/S and QL = 10σ/S where σ is the standard deviation of the MFI responses of blank samples and S is the slope of the calibration curve.

Spike recovery studies were performed adding a known amount of aP antigens to supernatants of centrifuged AlOH-containing vaccines (10 min, 3500 x g), thus gaining indication on assay accuracy. Spike values were expressed as percentages of aP antigens doses in TdaP vaccine: 1% spike corresponded to 80 ng/mL PT, 80 ng/mL FHA, 160 ng/mL 69K; 10% spike corresponded to 800 ng/mL PT, 800 ng/mL FHA, 1600 ng/mL 69K; and 100% spike corresponded to 8 µg/mL PT, 8 µg/mL FHA, 16 µg/mL 69K.

Specificity of the assay was evaluated through evaluation of the potential cross-reactivity of the tri-plex assay, defined as the ability of an antibody to react with similar antigenic sites in different proteins, thus providing non-specific responses.

Experiments were arranged to compare MFI generated by each specific assay in presence of the relative antigen at 0.78 ng/mL or of the two non-related antigens in combination (50 ng/mL each).

Inhibition experiments were performed to gain additional information on assay specificity. In detail, individual aP antigens in their own buffer (bulk) were diluted at concentrations ranging from 1.56 to 200 ng/mL and incubated in presence or in absence (absence of inhibition) of fixed concentrations of capture antibodies anti-PT (4 ng/mL), anti-FHA (2 ng/mL), and anti-69K (1.2 ng/mL) singularly or mixed together. Remaining non saturated antigens were then quantified by the tri-plex assay.

2.7 Assay application to TdaP vaccines

Assay application to TdaP vaccines followed three different procedures – illustrated in Figure 5 - to gain a complete perspective of the state of antigens in the vaccine. (A) After vaccine centrifugation (10 min, 3500 x *g*) formulation supernatants were recovered and directly analyzed; (B) Entire TdaP vaccine formulation was linearly diluted in PBS 4X + 0.01% v/v Tween20 to reach a concentration range for aP antigens between 50 and 0.78 ng/mL, and directly analyzed in presence of AlOH adjuvant; (C) TdaP vaccine was linearly diluted as described in procedure B, samples from each dilution step were then centrifuged (10 min, 3500 x *g*) to allow AlOH separation and supernatants were recovered for the analysis. The $80 < \%(C_{obs}/C_{exp}) < 120$ range for acceptability of antigens quantification was used.

2.8 Statistics

Statistical analysis was performed by the use of Graph Pad Prism 5.0 software (GraphPad Software Inc, San Diego, CA). Calibration curves were interpolated with a 5PL non-linear regression model. Comparison between single-plex and tri-plex methods was based both on a Deming regression study followed by evaluation of correlation coefficients using Pearson method. Significant differences ($p \leq 0.05$) among groups in cross reactivity experiments were ascertained using the two-tailed t test at the 95% confidence interval, while inhibition experiments were evaluated through ANOVA analysis followed by Bonferroni test of significance at the 95%

confidence interval. Linearity of the analytical procedure in the identified working range was evaluated by linear regression among data points.

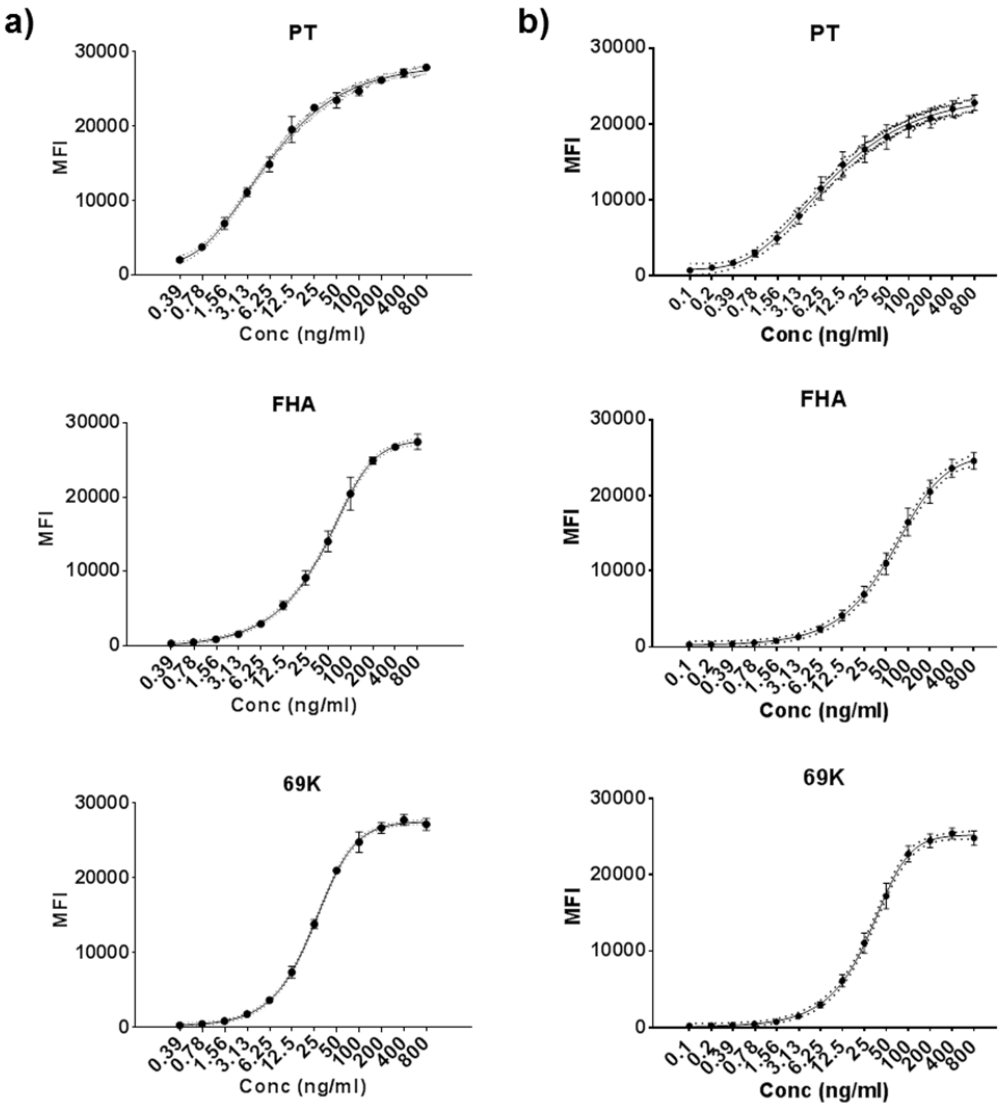

FIGURE 10. Calibration curves of **a)** mono-plex assays and **b)** tri-plex assay used to determine antigens concentration from median fluorescence intensities. Mean fluorescence values associated to each concentration are shown together with 95% confidence interval. Curves are fitted with a 5-PL nonlinear regression model (continuous connecting line), 95% confidence bands associated to the fitting are represented by the dots. Parameters describing 5-PL tri-plex curves: PT (d = 542.1, a = 23264.2, c = 0.57, g = 3.65, b = -0.61), FHA (d = 226.7, a = 25437.7, c = 109.2, g = 0.6, b = -1.5), 69K (d = 61.2, a = 25236.7, c = 54.7, g = 0.46, b = -2.1).

3. Results

The feasibility of Luminex technology for characterization of aP antigens was first tested by creating individual single-plex sandwich immunoassays for each of the three aP antigens. Three different digital signatures regions of magnetic beads (i.e., MC10033, MC10036, MC10039) were used for PT, FHA, and 69K, respectively. The 5PL nonlinear regression models were chosen to generate calibration curves for extrapolation of concentration values from MFI readings, as these models are commonly used to fit immunoassay or bioassay data [62].

As capture reagents, mouse pAb anti-PT, pAb anti-FHA and mAb-anti69K were selected and coupled to the respective Luminex beads, whereas rabbit polyclonal sera anti-PT, anti-FHA and anti-69K were used as detection reagents. Bulk aP antigens were then individually tested in the single-plex immunoassays at the concentrations present in TdaP vaccine (i.e., 8 µg/mL PT, 8 µg/mL FHA, and 16 µg/mL 69K). Since the used bulk antigens may present physical instability when formulated at the vaccine concentrations in absence of adjuvant (not shown), 0.01% v/v Tween20 (a surfactant commonly used in vaccine products [63]) was added to prevent precipitation events. Three independent calibration curves were generated in the concentration range 0.1 - 800 ng/mL for each individual aP antigen (Figure 10a). The working range of each single-plex assay was identified between 0.78 and 50 ng/mL since in this range the analytical procedure provided appropriate accuracy (deviation of ± 20% between C_{obs} and C_{exp}) and linearity ($R^2 > 0.89$).

We thus analyzed if multiplex conditions, i.e. the simultaneous detection of the three antigens (and thus of three bead sets), could lead to possible interference. To this aim, a sample containing the three antigens in assay buffer at 800 ng/mL was serially diluted 1:1 (v/v, with 4X PBS buffer) till 0.1 ng/mL and analyzed. As shown in Figure 10b, curves obtained under the tri-plex assay configuration were properly fitted into the 5-PL nonlinear regression model for each antigen (R^2 = 0.877, 0.929, 0.962, for PT, FHA and 69K, respectively), indicating that no major interference were occurring. Importantly, comparison of the MFI of the single-plex assays with those of the tri-plex assay through Deming regression model and the Pearson correlation coefficients (Figure 11) showed good correlation (PT = 0.9930,

FHA = 1.000, 69K = 0.9930). The confirmation that the two assay configurations (single-plex and tri-plex) lead to comparable results prompted us to further evaluate additional assay parameters.

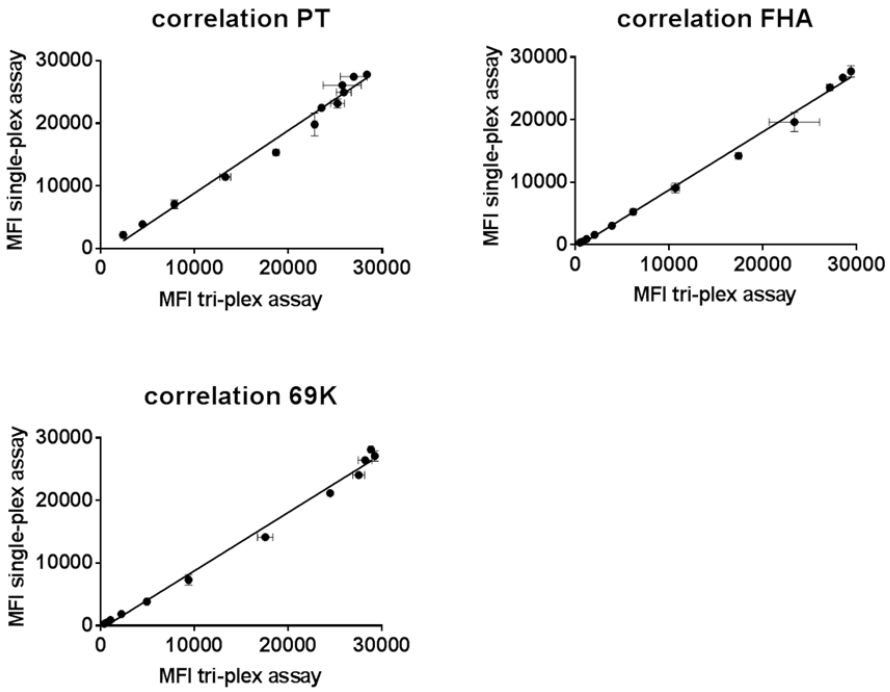

FIGURE 11. MFI generated by single-plex and tri-plex assays were compared through the Deming regression model obtaining the following best fit values: PT slope 1.002 ± 0.04109, y-intercept when x=0 -1164 ± 854.7; FHA slope 0.9286 ± 0.02081, y-intercept when x=0 -509.1 ± 352.0; 69K slope 0.9322 ± 0.02494, y-intercept when x=0 -569.1 ± 470.1. Comparability of single-plex and tri-plex assays was further confirmed by Pearson correlation which provided these correlation coefficients: PT r = 0.9917, FHA r = 0.9975,

3.1 Assay qualification

The repeatability of the tri-plex assay was determined through inter-assay variation data, calculated in the 0.78 - 50 ng/mL working range of eleven independent standard curves. Values of mean percentage coefficients of variation (Table V) resulted below 15% for all the concentrations considered and for all three antigens, thus showing repeatability of the multi-plex immunoassay [68].

The sensitivity of the tri-plex assay was assessed through the collection of MFI values of 80 blank wells analyzed in independent experiments. Detection limits (DL) and quantification limits (QL) were calculated as MFI values. QL were converted in concentration through data interpolation in the relative 5-PL calibration curve,

while DL (in MFI values) were lower than the lowest values of the respective curves (Table VI). Spike recovery studies were performed by adding to AlOH-adjuvanted vaccine supernatants known concentrations of aP antigens, corresponding to 1%, 10% or 100% of the vaccine doses. Results are listed in Table VII and showed a spike recovery equal or higher than 80% for all the condition tested, thus establishing assay accuracy across the specified range.

To assess the specificity of the tri-plex assay, cross-reactivity experiments were then conducted. To this aim, the MFI values were measured for each bead set in presence of the lowest concentration (0.78 ng/mL) of the related antigen and compared to the MFI signals measured in presence of the highest concentration (50 ng/mL) of both the other non-related antigens. For example, the signal obtained

TABLE V. Assay repeatability expressed as mean percentage coefficient of variation (% CV) of eleven tri-plex assays performed under the same operating conditions.

ng/ml	% CV		
	PT	FHA	69K
0.78	3	13	3
1.56	5	7	5
3.13	5	6	3
6.25	10	4	4
12.5	9	3	2
25	7	3	3
50	14	3	8

TABLE VI. Detection limit (DL) and quantification limit (QL) for each aP antigen expressed as DL = 3.3σ/S and QL = 10σ/S, where S is the slope of the calibration curve and σ is the standard deviation of the responses of blank samples. (n.a., not available, MFI lower than the lowest point of calibration curve).

	PT		FHA		69K	
	MFI	ng/ml	MFI	ng/ml	MFI	ng/ml
DL	320.6	n.a.	162.2	n.a.	36.7	n.a.
QL	971.5	0.188	491.5	0.691	111.3	0.087

TABLE VII. Assay accuracy reported as percent spike recovery of known amounts of aP antigens added to AlOH-adjuvanted vaccine supernatants: % aP are referred to % of PT, FHA, or 69K antigens doses in TdaP vaccine (1% correspond to 80-80-160 ng/ml; 10% correspond to 800-800-1600 ng/ml; 100% correspond to 8-8-16 µg/ml).

% aP	% spike recovery		
	PT	FHA	69K
1	95.7	98.8	80.4
10	80.2	80.7	80.0
100	80.4	86.2	85.6

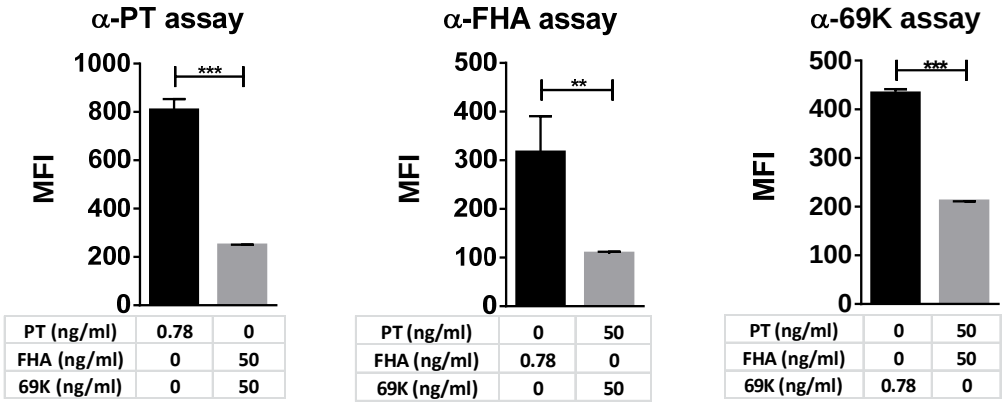

FIGURE 12. Specificity of the tri-plex assay was assessed through analysis of cross-reactivity. MFI signals generated by each bead of the tri-plex assay in presence of the related antigen at 0.78 ng/ml - the lowest concentration in assay working range – were reported in black. Cross-reactivity fluorescence (grey bars) derived from beads which recognize the corresponding non-related antigens both at 50 ng/ml - the highest concentration at which antigens are analyzed.
** $p < 0.01$, *** $p < 0.005$

with beads recognizing PT in presence of 0.78 ng/mL PT was compared with the signal generated in presence of 50 ng/mL of FHA plus 50 ng/mL 69K. As shown in Figure 12, the non-specific signal with high concentrations of unrelated antigens resulted in every case significantly lower than the signal generated by the lowest concentration of related antigen (0.78 ng/mL). To further evaluate specificity, inhibition experiments were also performed, meaning that the Luminex experiment was started after preincubation of each aP antigen with the respective capture antibody (as single or in mixture with the other two capture antibodies), resulting in MFI inhibition, as expected. On the contrary, a response similar to the one obtained without inhibition was found when using non-related antibodies, thus confirming tri-plex assay ability to provide information on antigens identity (Figure 13). Moreover, after exposure to a thermal stress (e.g., overnight incubation at a temperature superior than antigens melting point) antigens were no more properly recognized and quantified by the related antibody (data not shown).

3.2 Assay application to TdaP combination vaccine

As following step, tri-plex assay was applied to whole TdaP vaccine, to verify the possibility to use it for characterization purposes. The TdaP vaccines were assayed at linear dilutions from 50 to 0.78 ng/mL of aP antigens. To evaluate the accuracy

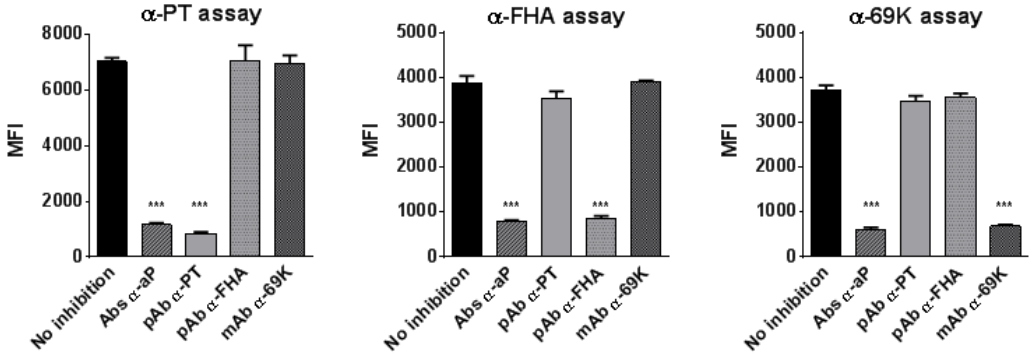

FIGURE 13. Linear dilutions of individual aP antigens were incubated in presence or in absence (no inhibition) of fixed concentrations of capture antibodies pAb α-PT, pAb α-FHA, mAb α-69K singularly or mixed together. MFI reduction was observed for each aP antigen when incubated with the respective capture antibody or with a mixture of the three capture antibodies. On the contrary, a response similar to the one obtained without inhibition was found when using non-related antibodies, thus confirming specificity of the tri-plex assay.
Statistical significance (*** $p < 0.05$) was calculated through ANOVA analysis followed by Bonferroni test using no inhibition as controls groups.

of calibration curves in quantifying antigens, control samples formulated at known concentrations and in the absence of AlOH adjuvant were added to the experiments. Samples containing AlOH only were also included in the analysis to explore the risk of artificial fluorescence readouts. However, no evidence of indirect binding of reagents used in the assay to AlOH surface was found (data not shown).

Assay application on TdaP vaccines followed three different procedures - illustrated in Figure 14 - to gain a complete perspective of the state of antigens in the vaccine: A. analysis of antigens in vaccine supernatant, to evaluate the amount of non-adsorbed antigens; B. analysis of antigens in TdaP vaccine adjuvanted with AlOH, to evaluate total antigen amount; C. analysis of vaccine supernatants after vaccine dilution and AlOH separation, to evaluate antigen desorption from AlOH occurring during dilution.

Interestingly, although aP antigens resulted almost completely adsorbed to AlOH adjuvant, the high sensitivity of Luminex assay allowed the detection and the quantification of very low amounts of not-adsorbed antigens. In fact, these values, calculated as percent ratio between the amount of non-adsorbed proteins and the total amount of formulated protein in several independent assays, were found to be 0.40 ± 0.10 % for PT, 0.18 ± 0.05 % for FHA and 0.16 ± 0.09 % for 69K. Efficient

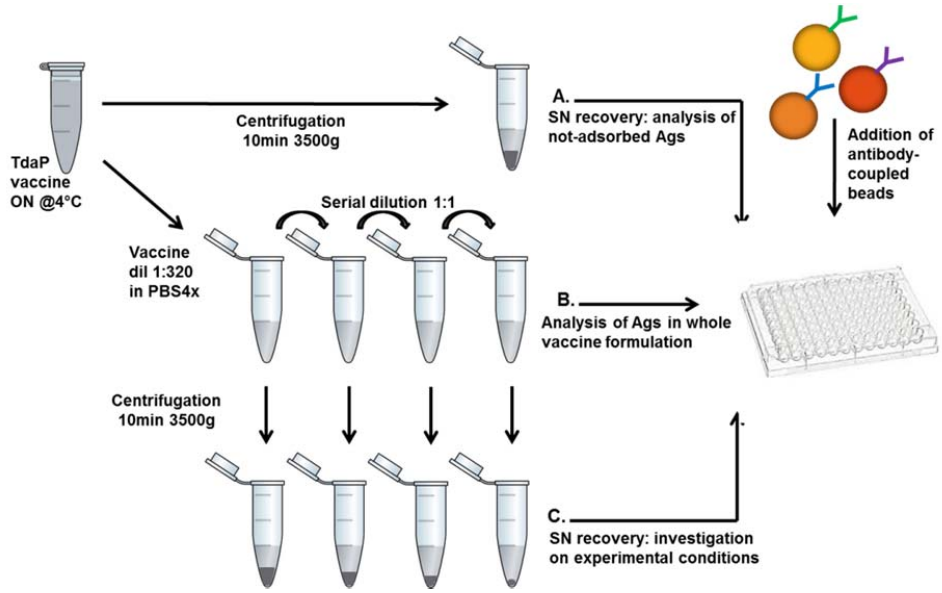

FIGURE 14. Assay application to TdaP vaccines followed three different procedures to gain a complete perspective of the state of antigens in the vaccine. A. analysis of non-adsorbed antigens in formulation supernatants after vaccine centrifugation; B. analysis of antigens in whole TdaP vaccine formulation linearly diluted and directly analyzed in presence of AlOH adjuvant; C. TdaP vaccine linearly diluted (as described in procedure B.) and centrifuged to allow AlOH separation and recovery of supernatants which were analyzed to evaluate the influence of experimental conditions on antigens adsorption to AlOH surface.

adsorption to AlOH was expected since the experimental isoelectric points of aP antigens (PT = 5-6 or 8.6-9 depending on the subunit, FHA = 6.7-7.7, 69K = 5.9-6.1) were compatible with the one of AlOH (pI = 11) when formulation pH was adjusted to the physiological range. Moreover, the results obtained were in agreement with the qualitative information obtained analyzing vaccine supernatants by Western blot analysis in which no antigens were detected (data not shown).

Quantification of antigens in TdaP vaccines in presence of AlOH adjuvant was performed by the analysis of six linear dilution steps of the whole vaccine, which resulted in observed antigens concentrations (C_{obs}) in agreement with nominal amounts (C_{exp}) with a deviation of ± 20%, indicating assay linearity (Figure 15). The only measure showing a deviation higher than 20% was recorded when analyzing PT at 25 ng/mL, likely related to the fact that this concentration is close to the upper plateau of PT calibration curve, where linearity is poorly satisfied (Figure 10). Assay performance for aP antigens quantification in TdaP vaccines adjuvanted with AlOH was also compared to the quantification of not-adjuvanted

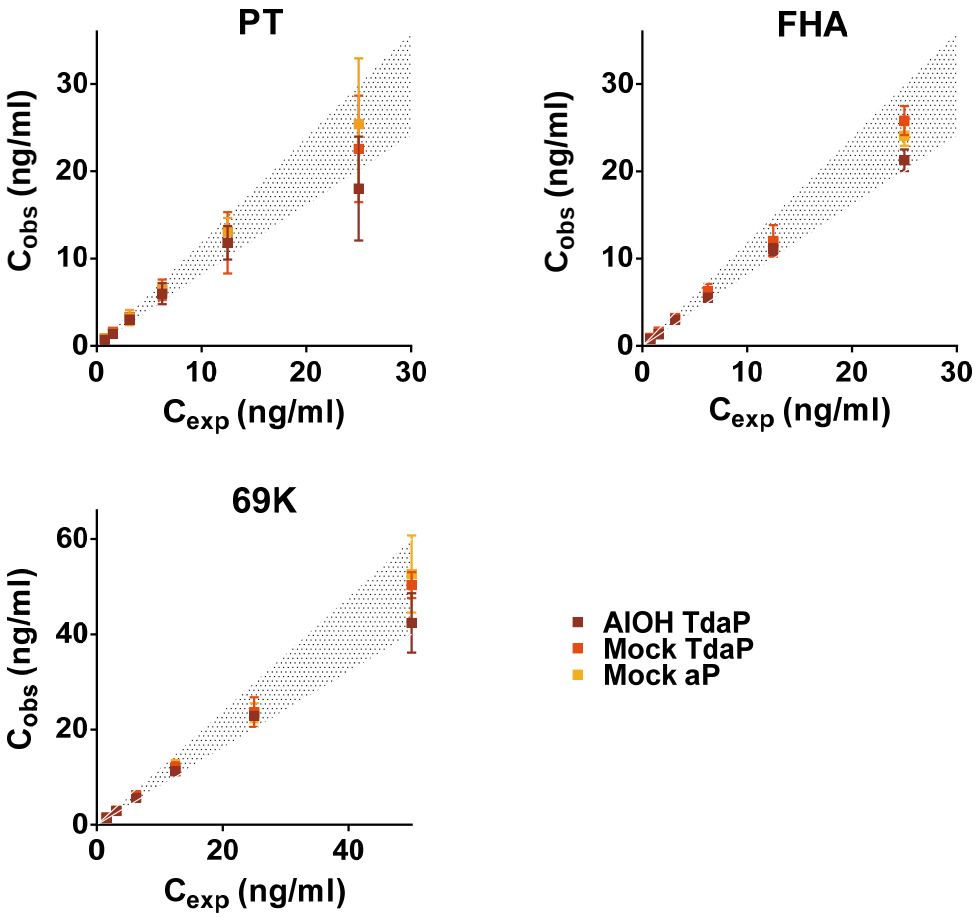

FIGURE 15. Analysis of PT, FHA, and 69K antigens in whole TdaP vaccine in presence of AlOH adjuvant (dark red) performed at six linear dilution steps of the vaccine. Analysis of aP antigens in TdaP mock (orange) and in aP mock (yellow) formulated without adjuvant was added to evaluate assay ability to unequivocally quantify aP antigens also in presence of potentially interfering vaccine components. The highlighted region indicates the accepted range for quantification (80 < %(Cobs/Cexp) < 120). Quantification of PT at 25 ng/ml showed a deviation higher than 20% which is related to the fact that this concentration is close to the upper plateau of PT calibration curve.

aP antigens in combination or not with TT and DT toxoids (mock formulations). As shown in Figure 13, assay specificity was confirmed under these conditions resulting in unequivocally assessment of the analytes, also in presence of potentially interfering vaccine components, such as AlOH adjuvant or other antigens.

Finally, the influence of experimental conditions on antigens adsorption to AlOH surface was evaluated by diluting TdaP vaccine in assay buffer, and analyzing vaccine supernatants after centrifugation. As shown in Figure 16, data indicated that aP antigens underwent a spontaneous desorption from AlOH surface during dilution,

probably due to the high concentration of the phosphate buffer used. Notably, while the quantification of PT and 69K antigens in the centrifuged diluted samples was complete, almost 40% of FHA resulted to be still bound to adjuvant particles (Figure 16). Nevertheless, this did not impact the overall antigens quantification in TdaP vaccine, which was achieved only by diluting TdaP vaccine in phosphate buffer.

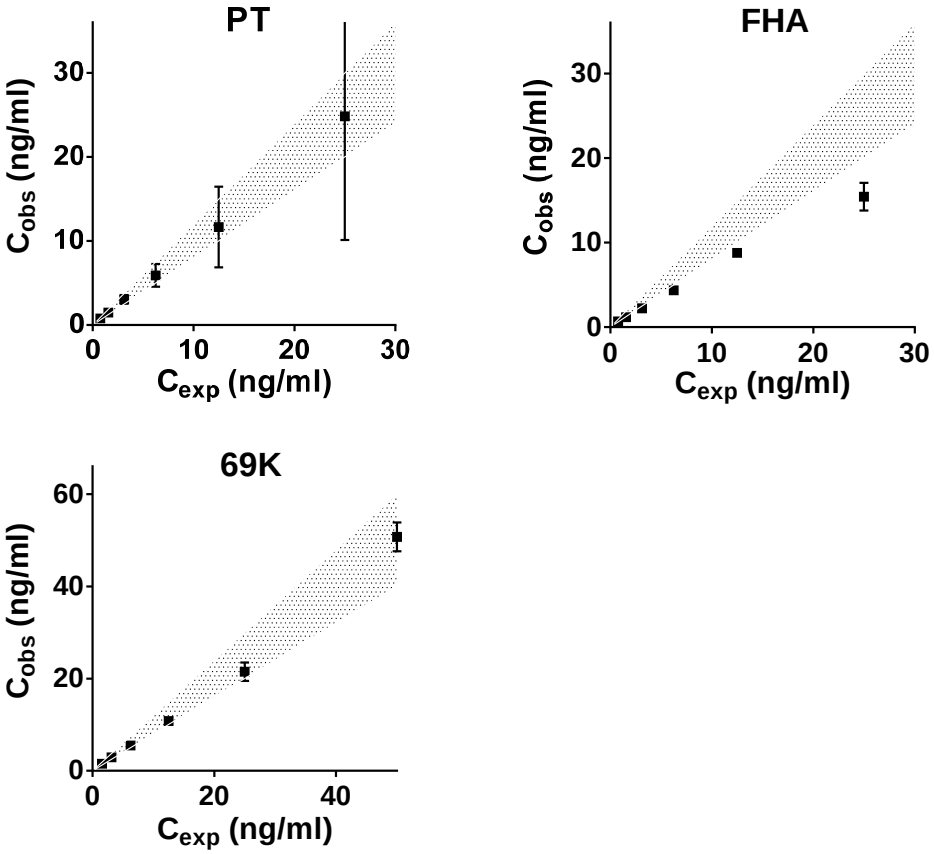

FIGURE 16. Analysis of PT, FHA, and 69K antigens recovered in supernatants after TdaP vaccine linear dilution and centrifugation. Supernatants analysis was performed to evaluate the influence of experimental conditions on antigens adsorption to AlOH surface: PT and 69K quantification in centrifuged diluted samples was complete while FHA appeared to be still partially bound to AlOH particles. The highlighted region indicates the accepted range for quantification ($80 < \%(Cobs/Cexp) < 120$).

4. Discussion

In this work the potential of Luminex technology to develop a multi-plex microsphere-based immunoassay for simultaneous analysis of acellular pertussis antigens (PT, FHA, 69K) in AlOH adjuvanted TdaP vaccines was exploited. Luminex technology is currently applied in various fields ranging from genetic disease diagnostic [69, 70] to bio surveillance [71, 72]. To the best of our knowledge, this is the first reported application for multi-target characterization of antigens in a complex vaccine formulation, such as the adjuvanted trivalent TdaP vaccine.

Since the assay relies on antibody recognition and fluorescence detection, it combines both the specificity and the sensitivity required for antigen characterization in the final vaccine formulation. Particularly, the assay specifically determines identity and quantity of antigens without laborious procedure for physical separation from AlOH adjuvant, thus preventing protein loss and structure alterations such as damage of critical epitopes by exposure to stringent experimental conditions required for desorption processes [23, 34, 35]. In fact, the simultaneous characterization of the three aP antigens was performed directly on the whole TdaP vaccine containing AlOH without any preliminary treatment of the adjuvant. Previous works reported that detection antibodies have the tendency to non-specifically adsorb to AlOH [40, 41] mainly through ligand exchange mechanisms and electrostatic interactions [73]. Despite that, no evidence of indirect binding to AlOH of the reagents used leading to unspecific signals was found in the Luminex assay, and analysis of adjuvant led to fluorescence readouts comparable to blank thus confirming assay specificity.

The analytical procedure reported here was developed as proof of concept for possible use of Luminex technology for vaccine characterization purposes, and thus to identify possible advantages and/or drawbacks to support early R&D application. In fact, the analysis of the current immunoassay for GLP-compliant pre-clinical safety study or for clinical purposes would require a full-validation process and in depth characterization of the reagent used [74]. For example, polyclonal antibodies were used as capture reagents in this work and additional analyses should be

addressed to appropriately select monoclonal antibodies capable to recognize antigen epitopes critical for vaccine immunogenicity. This would indeed have the potential to test vaccine quality and consistency among batches, potentially allowing correlations with the clinical efficacy of the product, which is commonly evaluated through *in vivo* potency test in animal models. It is notably that quantification of antigen content as a surrogate of potency has already been applied for viral vaccines [75-79] with the aim to find a close correlation between antigen amount, degree of adsorption to adjuvant and efficacy of the final product. The current study could then be considered as a model to further set up *in vitro* potency assays thus supporting the replacement of animal tests accordingly to the 3Rs concept (Refinement, Reduction and Replacement of animal testing) [80]. Nevertheless, we addressed a number of typical steps and parameters required for pre-validation analysis, in order to evaluate actual feasibility and possible challenges. Importantly, the method has proved to work in the multi-plex configuration with good specificity, accuracy and linearity, in a range between 0.78 and 50 ng/mL, which indeed allows simultaneous aP antigen characterization in a single raw of linear dilution of vaccine. Moreover, the Luminex method presented here provides a substantial improvement in terms of sensitivity as compared with other commonly used immunoassays [81, 82], which is achieved through antibody recognition, generation of a high resolution fluorescent signal and solution phase kinetics. In fact, sensitivity is often a critical parameter for vaccine characterization methods, since antigens are usually formulated in vaccines at significantly lower concentrations than those measurable with currently available analytical tools, especially when considering booster dosages [36]. For example, the o-Phthalaldehyde (OPA) fluorescent protein assay is extensively used to determine protein content in vaccine formulations with a moderate sensitivity (20 - 500 μg/mL), but it lacks specificity when multiple antigens are present in the samples [38, 83]. Recently, a chemo-luminescent method with similar sensitivity and specificity has been developed to quantify alum-adsorbed vaccine antigens through determination of total nitrogen content [39]. Importantly, the high sensitivity of the Luminex tri-plex assay allowed precise quantification of low amounts of antigens in

formulation supernatants after AlOH separation via vaccine centrifugation. Antigens adsorption on AlOH adjuvant surface is yet considered a very important parameter for the function of this adjuvant [26, 32] (e.g., WHO recommends adsorption values equal or greater than 80% for DT and TT [84]).

Additional analysis performed on centrifuged aliquots of TdaP vaccine after formulation dilution in assay buffer led to the conclusion that antigens undergo a spontaneous desorption from AlOH surface during the experimental procedure. Since antigens adsorption to AlOH is strongly influenced by pH, ionic strength, and presence of anions such as phosphate [85], it is reasonable that phosphate ions contained in assay buffer compete with proteins for AlOH binding [86]. While complete desorption from AlOH was observed for PT and 69K antigens, only partial recovery of FHA was possible in formulation supernatants, which could probably be related to its filamentous and repetitive structure[87, 88] and thus to stronger binding to AlOH. Nevertheless, quantification of antigen in the total formulation was confirmed to be accurate, essentially showing that a common characterization protocol including analysis of full vaccine formulation after linear dilution and of supernatant would allow concomitant detection of total amount of antigens and of percentage of adjuvant adsorption, respectively, through a simple workflow (A and B in Fig. 12), and in an individual assay format.

Taking together, a novel vaccine characterization method through the application of Luminex technology is here presented, with possible application in early research and potential for consideration in future development and validation processes. Importantly, this method could represent a significant improvement for the *in vitro* characterization of complex AlOH-adjuvanted vaccines because providing - in a single experiment - information on antigen identity, quantity, and degree of adsorption to adjuvant surface of each immunogen included in a multivalent combination. In principle, its use may also find application for larger vaccine combinations and in presence of other adjuvants and delivery systems such as emulsions and polymeric particles, and experiments are ongoing to evaluate this possibility. In conclusion, this novel application of Luminex technology may overcome certain challenges typically experienced with the available techniques for

antigens characterization, like sensitivity issues, laborious and potentially critical procedures for antigen desorption from AlOH and simultaneous detection of different antigens, with further potential for future development.

5. Acknowledgements

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ANTIGENS PRECIPITATION UPON CENTRIFUGATION

As previously described, accurate and reproducible quantification of aP antigens in TdaP vaccine has been achieved by the application of Luminex technology for the characterization of TdaP vaccine formulations. Through direct antibody recognition the developed assay provided information on antigen identity, quantity, and degree of adsorption to adjuvant surface.

Among various scientific questions concerning *in vitro* vaccine characterization, one of the major concerns for formulation scientists is the determination of protein structure and stability in presence of an adjuvant. What happens to antigenic structures when adsorbed to AlOH has been rarely characterized since this information is not easily accessible due to the turbidity of the vaccine formulation and adjuvant interference with many available analytical techniques. Another challenge is the evaluation of the degree of adsorption of antigens to AlOH surface. Usually if no antigen is recovered in the supernatant after vaccine centrifugation, AlOH adjuvant adsorption is assumed. However, co-precipitation of antigens with AlOH particles cannot be excluded. In this study, preliminary analysis of mock

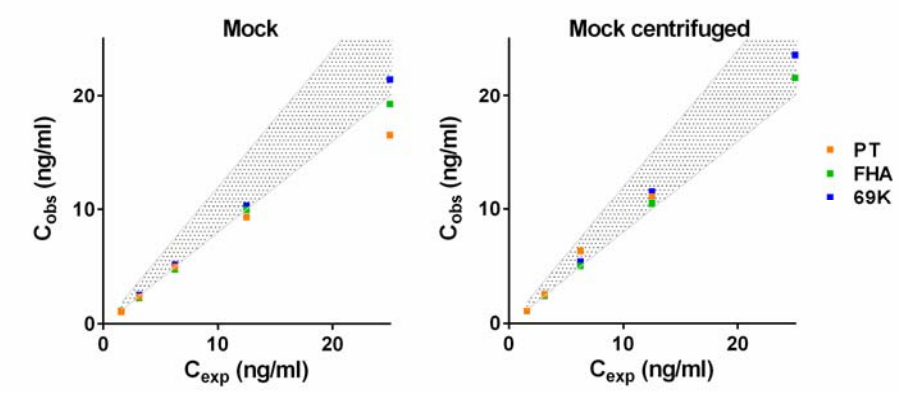

FIGURE 17. Analysis of PT, FHA, and 69K antigens formulated in mock TdaP vaccines (without AlOH adjuvant). Analysis was performed on five linear dilution steps of the mock vaccine (left) or of the supernatant of the mock vaccine recovered after centrifugation. Supernatant analysis was performed to evaluate antigens stability in formulation conditions when not adsorbed to AlOH surface. The highlighted region indicates the accepted range for quantification (80 < %(Cobs/Cexp) < 120).

formulations subjected or not to a centrifugation step was performed to evaluate protein precipitation in formulation conditions, with the conclusion that centrifugation *per se* does not have an impact on the antigen stability when formulated in absence of AlOH adjuvant (Figure 17). Despite that, phenomena observed in mock experimental conditions may not represent a real case in presence of the adjuvant, and antigens co-precipitation with AlOH upon centrifugation cannot be excluded yet remaining an un-resolved technical challenge.

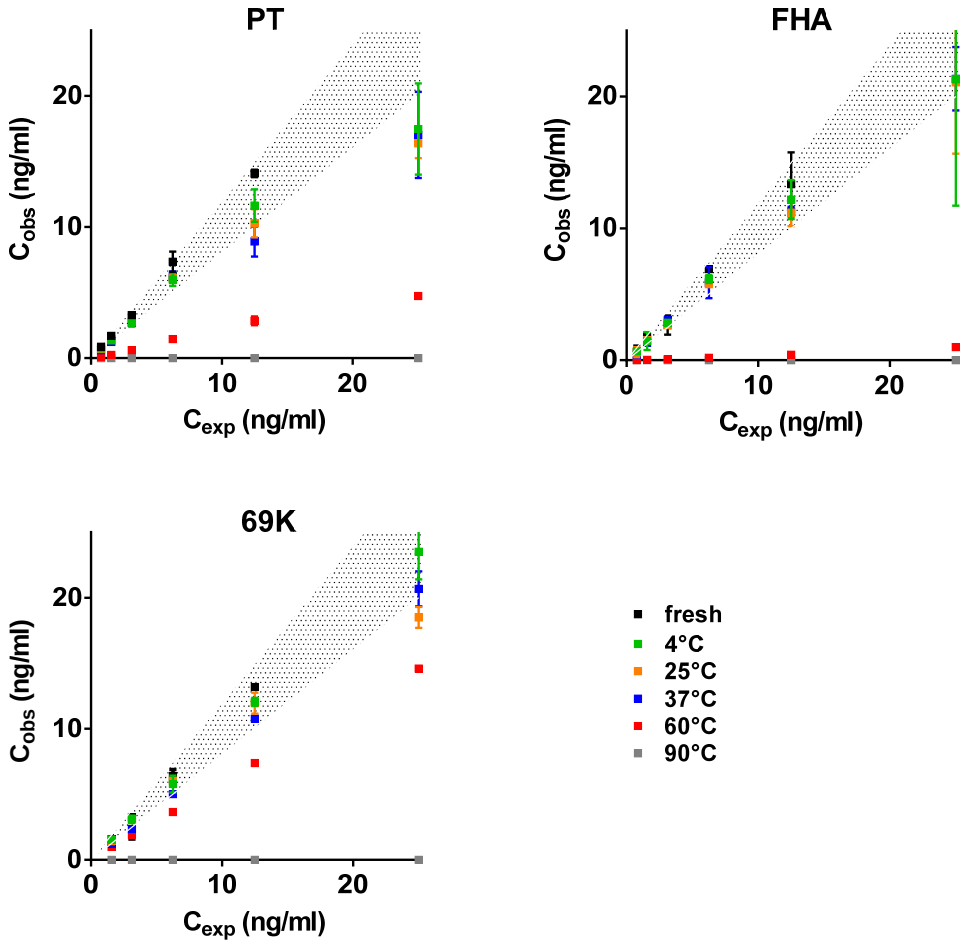

FIGURE 18. Analysis of PT, FHA, and 69K antigens after overnight exposure to different temperatures: 4, 25, 37, 60, 90 °C. Analysis was performed on six linear dilution steps of the stressed antigens mixed together to preliminary investigate the abilty of capture and detection antibodies used in the assay to recognize damaged antigen epitopes. The highlighted region indicates the accepted range for quantification (80 < %(Cobs/Cexp) < 120).

Individual acellular pertussis bulk antigens (in their buffer) were exposed overnight to different temperatures (4, 25, 37, 60 and 90 °C) and analyzed for the ability of antibodies to still recognize antigen epitopes.

The analysis proved that the developed analytical method was suitable to provide information about antigens integrity and stability. While increasing the temperature, observed antigen concentrations (C_{obs}) resulted less in agreement with nominal amounts (C_{exp}) (Figure 18). The highlighted regions in the graphs correspond to the selected acceptable range for antigen quantification $80 < \%(C_{obs}/C_{exp}) < 120$. After an overnight incubation at 60 or 90 °C, no points fit into respective regions any longer, and none of the three antigens was properly quantified confirming that antigens conformational changes could be assessed by this assay. Differential scanning fluorescence (DSF) experiments performed by Novartis Vaccines colleagues provided melting temperatures (Tm) of the antigens which resulted in agreement with the qualitative information obtained using Luminex (data not shown). In fact PT unfolding was identified as a multiple-transition process at 60, 72, and 85 °C, while FHA presented a single Tm at 45 °C. 69K was confirmed to be the most stable antigen showing a slight transition at 65 °C followed by a more marked structural rearrangement at 81 °C.

The ability of the antibodies used in this assay to recognize damaged antigen epitopes could be exploited in forced degradation studies - an essential step in vaccine formulation development - for the identification of potential vaccine stabilizers [89]. Additional work should be addressed to replace capture antibodies currently used in the assay with appropriately selected and characterized monoclonal antibodies capable to recognize antigen epitopes critical for vaccine immunogenicity.

The potential of the developed Luminex-based assay to be applied for characterization of vaccines in presence of different adjuvants was discussed in the previously reported manuscript and is here further investigated for the MF59 adjuvant. The MF59 oil-in-water (o/w) emulsion was licensed in 1997, being the first alternative adjuvant approved for human use after aluminum salts. The oil used in MF59 is squalene which is emulsified - involving the non-ionic surfactants Tween80 and Span85 - and then processed with a microfluidizer. The final o/w emulsion is stable and composed of oil droplets uniformly distributed at 160 nm [13].

In vitro characterization of vaccines adjuvanted with an emulsion normally requires separation of oil phase from the aqueous phase by high speed centrifugation. However oil droplets – even the little amount left after phase separation - might interfere with routine analytical procedures causing light scattering phenomena. In addition, the milky aspect of MF59 is not favorable when working with techniques based on light absorbance [34].

Since Luminex technology conceives an event as "positive" only if recognized both by the red and the green detection lasers, this investigation relied on the hypothesis that oil droplets would not be registered and would pass undetected through the

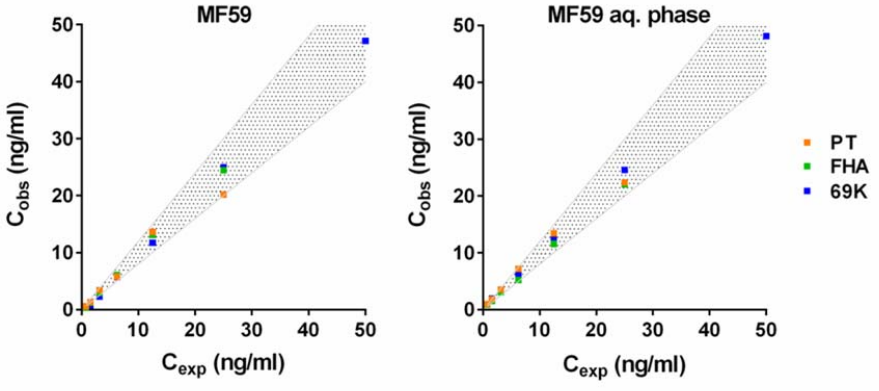

FIGURE 19. Analysis of PT, FHA, and 69K antigens in TdaP vaccine adjuvanted with MF59. Analysis was performed at six linear dilution steps of the vaccine in presence of the emulsion adjuvant (left) or on the aqueous phase only after vaccine centrifugation (right). The highlighted region indicates the accepted range for quantification (80 < %(Cobs/Cexp) < 120).

detection chamber. Based on this interpretation, Luminex direct assay was applied to TdaP antigens formulated in MF59. The quantification of aP antigens performed in the whole MF59 formulation resulted comparable to the one performed in the aqueous phase only (Figure 19) thus confirming the initial hypothesis.

It is known that antigens formulated in MF59 could encounter instability processes due to interactions with oil and water interfaces [34]. Despite that, no modification in quantification and identification of antigens were detected, suggesting that TdaP antigens are compatible with MF59 emulsion adjuvant.

CHAPTER III. *IN VIVO* EVALUATION OF NOVEL ADJUVANTS FOR IMPROVED aP COMBINATION VACCINES

"Messieurs, c'est les microbes qui auront le dernier mot"
Gentlemen, it is the microbes who will have the last word

- Louis Pasteur

BORDETELLA PERTUSSIS

Bordetella pertussis is a Gram negative bacterium of the genus Bordetella, firstly observed and isolated at the beginning of the twentieth century by Bordet and Gengou [90]. *B. pertussis* is the etiologic agent of pertussis, a disease which results in an acute respiratory tract infection and is usually known as *whooping cough* since it causes severe and incontrollable cough which ends with a whoop when no more air is present in the lungs. *B. pertussis* presents many virulence factors responsible for infection and pathogenesis, which are mainly expressed over bacteria surface or released in the surrounding environment (Figure 20) [91]. At first, bacteria interact and adhere to the cilia of respiratory epithelia cells mainly through the action of adhesins such as fimbriae, filamentous hemagglutinin (FHA), and pertactin (PRN or 69K). Then tracheal cytotoxin is produced to paralyze cilia causing inflammation of the respiratory tract and making the cleaning of the infection harder, while pertussis toxin (PT) is released to inhibit host immune system by inducing systemic pathological effects.

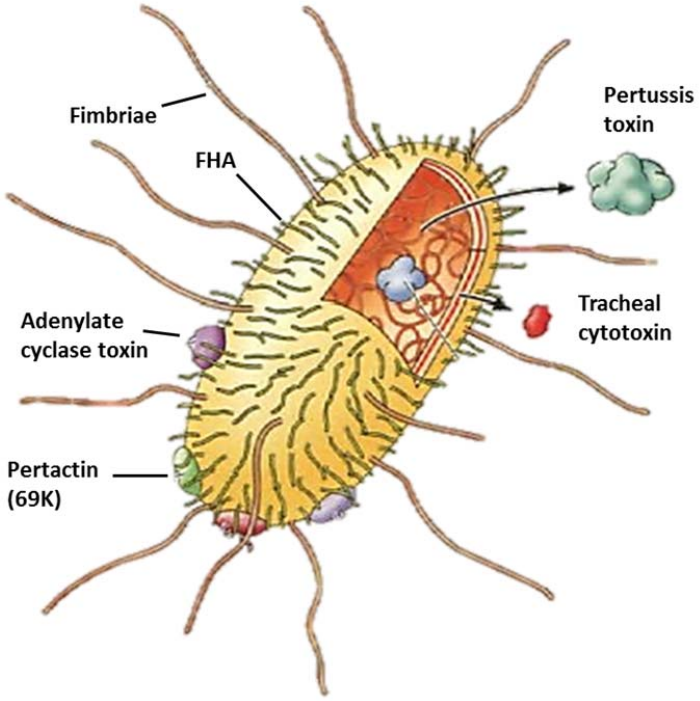

FIGURE 20. Schematic representation of *Bordetella pertussis* bacteria and its main virulence factors. Adapted from [91].

Pertussis is a highly contagious disease and vaccination is recognized as the mean to potentially eradicate the disease which is still endemic worldwide, despite vaccines against pertussis are actually available and recommended. Indeed, the first vaccine against pertussis was introduced during the 1940s and contained killed whole-cell *B. pertussis* bacteria (wP) in combination with diphtheria toxoid (DT) and tetanus toxoid (TT) inactivated with formaldehyde treatment [6]. The introduction of the so-called DTwP vaccine resulted in a drastic decrease in the number of reported cases of pertussis disease (Figure 21). However, reactogenicity issues and frequent adverse events reduced the confidence in wP vaccines and directed scientific efforts towards the development of safer vaccines which resulted in the replacement of killed bacteria with purified protective antigens (acellular pertussis, aP) [92]. DTaP vaccines were introduced during the 1990s and totally replaced whole cell inactivated bacteria (wP) in the USA and in many developed countries reaching a vaccination coverage superior than 95% [93, 94]. However,

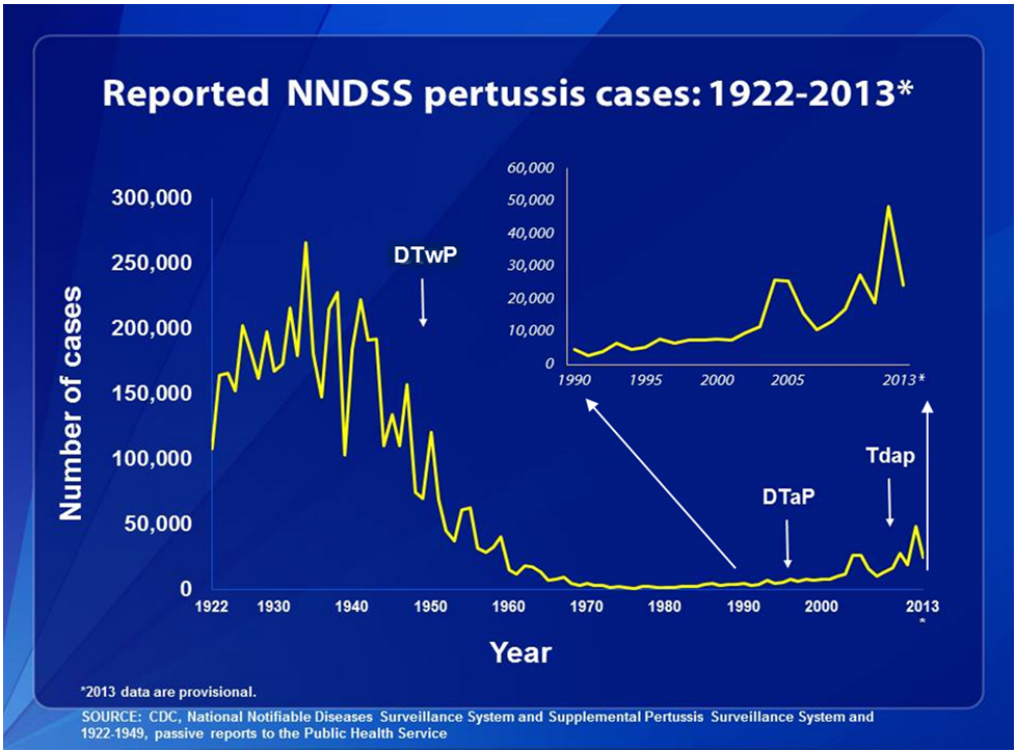

FIGURE 21. Trend of reported cases of pertussis disease following the introduction of DTwP and DTaP vaccines. Adapted from: CDC, National Diseases Surveillance System and Supplemental Surveillance System and 1922-1949 passive reports to Public Health Service.

following the DTaP vaccine introduction in immunization schedules, a widespread pertussis outbreak has been reported in many countries [50-54], despite the introduction of a booster vaccine (TdaP) formulated at different dosages for adolescents and adults to control herd immunity in all age groups (Figure 21) [95]. The observation that the rate of infection was significantly higher among children who have been vaccinated with only aP-containing vaccines led to the hypothesis that acellular antigens might not provide adequate long lasting protection against the disease. Other common hypothesis impute current vaccines to have imposed selective pressure on protective antigens thus resulting sub-optimal for current circulating strains which have for example evolved to express high levels of PT (ptxP3) [96] or to be 69K deficient [97]. Moreover, an increase in the number of reported cases could also be correlated with improved disease awareness, surveillance, and diagnostic tools [98]. Despite this, the most reliable theory for pertussis resurgence claims that aP-containing vaccines induce a waning immunity

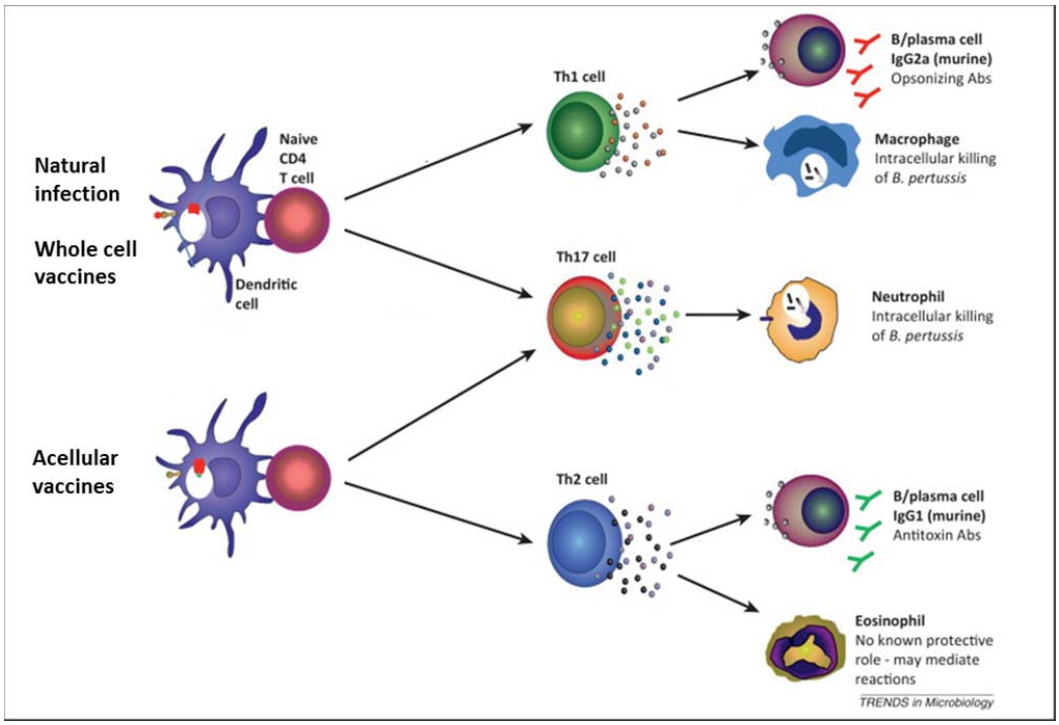

FIGURE 22. Immunogenicity of *Bordetella pertussis* and immune responses induced by natural infection, immunization with whole-cell vaccines, or immunization with acellular pertussis antigens. Adapted from [107].

[99-102] which protects against severe disease but fails to prevent colonization and transmission [103].

Each hypothesis comes along with different approaches to improve current vaccination strategies, among which the selection and inclusion of new antigens taken either from *B. pertussis* (e.g. adenylate cyclase toxin, [104]) or from other pertussis strains. In this way antigenic divergence and resistance induced by current vaccination would be overcome and the possibility to induce protective immunity increased [105]. Locht *et al* [106] even foresee a revival of whole cell vaccines attenuated by PT inactivation or by altering lipopolysaccharide (LPS) composition thus being less reactogenic. A more straightforward approach considers the optimization and improvement of existing aP-containing vaccines avoiding extensive work on the discovery of new antigens. The rational design of an improved formulation should depend on studies focusing on *B. pertussis* immunogenicity.

As clearly outlined in Figure 22, natural infections mediated by *B. pertussis* or immunizations with whole cell vaccines are known to induce mainly a cellular immune response mediated by Th1 and Th17 cells which is associated with an efficient and rapid clearance of *B. pertussis* and confer high levels of protection against subsequent infections. Using a mice infection model Kingston Mills' group showed that protection from *B. pertussis* colonization correlates with Th1/Th17 immune profile and subsequent induction of antibodies of the opsonizing IgG2a subclass, with activation of macrophages and neutrophils as immune effector cells for mediating intracellular killing of *B. pertussis* [107]. On the other hand acellular pertussis vaccines are known to induce a strong humoral immunity with Th2/Th17 profile, production of toxin neutralizer IgG1 antibodies and activation of eosinophils only, which have no known protective role in pertussis immunity [107]. This is a reflection of the presence of aluminum salts – known inducers of Th2 immunity - in aP-containing vaccines, which further polarize immunity towards the Th2–type.

EVALUATION OF ALTERNATIVE ADJUVANTS FOR IMPROVED IMMUNITY TO *BORDETELLA PERTUSSIS*

The approaches pursued here to enhance protective efficacy of acellular pertussis vaccines are based on the crucial role of Th1-type immune response in clearing *B. pertussis* infection [108, 109]. Thus the improvement of the current formulation through the delivery of aP antigens with an appropriate Th1 promoting adjuvant was explored. In particular, MF59 emulsion and the combination of a Toll-Like Receptor 4 agonist (TLR4a) or of a Toll-Like Receptor 7 agonist (TLR7a) with aluminum hydroxide (Alum) were investigated for their ability to enhance immunogenicity of a TdaP combination vaccine containing genetically detoxified PT toxin (PT-9K/129G) [60], filamentous hemagglutinin (FHA) and pertactin (69K) as *B. pertussis* antigens.

MF59 emulsion

MF59 oil-in-water emulsion [13] was firstly evaluated in a clinical trial for a vaccine against influenza in 1992 and after its licensure in 1997 it became the first adjuvant approved for human use in alternative to aluminum salts. Since then MF59 has been extensively evaluated in clinical trials with several different antigens [61, 110] showing excellent safety and tolerability profiles in all age groups, including the elderly and six months old children [111, 112]. In addition, the ability of MF59 to enhance the humoral or the cellular response - accordingly to the one naturally induced by the co-administered antigens [113] - appeared to be beneficial for raising a protective immunity against aP antigens.

Toll-like receptors and their agonists

As alternative strategy, the inclusion of Toll-like receptor agonists (TLRa) to Alum adjuvanted formulations was evaluated. TLRa are molecules which act as immune potentiators by directly stimulating antigen-presenting cells (APCs) through binding receptors of the innate immune system called pattern recognition receptors (PRRs). As a result, local inflammation is induced and additional immune cells are recruited to allow stimulation of antigen-specific B- and T-cells and consequently the activation of adaptive immunity [114]. Typical ligands of these receptors are highly conserved microbial and viral products and their derivatives, defined as pathogen associated molecular patterns (PAMPs). For example, TLR4 recognize lipopolysaccharide (LPS), TLR5 recognize flagellin, single stranded viral RNAs are ligand of TLR7 and TLR8, and CpG motifs are recognized by TLR9 [115]. Because of the role of TLRa as immune-potentiators and linker of innate and adaptive immune responses, in the past decade a strategy was pursued to exploit natural PAMPs for the generation of improved and well-characterized TLR ligands to be used as vaccine adjuvants, usually in combination with Alum as a delivery system.

For the aim of this thesis, Monophosphoryl Lipid A (MPLA) was selected as TLR4a. MPLA is a derivative of LPS which retains the immuno-stimulatory properties of the natural ligand of TLR4 while lacking toxicity. MPLA is included in the adjuvant AS04 [14] and is currently a component in two licensed vaccines against human papillomavirus (Cervarix® from GSK) [16] and hepatitis B virus

(Fendrix® from GSK) [15]. A synthetic small molecule property of Novartis Vaccines was instead used as agonist of TLR7 [116]. Despite differences in expression and intra-cellular signaling, TLR agonists have been evaluated in numerous preclinical models and are known to increase immunogenicity and redirect the immune response induced by Alum toward the stimulation of the Th1-type against a variety of antigens [117].

As mentioned MF59 and Alum+MPLA are the only two novel vaccine adjuvants approved for human use after the approval of aluminum salts [118]. Therefore these two adjuvants represent attractive options for quickly developing vaccines to fight pertussis resurgence, reducing the risk to have the product rejected during clinical evaluations. The results obtained with these two alternative adjuvants were gathered in a manuscript entitled "The potential of adjuvants to improve immune responses against TdaP vaccines: a preliminary evaluation of MF59 and Monophosphoryl lipid A" which was submitted to the *Clinical and Vaccine Immunology* (CVI) journal and is reported in the following pages.
Additional results generated with Alum+TLR7a are presented and discussed in a supplementary data section.

THE POTENTIAL OF ADJUVANTS TO IMPROVE IMMUNE RESPONSES AGAINST TdaP VACCINES: A PRELIMINARY EVALUATION OF MF59 AND MONOPHOSPHORYL LIPID A

Running title: "The potential of adjuvants to improve TdaP vaccines"

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Abstract

The successful approach of combining diphtheria, tetanus and pertussis antigens into a single vaccine has become the cornerstone of pediatric and adults immunization programs. Yet, even if vaccination coverage is high, a resurgence of pertussis has been reported recently in many countries suggesting that current vaccines may not provide adequate long-lasting protection.

In order to develop pertussis-containing vaccines able to induce a more durable and better tailored immune response different approaches have been proposed, including the use of novel adjuvants. Currently licensed pertussis vaccines contain aluminum salts, which are the most common adjuvants for human use. By inducing mainly humoral immune responses aluminum salts might not be ideal for providing protection against pathogens which require a cellular immune response, such as *Bordetella pertussis*.

Other adjuvants that induce more balanced T-helper profiles or even Th1-prone responses might be more adequate. In this study, we tested two different adjuvants: MF59 emulsion adjuvant – that induces a mixed Th1/2 response - as well as the combination of a Toll-Like Receptor 4 agonist (TLR4a) with aluminum hydroxide to induce even further Th1 polarization. The adjuvants were evaluated for their ability to improve immune responses against a TdaP vaccine containing three *B. pertussis* antigens: genetically detoxified pertussis toxin (PT-9K/129G), filamentous hemagglutinin (FHA) and pertactin (69K).

The quicker onset of serum titers and the changed quality of the antibody responses induced by the adjuvants evaluated here fully support the potential replacement of aluminum salts with alternative adjuvants to enhance pertussis immunogenicity in aP-containing combination vaccines.

Introduction

The highly contagious respiratory tract infection caused by the Gram negative bacterium *Bordetella pertussis* is still endemic worldwide, despite the fact that vaccines are available and recommended. The first pertussis vaccines based on whole, killed bacteria (wP) were developed in the 1930s and were later combined with diphtheria and tetanus toxoids. The derived DTwP vaccines have been a cornerstone of childhood immunization since the 1940s [6]. Effectiveness of DTwP vaccination has been documented in numerous clinical trials, but because of occurring adverse events the confidence in wP vaccines and vaccination compliance decreased with declining case numbers [106]. The efforts to develop safer vaccines have resulted in the replacement of inactivated bacteria with purified protective antigens of *B. pertussis* (aP) [7]. These DTaP vaccines have been introduced for both primary and booster immunizations [95] and depending on the manufacturer contain one to five *Bordetella* proteins.

Thanks to the reduced reactogenicity, aP vaccines have totally replaced wP in the USA and in many developed countries and vaccination coverage is in excess of 95% [93, 94].

However, the number of whooping cough cases started to increase soon after the introduction of aP vaccines. Attempts have been made to counter this with additional booster vaccines suitable for adolescents and adults (TdaP) since 2005. Yet, these just postponed the medium age of pertussis cases from infant to older children. Moreover total case numbers have still been increasing over the last years [50-54].

Common hypotheses for the resurgence of pertussis claim that aP vaccines are less effective than wP vaccines and that aP-induced immunity wanes more quickly [99-102]. Also changes in the genetic makeup of circulating pertussis strains have been observed [105, 119]. Another plausible explanation has been suggested by a recent study showing that aP vaccines protect against severe disease but do not prevent infection and transmission of *B. pertussis* in a nonhuman primate model [103]. Therefore little herd immunity should be conferred by aP vaccines on a population basis, in contrast to wP vaccines that were shown to shorten *B. pertussis*

colonization in the primate model [103]. An immunological explanation for this finding has been extensively assessed in a mouse aerosol challenge model [120]. Studying the protection from colonization with different vaccine formulations and in various knockout mice this group could show that protection from colonization correlates with a Th1/ Th17 immune profile and the induction of antibodies of the IgG2a subclass. IgG2a antibodies in mice are known for their better opsonizing capacities, in contrast to IgG1 antibodies. Classical aP vaccines induce mainly Th2 and IgG1 responses and work presumably mostly via toxin-neutralization activity [107]. Analysis of the pertussis-induced helper T-cell immune response demonstrated that aP vaccination promotes mostly Th2 responses, whereas wP and natural infection induce Th1 and Th17 responses [121-123].

In addition, the currently licensed DTaP vaccines are administered with insoluble aluminum salts, which preferentially induce Th2 response [23]. Based on the crucial role Th1 response has been found to play in clearing the infection [108, 109] one of the proposed approaches to enhance the protective efficacy of aP vaccines considers the inclusion of novel Th1-promoting adjuvants in the formulation [106, 124, 125]. Several studies have shown that alternative adjuvants to aluminum salts, such as micro and nanoparticles, reduced the bacterial loads in comparison to not-adjuvanted formulations [126] and induced enhanced and persistent responses in serum antibody titers against aP antigens [127, 128].

Here, the emulsion MF59 and the combination of the Toll-Like Receptor 4 agonist Monophosphoryl Lipid A (MPLA) with aluminum hydroxide (Alum), in a formulation similar to AS04, were evaluated for their ability to adjuvant a TdaP trivalent vaccine containing the genetically detoxified PT toxin (PT-9K/129G) [60], filamentous hemagglutinin (FHA) and pertactin (69K) as *B. pertussis* antigens.

These adjuvants were chosen since they are the only two novel adjuvants already included in approved vaccines for human use [118] and represent attractive options for fast development of vaccines to counter pertussis resurgence quickly. MF59 is an oil-in-water sub-micron emulsion [13, 129] which has been extensively evaluated in clinical trials with several different antigens including recombinant proteins [110] and bacterial toxoids [61]. The excellent safety and tolerability MF59

has demonstrated even in toddlers, infants, and newborns [111, 112] encouraged its evaluation in this work whose focus are combination vaccines for children and adolescents. In addition, MF59 adjuvant is able to enhance the humoral or the cellular response accordingly to the one naturally induced by the co-administered antigens, without biasing the immunological profile [113].

As alternative strategy, the inclusion of the MPLA [130] into an Alum-based TdaP formulation has been exploited. Acting as PAMPs, the TLRa have been shown to be strong vaccine adjuvants improving the immunogenicity of numerous antigens in preclinical models [117]. Similar adjuvants are currently licensed for adolescent and adult populations but the development for infants might be more challenging as compared to MF59. Yet TLRa in association with Alum, in particular the TLR9a CpG oligodeoxynucleotide, were found to be highly effective to stimulate Th1 type immune response against *B. pertussis* antigens, including PT and FHA and to enhance protective capacity [120, 123]. Therefore, the combination of Alum and TLRa might constitute the ideal adjuvant to prime the most adequate responses against pertussis.

The different TdaP-adjuvanted formulations were characterized and compared in a mouse model to assess the potential of MF59 and Alum+MPLA respectively to enhance and better tailor the immune response against TdaP antigens.

Materials and Methods

Antigens and Adjuvants

Tetanus toxoid (TT) and diphtheria toxoid (DT) were obtained from Novartis Vaccines (NV) manufacturing (Marburg, Germany). Genetically detoxified pertussis toxin (PT-9K/129G), filamentous hemagglutinin (FHA) and pertactin (69K) antigens were produced by Lonza Group (Basel, Switzerland).

Aluminum hydroxide (Alum) and MF59 adjuvants were obtained from NV manufacturing (Marburg, Germany). Toll-Like Receptor 4 agonist (MPLA, 699800) was commercially obtained by Avanti Polar Lipids, Inc. (Alabaster, AL).

Preparation of adjuvanted formulations

TdaP antigens 10 Lf TT, 4 Lf DT, 8 µg PT-9K/129G, 8 µg FHA, and 16 µg 69K in a volume of 1 ml were adsorbed onto 2 mg/ml Alum suspension [24]. When required, MPLA powder was resuspended in 0.5% v/v triethanolamine (TEoA) [131] and added to the formulation [132] at the final dose of 10 µg.

Formulations were adjusted by the addition of 10 mM Histidine buffer and 9 g/l NaCl to the recommended physiological ranges of pH and isotonicity.

For MF59-adjuvanted formulations the TdaP antigens mixture was diluted 1:1 (v:v) with MF59 and 10X phosphate buffered saline (PBS) was added to adjust formulation pH and isotonicity.

Adjuvanted formulations characterization

Antigens identity and integrity was evaluated by Western Blot (WB) analysis. Briefly, Alum-based formulations were centrifuged (3500 g for 10 min at 25°C) and the supernatant removed [61]. The pellet was then resuspended in SDS-extraction buffer, boiled and centrifuged. The resulting supernatant was loaded on NuPAGE® Novex 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA). The aqueous phase of MF59 emulsion was separated via Airfuge® Air-Driven (Beckman Coulter Inc., Pasadena, CA; 70000 rpm for 20 min) and loaded for SDS-PAGE. Gels were run for 1 hour at 150 V and then blotted to a nitrocellulose membrane via iBlot® (Life Technologies, Carlsbad, CA). Membranes were blocked for 1 hour in 5% w/v milk/TPBS (PBS 1X + Tween20 0.05% v/v) and later incubated with optimized dilutions of monovalent polyclonal mouse anti-sera generated at NV. Horseradish peroxidase-conjugated goat anti-mouse IgG (Southern Biotech, Birmingham, AL) was used as secondary antibody (1:5000). After 1-hour incubation at room temperature, membranes were washed and Opti-4CN™ Substrate Kit (Bio-Rad, Hercules, CA) was used for bands revelation.

Antigens adsorption onto Alum was evaluated via micro BCA analysis [133] on formulation supernatants. A 5-points standard curve was prepared and a formulation containing TdaP antigens and the components of the vaccine, except for the adjuvants, was included as positive control.

The size distribution of Alum adjuvanted formulations and MF59 emulsion was measured by laser light scattering on the LS 13 320 SW (Beckman Coulter Inc, Pasadena, CA) and on the Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, UK) instruments respectively. The zeta potential of the formulations was measured using the Zetasizer Nano ZS after sample dilution in water.

Animal experiments

Animal studies were performed in compliance with the Italian law, approved by the local Animal Ethics Committee, and authorized by the Italian Ministry of Health.

Two similar mouse studies with TdaP antigens formulated with Alum, Alum+TLR4a or MF59 respectively were carried out one month a part from each other. Groups of 8 female, 6-week-old, specific-pathogen-free BALB/c mice (Charles River Laboratories International Inc., Wilmington, MA) were used for the studies. Each animal received two intramuscular (i.m.) immunizations at days 1 and 21. Injections were performed in quadriceps of hind legs for a total dose volume of 100 µl (50 µl/leg).

In all studies control groups with non-immunized mice were included. Animals were bled two weeks after each immunization and serum samples were analyzed by Luminex immunoassay for circulating antibodies titers. Toxin neutralizing antibody titers, inhibition of FHA binding activity and IgG subclasses were analyzed after the second bleeding.

Specific IgG measurement using protein-coupled microspheres

A Luminex penta-plex immunoassay was developed to determine antibody titers in mouse sera. In detail, 20 µg of each antigen were coupled to the carboxyl groups of 1.25 million MagPlex microspheres (Luminex Corporation, Austin, TX) following manufacturer's instructions.

IgG titers were determined in individual animals after 30 min incubation of antigen-coupled beads with the sera diluted in PBS 1X 1:10000 (post-1) or 1:20000 (post-2) into 96-well Multiscreen HTS filter plates (Merck Millipore, Darmstadt, Germany). A phycoerythrin-conjugated secondary antibody was then used for detection (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:400; 15 min). The

obtained immune-complexes were resuspended in PBS 1X and analyzed on the Luminex system.

For IgG subclasses analysis a 30 min incubation step with biotin-labeled antibodies specific for IgG1 or IgG2a subclasses (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:200) was performed after the first reaction between beads and diluted sera (1:20000 post-2). Following the incubation with R-Phycoeritrin conjugated to extravidin (Sigma-Aldrich, St. Louis, MO; 1:200; 15 min) the obtained immune-complexes were resuspended in PBS 1X and analyzed on the Luminex system.

All reaction steps were carried out in agitation motion using a horizontal shaker in the dark at room temperature. Among each incubation step, plates are washed three times in PBS 1X by a multiscreen vacuum manifold and a Chemical Duty Pump (Merck Millipore, Darmstadt, Germany).

IgG measurements were determined as median fluorescence intensities (MFI) on a Luminex FLEXMAP 3D analyzer (Luminex Corporation, Austin, TX) using the Bio-Plex Manager 5.0 software (Bio-Rad, Hercules, CA). A hyper-immune antiserum specific for each antigen was used as reference to convert MFI values in RLU/ml (Relative Luminex Units) for total IgG quantification. The limit of quantification (LOQ) of the assay was determined for each antigen and was considered as threshold for positive results.

DT neutralization assay

Vero cell line (monkey kidney epithelial cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) and antibiotics. Cells were grown at 37°C with 5% CO_2.

The DT-induced cell death was tested by the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI), based on the measurement of cytoplasmic ATP in metabolically active cells. To test the minimal dose of DT needed to cause cell death in 16 hours (1 CTU_{100}), cells were plated at $2x10^4$/well in 96-well plates

in presence of serially diluted DT (Sigma-Aldrich, St. Louis, MO). The CTU_{100} was defined as 10 ng/ml in the tested experimental conditions.

Post-2 mouse sera were two-fold serially diluted in cell medium, pre-incubated with 4 CTU_{100} of DT toxin and then incubated for 90 min at 37°C. Subsequently, 50 µl of Vero cells at density of $2x10^5$cells/ml were incubated with 50 µl of sera and toxin mixtures for 16 hours at 37°C. Endpoint titers were defined as the reciprocal of the highest dilution able to inhibit cell death. Pre-immune sera were used as negative controls.

PT neutralization assay

CHO-K1 cell line (Chinese hamster ovary cells) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in F12-K medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) and antibiotics.

In vitro neutralization assay was optimized following the principles described previously [134, 135]. To test the minimal dose of PT needed to cause 100% cell clustering in 16 hours (1 CTU_{100}), cells were plated at $2x10^4$/well in 96-well plates in the presence of serially diluted PT (Sigma-Aldrich, St. Louis, MO). The CTU_{100} was defined as 8 ng/ml in the tested experimental conditions.

Post-2 mouse sera were diluted and incubated with PT as described for DT neutralization assay. Subsequently, 50 µl of CHO-K1 cells at density of $4x10^5$cells/ml were incubated with 50 µl of sera and toxin mixtures for 16 hours at 37°C. Cells were observed by light microscopy to evaluate morphological alterations (clustered phenotype). Endpoint titers were defined as the reciprocal of the highest dilution able to inhibit cell clustering. Pre-immune sera were used as negative controls. Sera of mice immunized with commercially available TdaP vaccine (Boostrix®, antigens doses 10 Lf TT, 5 Lf DT, 16 µg PT, 16 µg FHA, and 5 µg 69K in a volume of 1 ml) were included in the assay to evaluate the neutralizing effect induced by a chemically detoxified PT toxoid (PTd) included in Boostrix®.

FHA binding inhibition assay

A549 cells (Human lung epithelium) were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in F12-K medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco, Life Technologies, Carlsbad, CA) and antibiotics. Cells were seeded on 96-well plate ($2.5x10^4$/ well) and cultured for one day. To perform the serum-mediated inhibition assay, pooled post-2 mouse sera were two-fold serially diluted in F12-K medium containing 1% naïve mouse serum and pre-incubated with 2.5 µg/ml FHA for 1 hour at 37°C. The mixtures were then added to cells and incubated for 30 min at 37°C. After extensive washing to remove unbound protein, cells were fixed with 3.7% paraformaldehyde and incubated with sheep anti-FHA serum (1:500) (NIBSC, National Institute for Biological Standards and Control) for 1 hour at room temperature. After washings, samples were incubated with Alexa Fluor 488 donkey anti-sheep IgG (1:500) (Life Technologies, Carlsbad, CA) and fluorescence associated to cell surface was measured at excitation/emission 485/535 nm by Tecan Infinite F200PRO microplate reader.

Statistical analysis

Graph Pad Prism 5.0 software was used for statistical analysis. Serum antibody titers were reported as median while neutralizing titers were reported as mean and significant differences ($p \leq 0.05$) among groups were ascertained using the two-tailed Mann-Whitney test at the 95% confidence interval.

Results

Adjuvanted formulations characterization

Vaccines were prepared and characterized to guarantee the quality of the final formulations as specified in Materials and Methods. The average particle size of Alum suspension was determined to be around 10 µm before and after TdaP antigens adsorption (Table VII). When adding MPLA to the vaccine Alum particles size doubled and an evident broadening of size distribution was observed. Significant reduction of the zeta potential was registered during adsorption of

antigens and MPLA onto Alum suspension. MF59 emulsion showed comparable particles size, polydispersity and zeta potential at every stage of the formulation process (Table VII). TdaP antigens identity and integrity were confirmed by Western Blot analysis in Alum as well as in MF59-based formulations. Characterization of freshly prepared vaccines gave comparable results to vaccines stored up to three months at 4°C (data not shown). To discriminate between Alum-adsorbed and not-adsorbed antigens, adjuvant was separated by centrifugation. The micro BCA performed on the supernatants showed that the adsorption of TdaP antigens onto Alum was higher than 95% and that the inclusion of MPLA in the formulation did not alter antigens adsorption rate (Table VIII).

TABLE VIII. Physical characterization of various vaccine formulations. N.a. not applicable.

Adjuvant	**TdaP antigens**	**Particle size (µm± SD)**	**Zeta potential (mV ± SD)**	**Antigen adsorption (%)**
Alum	-	10.8 ± 0.17	25.6 ± 0.87	n.a.
	+	11.1 ± 0.23	9.50 ± 0.61	> 95%
Alum+MPLA	+	21.6 ± 0.75	-2.50 ± 0.81	> 95%
MF59	-	0.15 ± 0.30	-39.0 ± 0.40	n.a.
	+	0.15 ± 1.00	-38.1 ± 0.81	n.a.

In vivo adjuvant effect

To assess *in vivo* potency of the formulations, groups of Balb/c mice were vaccinated twice (day 0, 21) and serum antibody titers were measured two weeks after each immunization. The analysis of total serum IgG indicated that adjuvants were needed to elicit potent immune responses (Figure 22). When antigens were formulated in absence of any adjuvant, no response was detected after one immunization against TT, FHA and 69K, or in the case of DT neither after the second immunization. The only exception was constituted by PT, which was readily detected even without any adjuvant after the first immunization. Yet, this might be due to the specific properties of the genetically inactivated PT toxin, which is

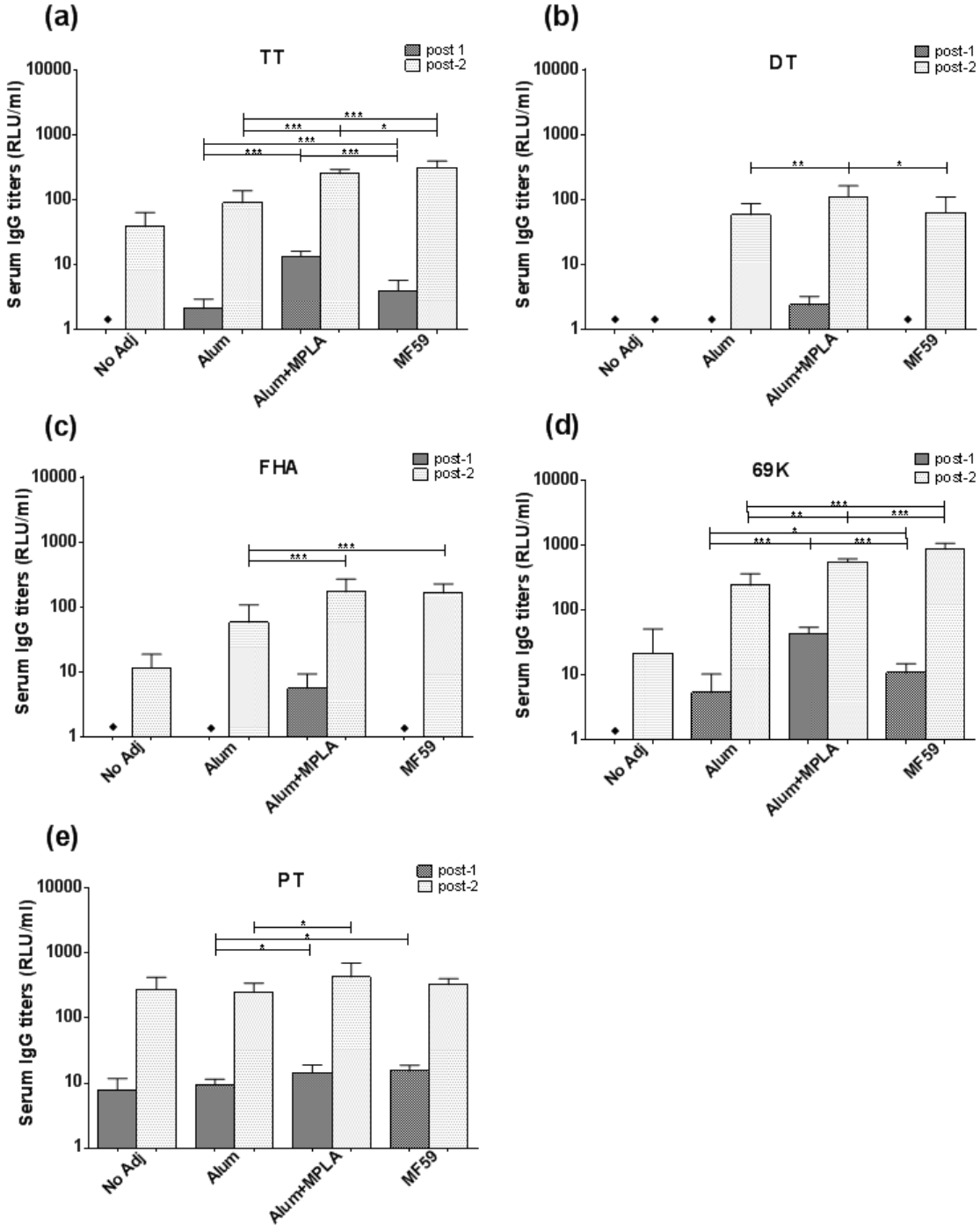

FIGURE 23. Serum (a) anti-TT, (b) anti-DT, (c) anti-FHA, (d) anti-69K, (e) anti-PT antibody titers in Balb/C mice after one and two immunizations at days 1 and 21. Averaged results of the two mouse studies are shown as median with interquartile range presented for each group.

- serum IgG titers below 1 RLU/ml; LLOQ ≤ 0.9 RLU/ml; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

known to be more immunogenic as compared to its chemically detoxified counterpart included in commercially available aP vaccines [60, 136]. Further explanation could be associated with the intrinsic adjuvant activity that the genetically detoxified PT was shown to harbor [137, 138]. Serum IgG analysis revealed that two immunizations with Alum-adjuvanted formulations were required to induce detectable IgG titers against DT and FHA antigens.

Similar results were also observed when including MF59 adjuvant in the vaccine. For the other antigens (TT, PT, 69K) MF59 was instead shown to be superior to Alum after a single immunization and the trend was confirmed after the second immunization. Serological analysis performed on the first bleeding for DT and FHA antigens indicated Alum+MPLA as the only adjuvant able to raise detectable responses. Overall, a single immunization with Alum+MPLA adjuvant induced IgG titers higher than the ones induced by Alum. As previously stated, PT-9K/129G was the only antigen to be immunogenic after a single immunization without any adjuvantsand resulted as effective as Alum. On the contrary, the alternative adjuvants tested were shown to have comparable adjuvant activity while raising significantly higher IgG responses in comparison to Alum. For the other TdaP antigens Alum+MPLA adjuvanticity was superior to MF59. As a whole, post-1 data depicted Alum+MPLA as the most potent adjuvant inducing quicker onset of the immune response and higher antibody titers against TdaP antigens, while MF59 performed at least as effectively as Alum.

After the second dose Alum+MPLA confirmed its stronger adjuvanticity as compared to Alum. In addition, Alum+MLPA was also more effective than MF59 for DT antigen confirming Alum as a successful adjuvant for this bacterial toxoid. On the contrary, formulations adjuvanted with Alum+MLPA or MF59 resulted in comparable responses against PT and FHA and MF59 was shown to be the most effective adjuvant for TT and 69K antigens.

On the basis of the serological responses at both vaccine doses, both the approaches investigated could be considered as promising alternatives to Alum in TdaP vaccines.

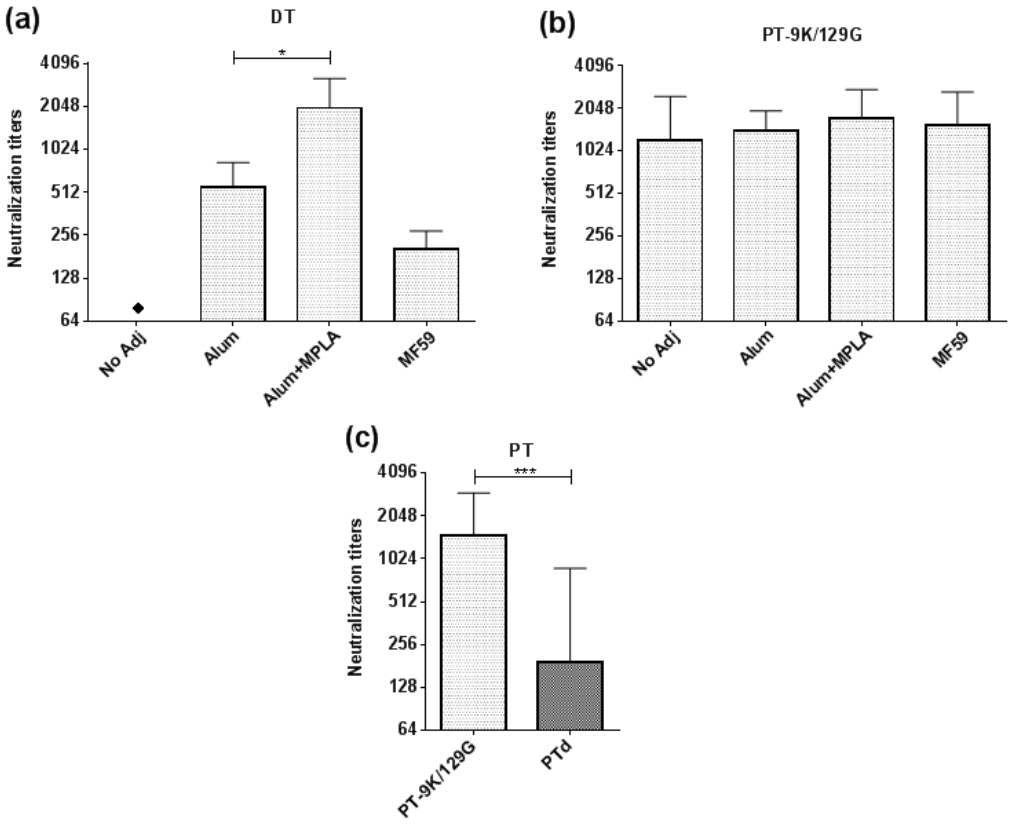

FIGURE 24. Post-2 in vitro neutralization titers (a) anti-DT and (b) anti-PT. (c) comparison of the neutralizing activity of genetically detoxified PT-9K/129G and chemically detoxified PT (PTd) included in Boostrix vaccine. Mean with standard deviation presented for each group.
• titers below detection limit. * $p < 0.05$, *** $p < 0.001$

DT and PT toxin neutralizing activity

To evaluate the ability of the induced antibodies to recognize antigenic functional epitopes, different toxin neutralization assays were performed after the second bleeding. The neutralization assay against DT indicated that Alum+MPLA was able to stimulate significantly higher neutralizing titers in comparison to Alum (Figure 24a).

In contrast, pertussis toxin neutralizing assay showed that the use of adjuvants in the formulation did not lead to significant enhancement of the neutralizing activity of anti-PT antibodies (Figure 24b).

As previously noticed for total serum antibody titers, this might be explained by the strong immunogenic properties of this antigen. In fact, evident differences in

neutralizing activity were observed when comparing TdaP formulations containing the genetically detoxified PT to Boostrix® vaccine in which a chemically detoxified PT (PTd) is used (Figure 24c). Notably, PT-9K/129G induced significantly higher neutralizing titers despite being formulated at half of the dose of PTd, further supporting the potential of PT-9K/129G as candidate antigen to develop improved vaccines against pertussis.

FHA binding inhibition

FHA is an important adhesion factor of *Bordetella pertussis* and the inhibition of its binding to ciliary epithelial cells is of significant relevance to prevent pathogen adherence to the host respiratory tract. Achieving higher adhesion blocking antibodies should therefore contribute to greater protection from bacterial colonization and so from contracting the disease [139].

The adjuvant effect on the induction of antibodies able to block FHA binding activity was evaluated on pooled sera after the second immunization and indicated that Alum+MPLA and MF59 equally improved the inhibition activity of anti-FHA antibodies in comparison to Alum (Figure 25).

Shift in the IgG1/ IgG2a profile

Alternative adjuvants to Alum were investigated for their ability to induce a more Th1 oriented immune response against aP antigens using TdaP as a model vaccine.

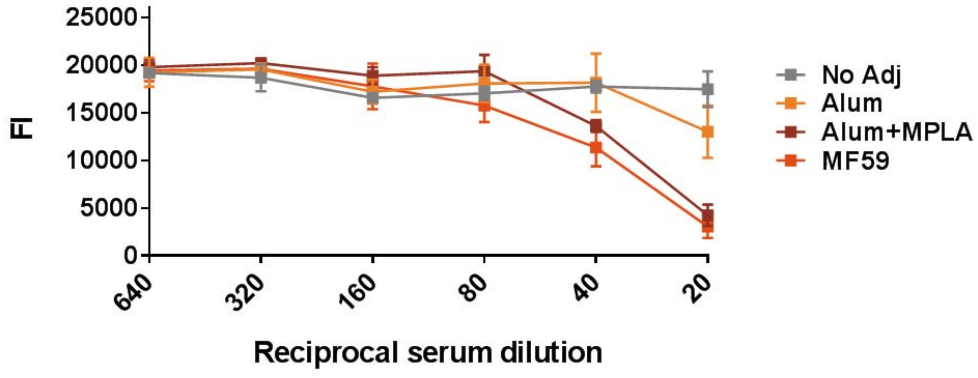

FIGURE 25. FHA binding activity of pooled post-2 sera of mice immunized with different formulations. Mean with standard deviation presented for each group. FI: fluorescence intensity at excitation/emission 485/535 nm.

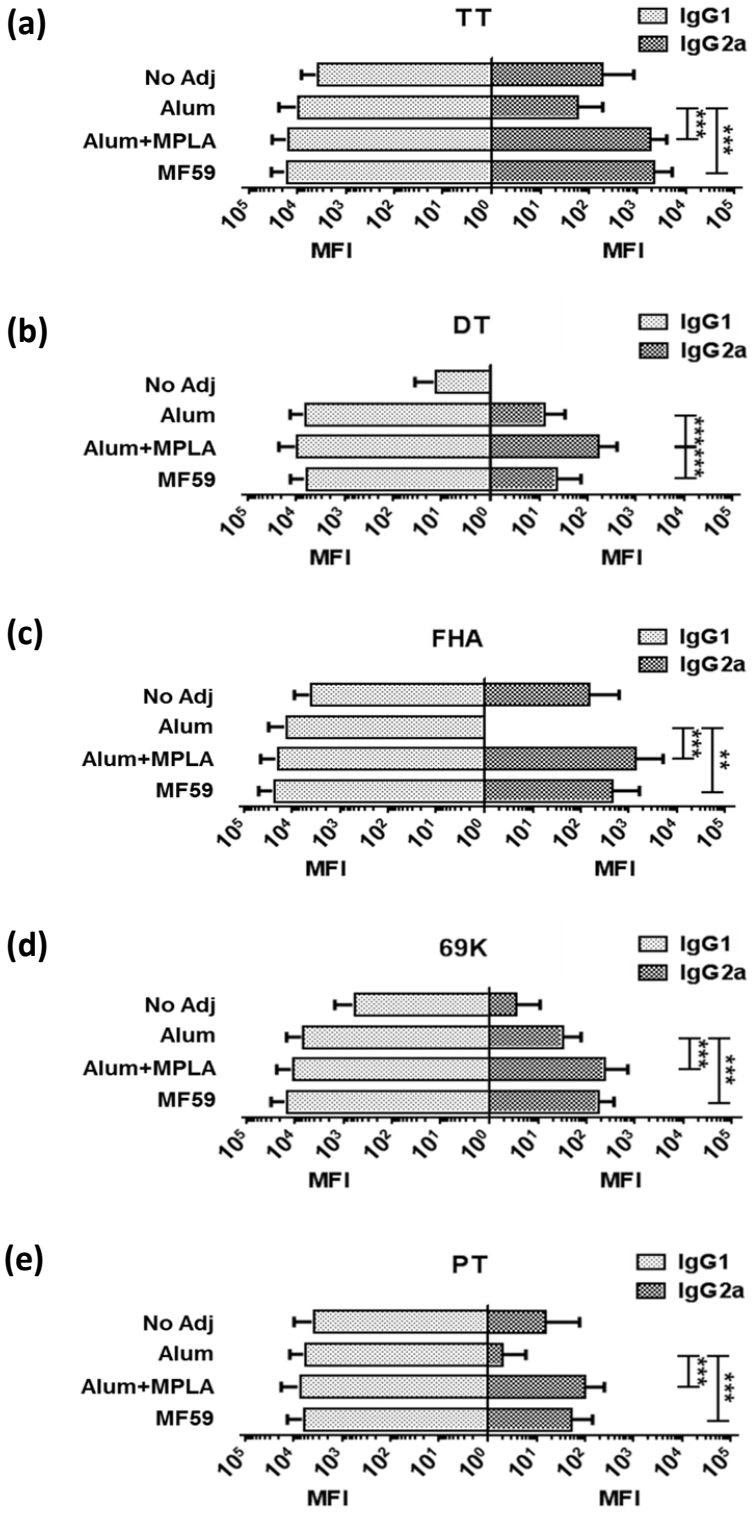

FIGURE 26. Median fluorescence intensities (MFI) relative to specific IgG1 and IgG2a responses (a) anti-TT, (b) anti-DT, (c) anti-FHA, (d) anti-69K, (e) anti-PT in Balb/C mice after two immunizations. Median with interquartile range presented for each group. • values below 1 MFI.

In the mouse model, a Th2 response is correlated to IgG1 antibodies, while Th1 responses are characterized by the subclass IgG2a, b or c (depending on the individual mouse strain). Classical aP vaccines induce mainly Th2 and IgG1 responses and work presumably mostly via toxin-neutralization activity [107]. In contrast, IgG2a, b or c antibodies are known for their better opsonizing capacities and should account, together with the phagocyte-activating-cytokine-secreting Th1 cells, for the improved bacterial clearance shown in the mouse model [120, 140]. To assess a potential shift in the IgG1/IgG2a balance and hence quality of the immune response, antibody subclasses were analyzed after the second immunization. As expected Alum induces a more Th2-prone response with reduced IgG2a and enhanced IgG1 titers in comparison to the not-adjuvanted formulation, as can be readily observed for FHA and PT. For the other antigens the overlaying adjuvant effect of Alum, enhancing all titers, make a comparison of a potential shift less assessable. Importantly a shift to a more IgG2a/Th1 prone profile was observed as compared to Alum when combining Alum and MPLA or using MF59 emulsion. Both adjuvants clearly enhanced IgG2a titers against the five TdaP antigens and, as expected, MF59 was shown to maintain the natural immunological profile of the antigens (Figure 26). On the contrary, Alum adjuvant completely reset the intrinsic Th1 inducing effect showed by FHA and PT antigens. The ability of MPLA to redirect the immune response against these antigens appeared evident through the reversion of the quality of the immune response when the immune-potentiator was combined with Alum.

Discussion

Despite a generally high vaccination coverage there is a recent resurgence of pertussis in many countries worldwide. Several hypotheses have been formulated but most experts agree that waning immunity and inadequate immune profiles induced by acellular vaccines are to blame, next to changes in the genetic makeup of circulating pertussis strains. Induction of a Th1 profile – as induced by wP vaccines or actual infection - is seen as beneficial to protect from bacterial

colonization and to enhance herd immunity. In contrast, currently available acellular vaccines mainly induce a Th2-prone response.

Future pertussis vaccines should have a safety profile similar to current acellular pertussis-containing vaccines, but ideally induce longer lasting immunity and more adequate T-helper response profiles. Therefore in this study the potential of alternative adjuvants to aluminum salts to enhance the efficacy of aP vaccines was assessed, and two different approaches that have already been successfully used in human vaccinations were chosen.

On the basis of the well-established safety of MF59 emulsion [141] and of the strong immunological stimulation induced by TLRa [142], TdaP formulations adjuvanted with MF59 or with the TLR4 agonist Monophosphoryl lipid A (MPLA) in combination with Alum have been studied. The possibility to develop stable and defined formulations has been evaluated through the physical characterization of TdaP formulations and the investigation of their *in vivo* immunogenicity and individual immune profile.

With respect to quantity, quality and functionality of the induced immune responses both alternative approaches created a promising scenario for the development of next generation aP containing vaccines.

The adjuvant effect of MF59 resulted in immune responses against TdaP antigens higher or at least comparable to Alum. This finding, along with the demonstrated enhancement of antibody responses against other antigens routinely included in the pediatric immunization schedules, such as those from *Haemophilus influenzae* type b (34) and hepatitis B virus (35), show the strong potential of MF59-adjuvanted TdaP vaccines to be readily further developed to improve current infant-pertussis combination vaccines. As revealed from the unchanged results of size distribution and zeta potential pre- and post-formulation, TdaP antigens remained mostly free in the aqueous phase of MF59 emulsion. Despite this condition could possibly induce instability processes [34], no modification in the antigenic electrophoretic profiles were detected suggesting TdaP antigens are compatible with MF59 adjuvant.

Antigens adsorption on adjuvant surface is instead one of the main feature of formulations based on aluminum salts and it is considered a very important

parameter for their function [26, 32], such that WHO recommended adsorption values equal or greater than 80% for diphtheria and tetanus toxoids [84]. The reduction of the zeta potential registered during formulation procedure confirmed TdaP antigens are adsorbed onto Alum adjuvant, condition known to ensure antigens stability in liquid vaccine formulations. Further proof came from micro BCA analysis, which measured in supernatants of formulation less than 5% of the total antigenic amount formulated, thus indicating only a minor part of antigens remained not-adsorbed to the adjuvant. In addition, TdaP antigens adsorption rate onto Alum was not modified when including MPLA in the formulation. Aluminum salts have been previously shown to induce enhanced and better modulated immune responses when combined with TLR agonists through the co-administration of both antigens and agonists to the same antigen presenting cell [14, 143, 144]. In addition Geurtsen *et al.* [145] demonstrated that aP vaccine adjuvanted only with MPLA were more protective than aP vaccines containing Alum. Yet, safety concerns have been raised for formulations where MPLA is not adsorbed to a delivery system like Alum or nanoparticles. Moreover, several other studies demonstrated that MPLA in combination with Alum adjuvant resulted in more potent immune responses and recommended MPLA adsorption onto Alum surface to prevent potential systemic toxicity [22]. In this context, the adjuvanticity of Alum in combination with the immune-potentiator MPLA for a TdaP trivalent vaccine has been exploited. Beyond the overall higher serum titers raised by the inclusion of MPLA in the formulation in comparison to Alum, Alum+MPLA was also shown to accelerate the onset of the immune responses suggesting that the addition of a TLR agonist to Alum-based formulations could potentially reduce the number of injections required to ensure aP vaccine immunogenicity.

A crucial parameter to develop a highly effective vaccine against pertussis is to induce high antibody titers against PT, one of the major virulence factors of *B. pertussis*. In this perspective, the inclusion of PT 9K/129G in future pertussis combination vaccines appears promising also considering that the genetically modified toxin has already been shown to retain higher immunogenicity than the chemically detoxified PT (PTd) included in the currently licensed vaccines [60,

146]. Furthermore, PT 9K/129G has been identified as ideal vaccine candidate since it cannot revert to toxicity while conserving the native B and T cell epitopes which may be lost during a chemical detoxification [147] and has been reported to have an intrinsic adjuvant activity in mice [137, 138]. The adjuvant effect for PT 9K/129G was less evident in comparison to what was observed for the other antigens of the combination, however significant improvement in terms of total IgG antibody titers was detected in presence of MPLA. Dose titration studies of PT antigen are planned to provide more detailed insights on the direct effect of TLRa and MF59 adjuvants on its immunogenicity.

To assess if the alternative adjuvants tested in the current work were effective in inducing Th1 response, the induction of opsonizing IgG2a was evaluated and taken as indirect evidence of enhanced cell mediated responses. In literature those responses are associated with an efficient and rapid clearance of *B. pertussis* [120] and with the superior long term protection of wP vaccination [148]. As expected, Alum+MPLA strongly potentiated the Th1 immune response leading to increased IgG2a antibody titers. Interestingly, an appreciable enhancement of IgG2a titers against TT and *B. pertussis* surface adhesins was also observed with MF59 emulsion thus confirming the peculiarity of this adjuvant to strengthen the immune responses naturally induced by the co-administered antigens [113]. The observed stimulation of Th1-type immune response against *B. pertussis* surface adhesins is encouraging for the improvement of aP-containing vaccines efficacy since these antigens are reported amongst the main mediators of pathogen colonization and transmission [103].

Considering that both Alum+MPLA and MF59 adjuvants have been proven to be valuable approaches in the mouse model, it would be interesting to further investigate if the adjuvant effect on pathogen colonization and immune response persistence could be answered in the baboon model which has emerged as an appropriate animal model to reproduce all aspects of pertussis clinical disease [103]. Overall, the high antibody titers against PT and the promotion of a Th1-oriented response against *B. pertussis* surface adhesins obtained with the alternative formulations developed are initial encouraging data fully supporting the potential of

adjuvants to raise better tailored immunological responses against aP-containing combination vaccines and to effectively control pertussis spread and overcome waning immunity issues. The critical adjuvant selection relying on adjuvants already licensed in vaccines for infants and toddlers, such as MF59, or on the addition of a TLR agonist to existing formulations, emerged as a simpler and faster approach for better vaccine development than the identification of entirely new vaccine candidates.

Acknowledgments

The authors are grateful to Marco Tortoli and the animal facility personnel for the precious help with the *in vivo* studies.

Contributions

V.A. actively contributed to perform IgG measurement experiments through the development of the Luminex penta-plex immunoassay. V.A. and C.B. analyzed and interpreted the results and drafted the manuscript.

EVALUATION OF A SYNTHETIC AGONIST OF TOLL-LIKE RECEPTOR 7

Pursuing Novartis Vaccines' strategy of using SMIP (small molecule immune-potentiators) as agonist to activate immune cells through the targeting of Toll-like Receptor 7 (TLR7), a synthetically obtained TLR7 agonist (TLR7a) was evaluated for its adjuvant effect when included in Alum adjuvanted vaccine formulations. A soluble agonist was designed and functionalized with PEG linker and a terminal phosphonate group to allow adsorption to aluminum hydroxide via ligand exchange with the aim of limiting SMIP's systemic exposure while ensuring co-localization of antigens and adjuvants and favoring retention time in the muscle for interaction with immune cells [116].

Materials and Methods

Only deviations from the procedures described in the previously reported manuscript are included in this section.

Antigens and Adjuvants

Toll-Like Receptor 7 agonist was internally synthetized by the Genomic Institute of the Novartis Research Foundation (GNF).

Preparation of adjuvanted formulations

TdaP antigens (10 Lf TT, 4 Lf DT, 8 µg PT, 8 µg FHA, and 16 µg 69K) were adsorbed onto 2 mg/ml Alum suspension [24] to a final volume of 1 ml. When required, TLR7a was added to the formulations at 50 µg per dose. Animals were immunized intramuscularly with a total volume of 100 µl (50 µl/leg).

TLR7a identity and degree of adsorption onto Alum adjuvant

TLR7a identity and adsorption rate onto Alum were assessed via reverse phase ultra-performance liquid chromatography (RP-UPLC) analysis. Briefly, after spinning down the vaccine, the supernatant was removed and aliquots of 7.5 µl were injected in the UPLC system. The pellet was resuspended in 500mM

potassium phosphate buffer (P3786 Sigma Aldrich) pH 9.0 and incubated at 95°C for 10 min. After Alum separation via centrifugation, the resulting supernatant was appropriately diluted and 7.5 µl were used for UPLC analysis. TLR7a separation was achieved by using a 50mm x 2.1mm, 1.7µm particle size, Acquity UPLC® BEH-C18 column (Waters). The mobile phase was a 10 to 90% acetonitrile gradient prepared in water added of 0.1% trifluoroacetic acid (TFA) at a flow rate of 1.2 mL/min. 268 nm UV was used for TLR7a detection. Detection and quantification limits of the method were found to be 0.39 µg/ml and 0.78 µg/ml respectively.

Results and Discussion

Similarly to what observed with Alum+MPLA and MF59 adjuvants, Alum+TLR7a proved to be a valuable approach for improving pertussis immunogenicity in the mouse model, too. Antigens adsorption onto Alum was estimated to be higher than 90% by WB analysis: none of TdaP antigens was in fact detectable in the supernatants of Alum or Alum+TLR7a formulations even after protein concentration via TCA (data not shown). Overall the inclusion of TLR agonists in Alum formulations was shown not to modify TdaP-antigens adsorption profile. Adsorption rate of TLR7a onto Alum was calculated by using a five-point calibration curve and was found to be higher than 95%. TLR7a identity and integrity was confirmed after desorption from Alum. In addition, RP-UPLC analysis of the desorbed TLR7a allowed a recovery higher than 95% of the agonist (data not shown). The agonist was always tested in combination with Alum adjuvant to prevent systemic adverse events. Moreover, previous studies showed that SMIP alone was inefficient in the induction of protective antibodies against tested antigens [116].

In vivo adjuvant effect

To assess *in vivo* potency of Alum+TLR7a, groups of Balb/c mice were vaccinated twice (day 0, 21) and serum antibody titers were measured before and two weeks after each immunization. Observations made for Alum+MPLA resulted to be valid also for Alum+TLR7a and serological analysis for DT and FHA antigens performed

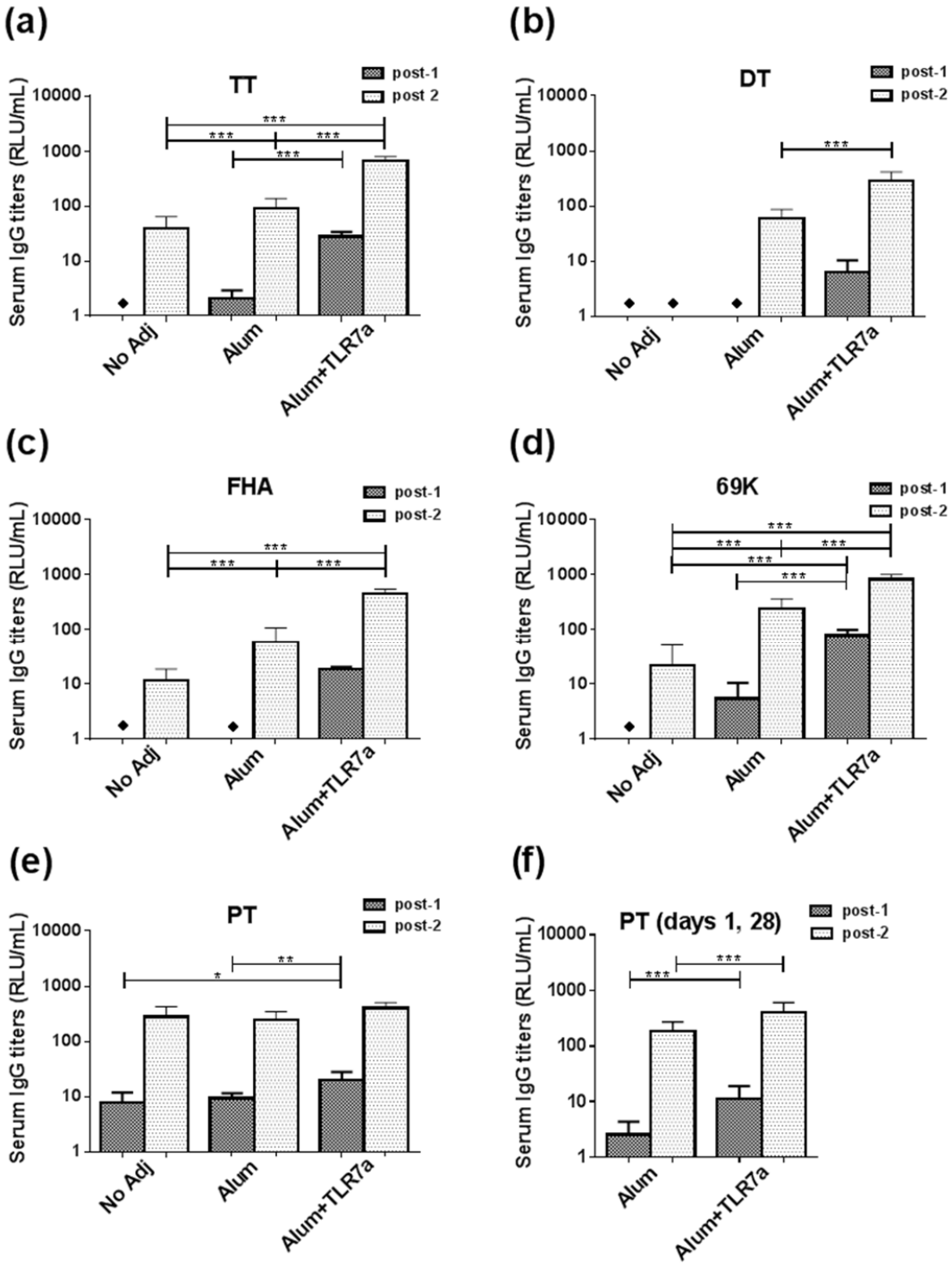

FIGURE 27. Serum (a) anti-TT, (b) anti-DT, (c) anti-FHA, (d) anti-69K, (e) anti-PT antibody titers in Balb/C mice after one and two immunizations at days 1 and 21. (f) anti-PT antibody titers in Balb/C mice immunized at days 1 and 28. Results are shown as median with interquartile range presented for each group.

- serum IgG titers below 1 RLU/ml; LLOQ ≤ 0.9 RLU/ml; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

on serum samples taken after the first immunization revealed that the combination of Alum with the agonist was the only strategy able to induce detectable antibody responses. Overall, a single immunization with Alum+TLR7a adjuvant raised IgG titers higher than the ones induced by Alum. The immune potentiator role of the TLR7 agonist was particularly evident for aP surface adhesins leading to titers comparable to those raised by two immunizations with the non-adjuvanted antigens. After the second dose Alum+TLR7a confirmed its stronger adjuvanticity in comparison to Alum for all the tested antigens with the exception of PT. The observation about the less pronounced adjuvant effect for PT was already described in the manuscript above and justified by the strong immunogenic properties of this antigen. Follow-up studies performed with Alum+TLR7a adjuvant increasing the number of immunized animals and extending the injection schedule from day 0, 21 to day 0, 28 made the adjuvant effect more evident and significantly higher than the on induced by Alum (Figure 27).

Neutralizing activity

To evaluate the ability of the induced antibodies to recognize functional epitopes of the antigens, toxin neutralization assays were performed after the second bleeding. Again, the combination of TLR7 agonist with Alum adjuvant stimulated significantly higher neutralizing titers against DT in comparison to Alum, while not providing an enhancement of neutralizing activity against PT. As previously noticed for total serum antibody titers, increasing the number of immunized animals and the interval between the two injections allowed the observation of an adjuvant effect for PT antigen (data not shown).

Shift in the IgG1/ IgG2a profile

Alum+TLR7a was investigated as alternative adjuvant to Alum for its ability to induce a more Th1 oriented immune response against aP antigens in TdaP vaccine. Classical Alum adjuvanted aP vaccines induce mainly Th2 responses with antibodies of the IgG1 subclass and work presumably mostly via toxin-neutralization activity [107]. In contrast, Th1 responses are characterized by the subclass IgG2a, b or c (depending on the individual mouse strain) which are known

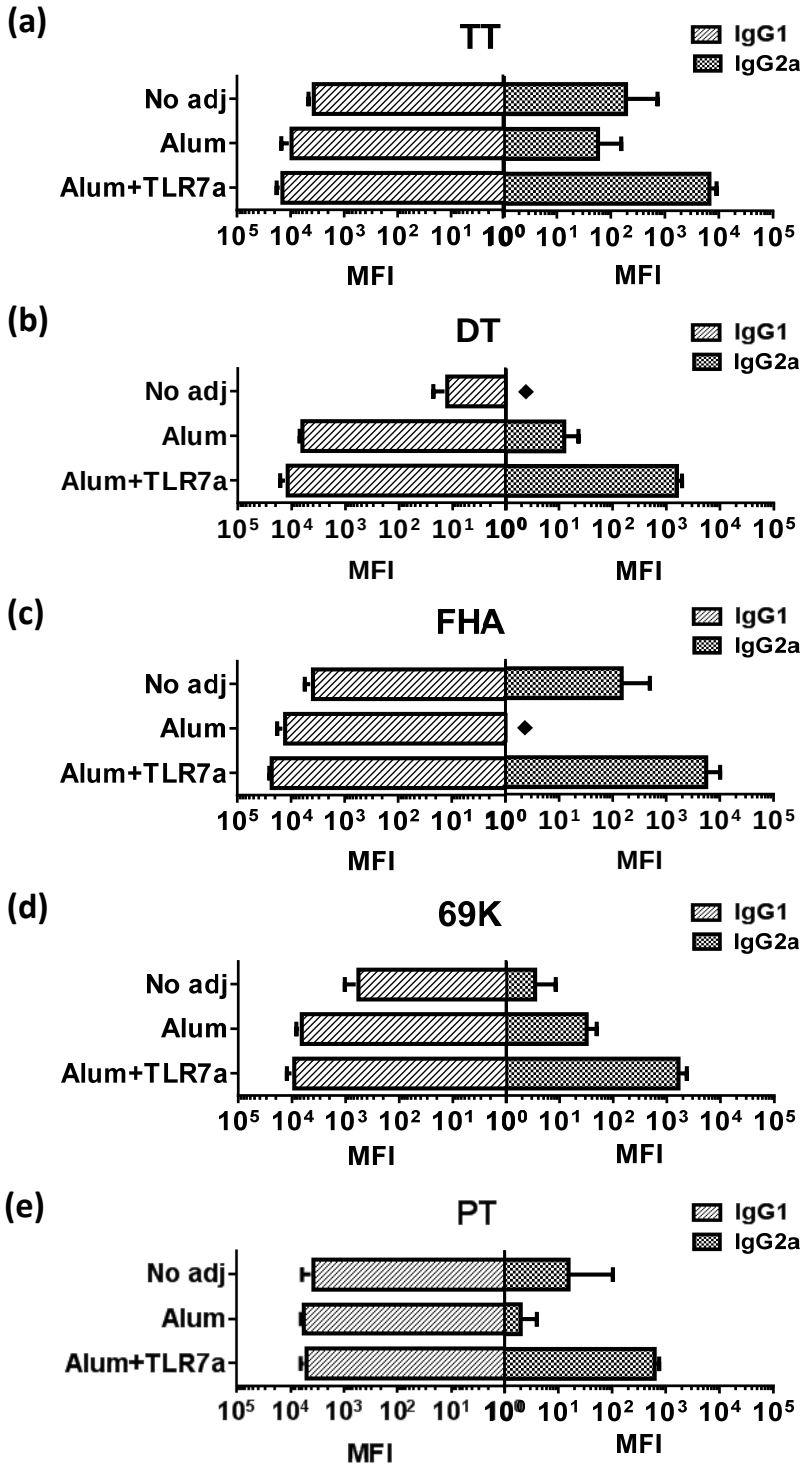

FIGURE 28. Median fluorescence intensities (MFI) relative to specific IgG1 and IgG2a responses (a) anti-TT, (b) anti-DT, (c) anti-FHA, (d) anti-69K, (e) anti-PT in Balb/C mice after two immunizations. Median with interquartile range presented for each group. • values below 1 MFI.

for their better opsonizing capacities and should improve bacterial clearance [120, 140]. To assess a potential shift in the IgG1/IgG2a balance and hence quality of serum antibody responses, subclasses were analyzed after the second immunization. The immune-potentiator role of TLR4a in inducing a shift to a more protective Th1 (IgG2a) profile was observed also for TLR7a when combined to Alum (Figure 28). As already stated, an enhancement of the Th1-type immune response is associated with a more efficient clearance of *B. pertussis* and with an extended protection like the one induced by wP vaccines or natural infection [120, 148].
In addition, preliminary evaluation of the stimulation of memory B cells was performed. However despite a visible trend indicating the immune-stimulatory effect of TLR7a the assay needs to be further improved – data not shown.

Dose-titration and antibody duration studies

Alum+TLR7a was selected as internal strategy for the development of improved acellular pertussis combination vaccines thus being used as main alternative adjuvant in follow-up studies. Immunization schemes were arranged vaccinating Balb/c mice twice (day 0, 28) with different doses of TdaP antigens and of TLR7 agonist in combination with Alum. Dose-titration studies revealed that similar results could be obtained using ten times less antigen doses with respect to the doses used in the previous studies (see paragraph *Preparation of adjuvanted formulations*). Also the dose of the immune-potentiator could be reduced to 10 μg/injection while maintaining comparable results. Importantly, together with an enhancement in quantity, total antibody titers as well as IgG2a-specific antibodies were found to persist up to ten months after the last immunization (data not shown).

Evaluation of larger TdaP-based combination vaccines

The feasibility to develop larger TdaP-based combination vaccines was evaluated through the co-administration of TdaP antigens together with three recombinant antigens conferring protection against *Neisseria meninigitidis* type B. Analysis of *in vivo* immune responses conveyed that the presence of additional antigens in the vaccine combination had no negative interference on the quantity of antibody titers raised against TdaP antigens (Figure 29).

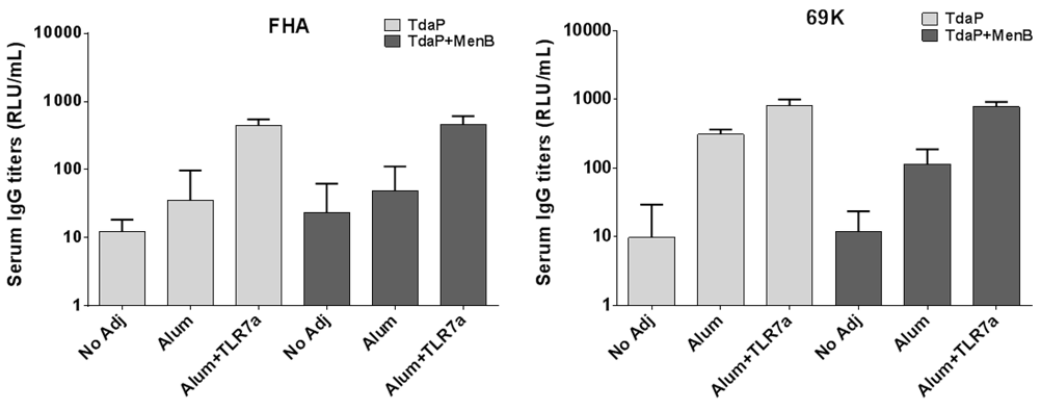

FIGURE 29. Serum anti-FHA and anti-69K antibody titers are taken as representative of all the five TdaP antigens. Results were obtained in Balb/C mice after two immunizations with TdaP or TdaP+MenB antigens. Median with interquartile range is shown for each group.

Conclusions

Results obtained using Alum+TLR7a as improved adjuvants for TdaP vaccine provided encouraging prospective of raising enhanced and better tailored immunological responses against aP-containing combination vaccines. TLR7a molecule is chemically and structurally defined and was specifically designed to be recognized by specific receptors thus stimulating the immune response. In comparison to MPLA, the synthetically obtained molecule TLR7a has the advantage of being structurally simpler thus potentially leading to more reproducible results, both in terms of vaccine formulations and induced immune responses.

CHAPTER IV. CONCLUSIONS

The objective of this PhD work was to investigate the possibility of improving quality and immunogenicity of current acellular pertussis combination vaccines, which are combined with diphtheria and tetanus toxoids in the keystone of combination vaccines. Vaccines and combination vaccines in particular, are complex multi-component products whose efficacy relies on the concomitant inclusion of antigens, adjuvants, and excipients. Currently licensed aP vaccines are typically adjuvanted with aluminum salts, known to induce mainly a humoral immune response which has been associated with the recent pertussis outbreaks reported in many countries. Therefore, existing vaccines are perceived as not ideal to provide protection against the disease. A formulation science approach was here proposed and developed to investigate the potential improvement of acellular pertussis vaccines by working on the adjuvant component. In particular, the promotion of the vaccine analytical characterization and the rational polarization of the quality of immune responses were suggested as effective strategies to be pursued for the development of improved combination vaccines.

Improved *in vitro* characterization assays for combination vaccines adjuvanted with aluminum hydroxide

In Chapter II significant progresses for the *in vitro* characterization of complex aluminum salts-adjuvanted vaccines were introduced. In particular, the possible use of Luminex technology for vaccine characterization purposes was exploited and developed in a multi-plex microsphere-based immunoassay for simultaneous analysis of aP antigens in TdaP vaccines. In a single experiment, the proposed assay provided - information on antigen identity, quantity, and degree of adsorption to adjuvant surface of each immunogen included in a multivalent combination. The simultaneous characterization of the three aP antigens was performed directly on the whole TdaP vaccine without any preliminary treatment of the adjuvant or laborious procedures for adjuvant separation, as commonly required for available analytical methods. The assay relied on antibody recognition and fluorescence detection, thus demonstrating specificity and providing a substantial improvement in terms of sensitivity as compared with other commonly used immunoassays. Importantly, the high sensitivity of the Luminex assay allowed precise quantification of low amounts of antigens not-adsorbed to adjuvant surface. Polyclonal antibodies were used as proof of concept in this work. Their replacement with appropriately selected monoclonal antibodies could allow the qualitative analysis of antigenic epitopes critical for vaccine immunogenicity. In this perspective, the possibility to correlate quality of antigenic components with the efficacy of the product could lead to the set-up of an *in vitro* potency assays accordingly to the 3Rs concept. Moreover, the Luminex-based approach has the potential to be implemented for larger vaccine combinations and to be applied in presence of other adjuvants and delivery systems, such as emulsions and polymeric particles.

***In vivo* evaluation of novel adjuvants for improved aP-containing combination vaccines**

Chapter III was dedicated to study aP vaccine efficacy and the possibility of tailoring immune responses by the modulation of the vaccine formulation. Despite generally high vaccination coverage a pertussis outbreaks are registered in many

countries worldwide. The most reliable hypothesis for pertussis resurgence claims that waning immunity and inadequate immune profiles induced by acellular vaccines are to blame. Induction of a Th1-type immune response - as induced by wP vaccines or natural infection - is seen as beneficial to protect from bacterial colonization and to enhance herd immunity. In contrast, currently available acellular vaccines mainly induce a Th2-type response. In this perspective, MF59 emulsion and two different TLR agonists in combination with aluminum salts were critically selected as alternative adjuvants for acellular pertussis antigens. Alternative approaches tested created a promising scenario for the development of next generation aP containing vaccines with respect to quantity, quality and functionality of the induced immune responses. Importantly, high antibody titers against PT and the promotion of a Th1-oriented response against *B. pertussis* surface adhesins were obtained with the alternative formulations developed. Moreover, the addition of a TLR agonist to Alum-based formulations was shown to accelerate the onset of the immune responses suggesting the potential to reduce the number of injections required to ensure aP vaccine immunogenicity. Encouraging data were obtained supporting the potential of adjuvants to raise better tailored immunological responses against aP-containing vaccine combinations and to effectively control pertussis spread and overcome waning immunity issues. The modulation of vaccine formulation through careful selection of adjuvants has thus emerged as a straight forward approach for the development of effective vaccines.

Overall, advancing the analytical characterization of vaccines on one side and inducing rational polarization of immune responses on the other were presented as suitable approaches to improve both quality and efficacy of future vaccines. Accordingly, the optimization of complex vaccine products through the rational use of adjuvants was proposed as a promising strategy for the development of next generation vaccines.

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