In vitro characterization of pertussis vaccines

Functional analysis as part of the Consistency Approach

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Colofon

ISBN: 978-90-393-6661-5

layout: proefschrift-aio.nl
cover: Marieke Esther Hoonakker

This thesis was financially supported by the Dutch ministry of Health, Welfare and Sports and the Dutch ministry of Economic Affairs

The printing of this thesis was financially supported by Intravacc and the PhD programme Infection and Immunity Utrecht.

About the cover: The pattern of hexagons reflects consistency, while the figures inside them represent several methods used within the Consistency Approach. From the top to the bottom, mice are gradually replaced by cells and the colour variation of *Bordetella pertussis* bacteria gradually declines, illustrating the movement from animal testing towards use of *in vitro* methods and increased consistency in production of vaccines.

Printed by: Ridderprint

In vitro characterization of pertussis vaccines

Functional analysis as part of the Consistency Approach

In vitro karakterisatie van kinkhoest vaccins

Functionele analyse als een onderdeel van de Consistency Benadering

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

donderdag 12 januari 2017 des ochtends te 10.30 uur

door

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geboren op 2 juli 1985 te Spijkenisse

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Chapter 1

General Introduction

Introduction into traditional quality control of vaccines

Vaccine history goes hand in hand with animal experimentation. In the 19th century, Louis Pasteur and his contemporaries like Robert Koch and Paul Ehrlich were the first who clearly formulated the concept of vaccination. They demonstrated that susceptible animals could be protected against a challenge with a virulent micro-organism after immunisation with the respective inactivated or attenuated micro-organism or, in some cases, with the weakened or inactivated toxin(s) of the micro-organism.[1] Since then, numerous vaccines have been marketed and vaccination has shown to be a highly efficient strategy to protect humans and animals against a range of infectious diseases. Animal models have been instrumental not only to R&D and preclinical testing of these vaccines, but also to monitor the quality of routinely produced vaccine lots. As such, animal models have become part of both the licencing process of new vaccines, as well as of routine lot release testing of marketed vaccines, since the models are believed to provide relevant information of the immunological properties and possible adverse effects. It is expected that animal models will remain necessary in the near future for R&D and preclinical testing, as questions on immune responses and safety in the context of interacting organ systems will require the complexity of an *in vivo* model.

The overall objective of this thesis was to contribute to the reduction of laboratory animals used for evaluation of vaccines with a focus on lot release testing. This becomes more urgent since the use of animal models for lot release testing is subject of debate for two reasons: 1) the relevance of the models used and 2) the ethical dilemma of using animals.

The relevance is disputed because:

- the animals are not always naturally susceptible to the pathogen against which the vaccine is directed (e.g. the mouse model for *Bordetella pertussis*[2]),
- the animal models do not always accurately reflect the pathophysiology and the immune response of the vaccine target species (as often is the case for human vaccines[3]), and
- mechanistic information on the toxicological- and/or immune response is generally lacking (e.g. the *in vivo* model for residual pertussis toxin).

The ethical dilemma is an issue since:

- large numbers of animals are often required (up to 100-150 animals are required for the potency testing of each lot of e.g. diphtheria, tetanus, pertussis and polio vaccine). Consequently, the accumulated number of animals used for quality control testing is substantial, e.g. about 17% of all animal experiments in the Netherlands are performed

for the purpose of "development, production and quality control of immune sera, vaccines and other biologicals" (Figure 1).[4]

- test procedures used may inherently inflict severe pain and distress to the animals when the dose of the vaccine is insufficient to protect against a challenge with the virulent microorganism/toxin, or when the vaccine lot contains a harmful contaminant or residual toxin.

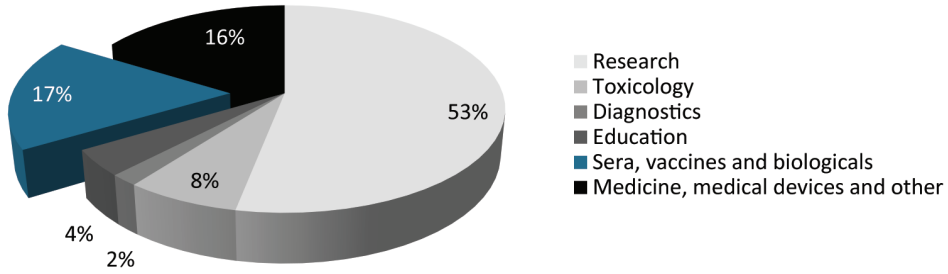

Figure 1 Purpose of animal experiments in the Netherlands. The pie chart represents the percentage of animals experiments performed for the indicated purpose (data adapted from Zodoende 2013).

Additional arguments to dispute the use of animal models are that virulent microorganisms or toxins used for challenge pose a risk to the employees in the laboratory and the fact that animal testing is time consuming and expensive. Despite these critical comments, it should be noted that animal models used for vaccine quality control purposes have ensured the release of safe and efficacious vaccine lots for many decades, although it is a matter of discussion whether this is due to the of the animal models or because of the consistent production of vaccine lots.

The aim of the studies described in this thesis was to evaluate cellular and other alternative methods to replace and reduce the use of animals for vaccine lot release testing. Particularly, these methods might play a role in the Consistency Approach, which is a new paradigm in vaccine lot release testing and which will be described in paragraph 1.2. The focus of this introduction will be on the role of current animal models, the ability of these animal models to assess relevant

Box 1 Alternatives, refinement, replacement and reduction (3R's)

Alternatives is the term that covers any approach or strategy that reduces the number of laboratory animals in a test, reduces pain and distress or increases welfare of the animals under study or replace the use of animals altogether.

Refinement alternatives refers to all procedures that reduce the pain or distress caused to the animals or improve animal welfare.

Reduction alternatives refers to strategies that reduce the number of animals required to answer the (scientific) question or increases the information obtained from each animal, thereby limiting the need for additional animal experiments.

Replacement alternatives refers to techniques or methods that will replace animal experiments or with testing strategies that exclude the performance of animal experiments.

immunological and toxicological properties and alternative 3R approaches. The concept of the 3Rs is explained in Box 1.

An outline will also be presented of the various chapters in this thesis. Our work will centre on animal models and alternatives for the lot release testing of *Bordetella pertussis* vaccines, but the principles generally apply to other human and veterinary vaccines.

1.1 Traditional animal models for vaccine quality control and the first generation of 3R alternatives

As vaccine production depends on biological processes, many variables may influence the quality of the final product. This warrants extensive quality control of the intermediates and final product of each production run, considered as a lot. Particularly, determination of the efficacy/potency and safety of these lots may require testing by *in vivo* models. Vaccine efficacy and potency are two terms used to describe a vaccine's capacity to induce a protective immune response, either in the target animal species (efficacy) or in a surrogate animal model (potency). Safety is defined as the absence of components that induce specific and non-specific adverse effects.[5] The requirements vaccines have to fulfil are laid down in national

and international guidelines (e.g. monographs of the Pharmacopoeia, FDA documents and WHO publications) and vaccine lots are only released for the market after approval by national authorities.

Potency tests can be divided into indirect and direct animal models that assess the immunological properties of a vaccine. In the indirect potency tests, animals are immunised with the vaccine under study and bled several weeks thereafter. The protective properties of the induced antibody responses are determined by mixing dilutions of the sera with a fixed dose of virulent microorganism or toxin and injecting these mixtures in a second group of naïve animals of the same or different species, or exposing cells to these mixtures (e.g. inactivated polio vaccine). In the indirect method, cytopathogenesis, lethality or severe clinical signs in the unprotected animals are used for estimating potency. This indirect design is frequently applied for veterinary vaccines, such as Clostridium toxoid vaccines.[6-8]

In the direct potency test, assessment of potency is based on immunisation of several groups of animals with increasing doses of the vaccine under study, followed by a challenge of the animals with a fixed (and often lethal) dose of the virulent microorganism or toxin. Animal models based on this direct design are used for some inactivated human vaccines, such as the whole-cell *Bordetella pertussis* vaccine.[9] For vaccines that have been more recently approved (e.g. pneumococcal vaccines) and for live-attenuated vaccines (e.g. varicella, measles or rubella), evaluation of antigen levels using immunochemical methods or titration of the micro-organisms suffice and animal tests are not required.[10-13]

Vaccine safety tests aim to assess a variety of aspects, including general toxicity, specific toxicity, contamination with extraneous agents (microorganisms, such as viruses, fungi and mycoplasma) and residual virulence or toxicity of the inactivated relevant antigens. In a safety test, animals are injected with the vaccine under study, frequently using relatively large volumes, followed by determination of safety by observing clinical manifestations of toxicity or contamination, such as a change in body weight, signs of illness, increase or decrease of body temperature or clinical signs of virulence. Because of the variety of purposes, test designs are rather diverse and the opportunities to refine, reduce and replace animal models are test- and vaccine specific.

Starting from the 1980's, the use of these traditional potency and safety tests is being increasingly questioned. This has resulted in numerous 3R's initiatives (Figure 2). Nevertheless, potency and safety testing of conventional vaccines (e.g. diphtheria, tetanus, pertussis and polio) still relies primarily on animal models. In contrast, new generation vaccines provide evidence that *in vitro* methods can be used for lot release testing, though it should be noted

that the nature of these 2nd and 3rd generation* vaccines is different from conventional vaccines, as they are generally based on fully characterised antigenic components of the virulent microorganism.[14,15] Nevertheless, we feel that also for the 1st generation of vaccines, increased scientific knowledge, standardised production processes and the implementation of process-monitoring systems such as Good Manufacturing Practice (GMP) and Quality Assurance (QA), urges us to re-evaluate the current testing strategy for quality control of vaccines and to search for new approaches.

1.2 A new paradigm in vaccine quality control: the Consistency Approach

Vaccine quality is related to a complex set of factors, including starting materials (media, cell lines, bacterial and viral seed lots), the production equipment, and the manufacturing protocol (including the techniques used for the culturing of the relevant microorganism, the purification or other processing of the antigens and the formulation of the final product). Since variations in all of these factors can affect vaccine quality, the historical paradigm in vaccine lot release testing is that each lot produced is a unique product of which the quality (including potency and safety) has to be demonstrated. Additional arguments for maintaining this paradigm are the complexity of the final products (many vaccines contain multiple antigens and one or more adjuvants) and the unknown effects of the combination of these components. However, since the widespread introduction of vaccination in the '50-'60, standardisation of vaccine production processes have improved significantly.[16] Key components of vaccines are now fully characterised, optimised and standardised (bacterial and viral seeds, cell lines, medium components), while quality control systems such as Good Manufacturing Practice (GMP) and Quality Assurance (QA)[17] have been implemented and production processes have been validated and laid down in Standard Operating Procedures (SOPs) and batch production and control records. In addition, there has been a significant improvement in non-animal techniques to characterise intermediate and final products. The combination of these aspects enables extensive monitoring and control of the production process and quality of the product from seed vial to final lot.[18,19] When these parameters are monitored for each newly produced vaccine lot and shown to be comparable to those of a vaccine lot of proven efficacy and safety, a vaccine manufacturer can show that

* Second generation vaccines consist of isolated subunits of antigenic components of the microorganism that are essential for protective immunity or bacterial polysaccharides chemically linked to immunogenic proteins. Third generation vaccines consists of recombinant DNA (rDNA) technology derived immunogenic proteins expressed in host systems or (rDNA induced) mutated microbes lacking genes that encode for pathogenicity. There is increased interest in chemically synthesized polypeptide vaccines and DNA vaccines that hold genes of pathogens important for immune responses. DNA vaccines contain recombinant plasmids that are taken up in the cells of the vaccinated person or animal, induce production of pathogen protein(s) and elicit immunity.

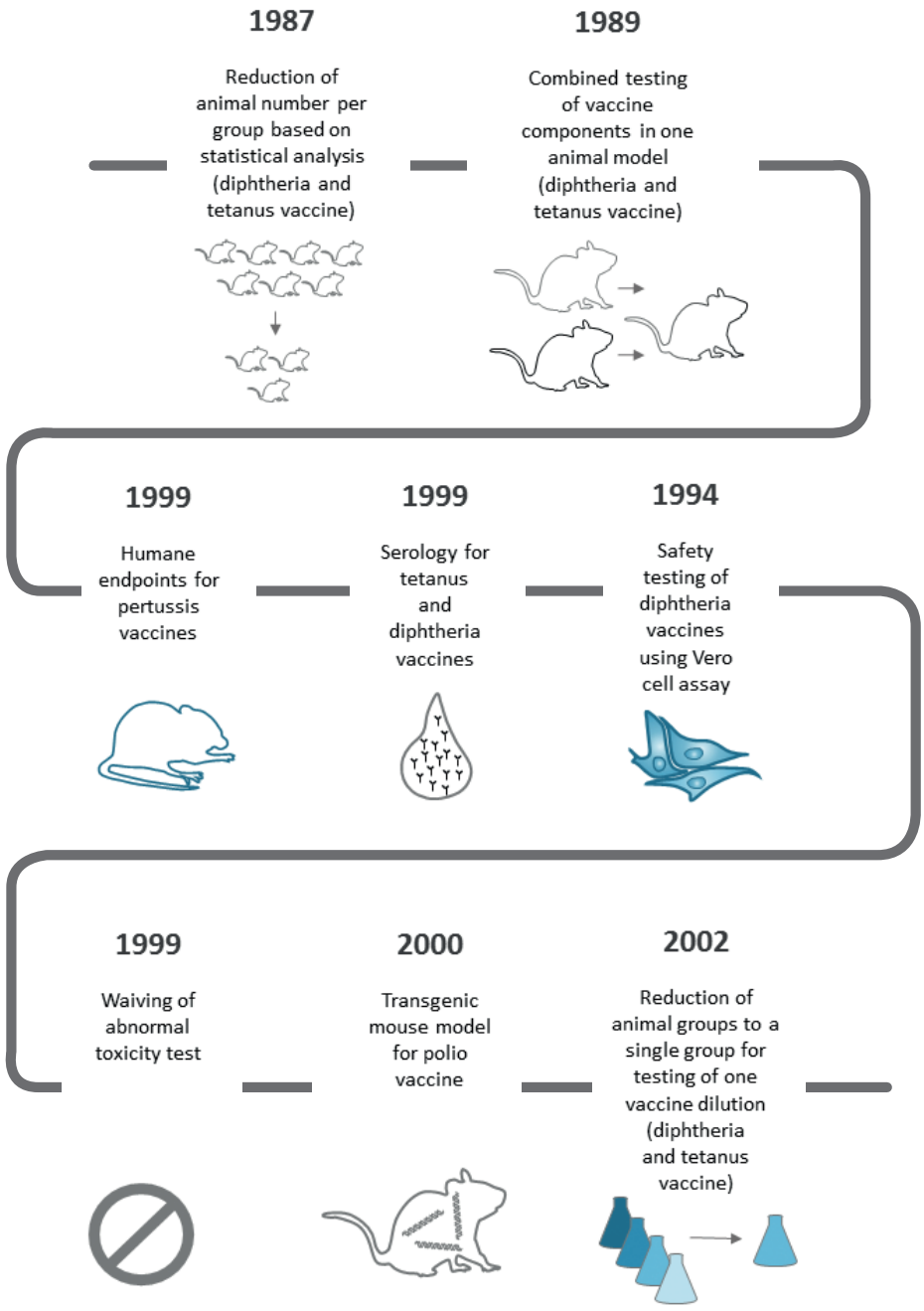

Figure 2 Validated and accepted alternatives for vaccine lot release testing

vaccine lots are produced in a consistent and reproducible way. Consequently the quality of the newly produced vaccine lots is equivalent to the quality of the vaccine lot with proven efficacy and safety. This testing strategy forms the basis for the so-called Consistency Approach.[18,20] When this approach is used, testing using animal models may only be needed for the first few lots of the vaccine to set up a product profile, whereas subsequent lot release can be based on a combination of GMP and QA-based in-process monitoring and thorough physicochemical, immunochemical and *in vitro* analysis of the intermediate and final products (Figure 3). This type of approach is already in place for newer generation vaccines. To adapt this approach for legacy vaccines, the elements critical for production need to be identified and evaluated, which can be done according to the following principles (also summarized in Figure 3).

The characterisation and standardisation of the production process starts with the identification and evaluation of the elements essential for vaccine quality, such as the antigens relevant for inducing protection, as well as a careful monitoring of the production process (e.g. oxygen concentration, nutrient consumption and temperature). In this phase, vaccine specific physicochemical, immunochemical and cellular methods that enable characterisation of relevant vaccine characteristics need to be developed. To establish a thoroughly characterised production process, a selective number of experimental production runs are required to set the limits of the production process in order to determine alert and acceptance criteria for crucial production parameters. After having established a consistent production process, a selective number of vaccine lots have to be evaluated *in vivo* and - in the case of a new vaccine - in clinical studies. Provided that these steps are successfully completed, subsequently produced vaccine lots can be released to the market based on the demonstration of a consistent production process and the manufacturing of intermediate and final products of consistent quality.[16,21,22] Crucial for the application of the Consistency Approach are appropriate physicochemical and immunochemical methods that enable thorough characterization of the composition and conformation of the intermediate and end products, while cellular *in vitro* methods enable evaluation of the immunological and adverse properties of the vaccines.

1.3 Analytical methods to characterise intermediate and final products

A variety of analytical methods have been developed to characterise intermediate and final vaccine products. These methods can be divided in:

- Colorimetric and separation techniques, including electrophoresis, chromatography and mass spectrometry. These techniques are applied for example to measure protein, polysaccharide and DNA concentrations.

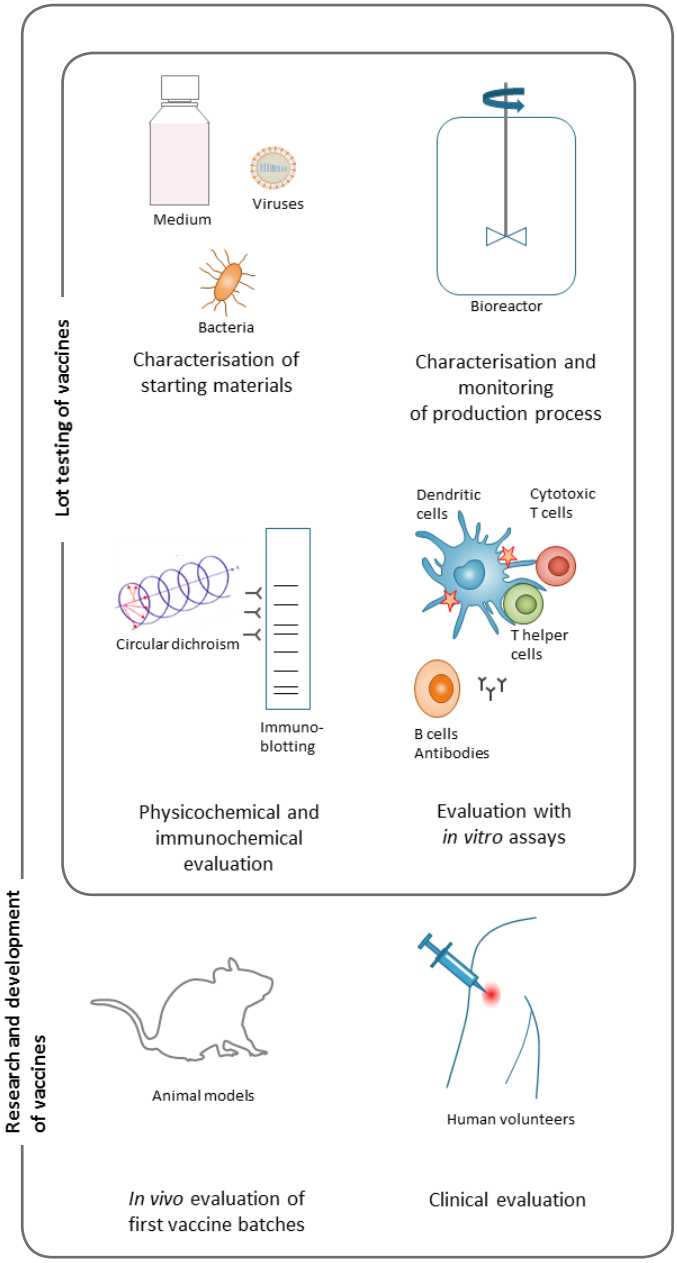

Figure 3 The principles of the Consistency Approach

- Physicochemical and immunochemical techniques, including fluorescence and infrared spectroscopy, and circular dichroism.[23,24] These techniques are used for the analysis of antigen conformation and for the characterisation of epitopes and antigens. Immunochemical techniques such as ELISA, Biosensor analysis and immunoblotting can be used to determine the presence of specific epitopes/antigens in the vaccine.

A major obstacle of the techniques for antigen characterisation is that most vaccine antigens are absorbed to an adjuvant. Desorption techniques have been used to circumvent this issue in the past, but the techniques used entail the inherent risk of changing antigen integrity. Fortunately, several new and more sensitive techniques are now available that enable direct detection and characterisation of antigens while adsorbed to aluminium salt, such as the Direct Alhydrogel Formulation Immunoassay and fluorescence spectroscopy, circular dichroism, infrared spectroscopy and differential scanning calorimetry.[23,25-28]

Table 1 Cell assays for *in vitro* evaluation innate immune responses

Cell type and orginin		Availability	Application	T cell stimulatory capacity		Ref.
Cell lines	HEK expressing PRR	n.a.	evaluation of individual PRRs	n.a.		34,35
	MM6, human leukemia	n.a.	monocyte type of response	n.d.		36-38
	MUTZ-3, human leukemia	n.a.	DC type of response	allogeneic: high		39,40
	THP-1, human leukemia	n.a.	monocyte/ macrophage type of response	allogeneic: low/ intermediate		41,42
	KG-1, human leukemia	n.a.	monocyte/ macrophage type of response	allogeneic: intermediate		42-46
	U-937, lymphoma	n.a.	monocyte type of response	n.d.		47-49
	HL-60, human leukemia	n.a.	monocyte/ macrophage type of response	allogeneic: intermediate		50-52
	K562, human leukemia	n.a.	monocyte type of response	n.d.		53-55
Primary cells	Monocytes	high	Monocyte response	allogeneic: low	autologous: low	56,57
	Macrophages	low	Macrophage type of response	allogeneic: high	autologous: high	58
	moDC	high	DC response	allogeneic: high	autologous: high	58-62
	pDC	low	DC response	allogeneic: n.d.	autologous: intermediate	63
	CD34+ DC	low	DC response	allogeneic: high	autologous: n.d.	59,64

n.a. not applicable

n.a. not applicable

1.4 *In vitro* assays to assess innate immune activation by vaccines

The physochemical and immunochemical techniques provide a detailed characterisation of the structure, conformation and presence of specific epitopes on the vaccine antigen, but do not assess the immunological properties of the vaccine. Though the capacity of a vaccine to induce an appropriate immune response traditionally has been studied using animal models, innovative techniques nowadays provide the opportunity to evaluate several functional characteristics of the vaccine without using animals.[29,30] Figure 4 illustrates that immune responses involve a large variety of cells and cascades of dynamic interactions in a complex organ system.[31-33] Multiple *in vitro* methods are available that can mimic parts of this immune response. These methods can largely be divided in two groups, those dedicated to assess initiation of innate immune responses (Table 1[34-64]) and those that assess the induction of adaptive immune responses (addressed in **Chapter 9**). The first category of methods may be instrumental to evaluate vaccine-induced innate immune cell activation, and includes methods indicative of a) binding to and signalling through specific pathogen recognition receptors (PRR), innate cytokine secretion and upregulation of co-stimulatory receptors, and b) of the induction of migration through a chemokine gradient.

Activation of cells through PRRs involved in the recognition of pathogen-associated molecular patterns (PAMP) and damage-associated molecular patterns (DAMP) can be studied by a range of reporter cell lines, expressing one or several of these receptors.[65-67] These methods provide information essential for understanding the role of one or a couple of receptors, but the integrated response can only be studied using primary-derived or cell line antigen presenting cells (APC), cells that endogenously expressing a range of PRRs.

There is a range of human cell lines derived from cancer patients with a monocytic and dendritic cell phenotype. Comprehensive overviews of most of the available cells lines and their properties are published elsewhere.[39,68,69] In general, phenotypically stable cell lines are derived from a single source and therefore contribute to reproducible results compared to primary immune cells that are derived from multiple donors with diverse immune status. However, cell lines frequently have deficiencies in functional properties that are characteristic for APC.[39] Despite these limitations, APC cell lines have proven to be effective tools to study DC physiology[39] and vaccine innate immune responses.[70] In addition to cell lines, primary monocytes or dendritic cells derived from the circulation have been used to investigate immunological responses *in vitro*/*ex vivo* (Table 1). Although primary monocytes and DCs are of a higher clinical relevance than cell lines, differences in response of these cells *ex vivo* compared to *in vivo* need to be considered.[71,72] Since monocytes are more abundant in the circulation, they are often used to induce monocyte-derived dendritic cells (moDC) by their culturing in the presence of growth

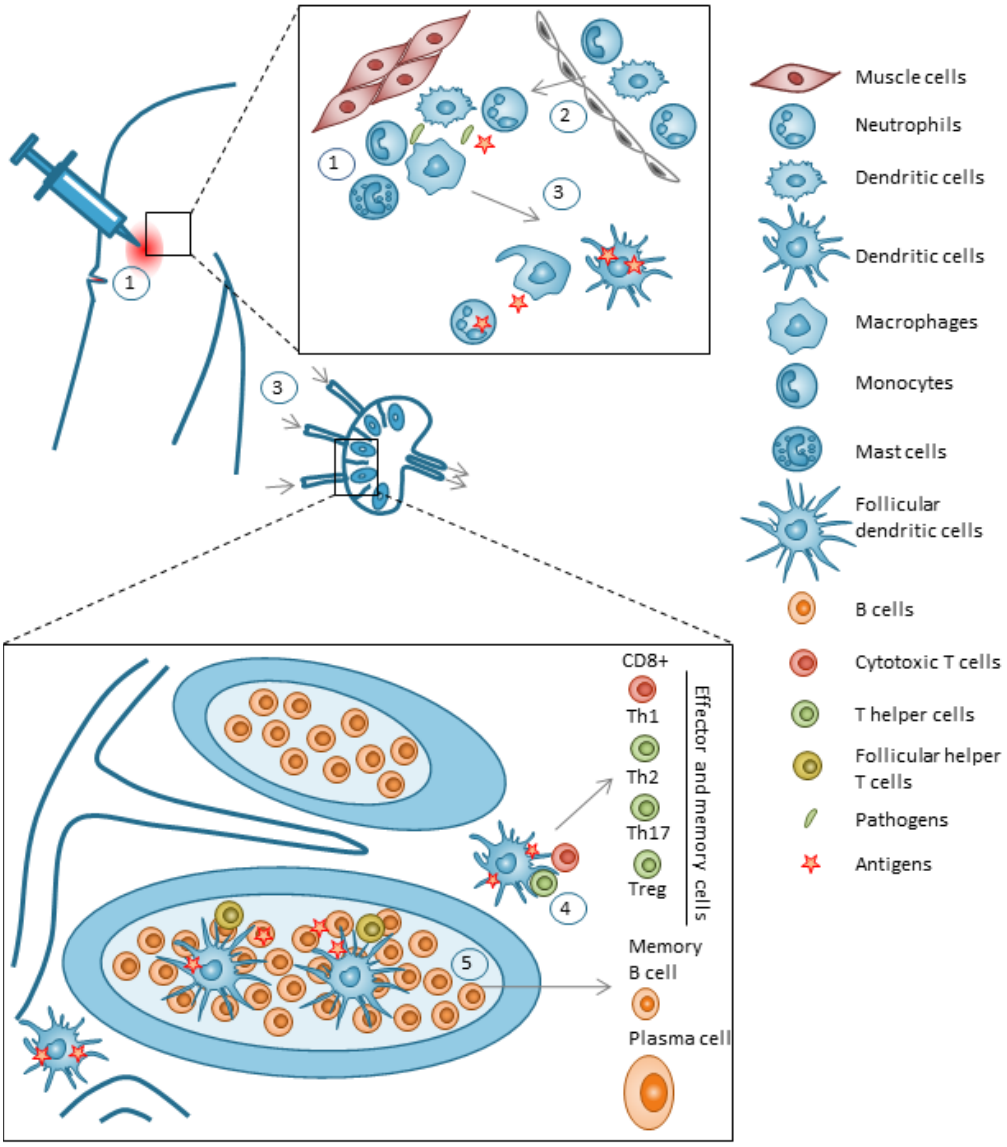
1
2
3
1
3
4
5
CD8+
Th1
Th2
Th17
Treg
Effector and memory cells
Memory B cell
Plasma cell
Muscle cells
Neutrophils
Dendritic cells
Dendritic cells
Macrophages
Monocytes
Mast cells
Follicular dendritic cells
B cells
Cytotoxic T cells
T helper cells
Follicular helper T cells
Pathogens
Antigens

Figure 4 Events in immunological response after pathogen invasion or administration of a vaccine (adapted from [31]). The immune system is dedicated to protect the body from the harmful effects of pathogens such as bacteria, viruses, fungi, parasites. The first line of defence is formed by the innate immune system, consisting of the complement system, natural killer (NK) cells, γδ-T cells, mast cells, lymphoid stromal cells, antigen presenting endothelial cells, dendritic cells (DC), macrophages and neutrophils. The second line of defence is formed by the adaptive immune response. This response is pathogen- and antigen specific and comprises helper T cells, cytotoxic T cells and B cells. Cells of the innate immune system have evolved in a highly specialised way to recognise a broad range of pathogens. Upon a pathogenic invasion or injection with a vaccine, mast cells and tissue cells immediately start to secrete cytokines and chemokines (**label 1**). This attracts neutrophils and NK cells from the circulation to the site of infection (**label 2**). These cells are specialised in the elimination of pathogens (neutrophils) and virus-infected cells (NK cells) by phagocytosis and secretion of cytotoxic proteins, respectively. In addition, resident macrophages, monocytes and DC, as well as recruited dendritic and monocytic cells, phagocytose the remaining pathogens or antigenic structures (**label 2**). Upon uptake of the pathogen or antigen, proteins are processed and the ensuing fragments coupled to MHC molecules are transported back to the cell membrane. DC are considered to be the professional APC, though monocytes, B cells and macrophages also have antigen-presenting properties. APC recognise pathogens by their conserved pathogenic structures, i.e. PAMP, as well as local cell damage by DAMPs. These structures are recognised by PRR, including Nod-like receptors, C-type lectin receptors and Toll-like receptors. Upon activation, APC secrete cytokines and concomitantly upregulate co-stimulatory receptors, migrating toward draining lymph nodes (**label 3**). Within the lymph node the APC are scanned by $CD4^+$ helper T (Th) cells (**label 4**) that recognise specific MHC-bound peptides with their T cell receptor (TCR). The combination of the MHC-presented peptides, expression of co-stimulatory receptors and secretion of pro-inflammatory cytokines primarily orchestrates the type of Th response, generally divided in Th1, Th2, Th17 or Treg (referring to regulatory) response. A different pathway is required for optimal activation of antibody-producing B cells. B cells are activated by freely diffusing antigens or pathogens. Binding to the B cell receptor initiates intracellular signal transduction, but it can also result in internalization of the pathogen or antigen, degradation and presentation of the peptides in a MHCII molecule. Depending on the type and intensity of this binding and the co-stimulatory signal derived from APC and Th cells, B cell migrate toward B cell follicles in the lymph nodes and initiate formation of germinal centres (**label 5**). The subsequent process of proliferation, hypermutation, differentiation and affinity maturation determines whether the B cell become antibody secreting plasma B cells or memory B cells. In the case of an insufficient innate immune response, appropriate T cell responses and antibodies are required for complete elimination of the pathogen. In addition, functional memory T and B cells aid mammalian species to respond more rapidly upon a subsequent encounter with the same pathogen. Vaccination is based on the formation of a functional memory immune response though administration of inactivated pathogens or immunological relevant parts of the pathogen mimicking a similar sequence of events induced by a complete pathogen.

factors and cytokines. These moDCs have proven to be a useful tool for studying vaccine-induced APC activation *in vitro*[70,73], though it has been recognised that moDC exhibit limited migratory and protective properties.[74] Other DC types, either circulating plasmacytoid DC or DC produced from $CD34^+$ progenitors *in vitro*, are considered to possess characteristics more similar to classic DC[62,75], though their low numbers in the circulation limit the use of these cells. Another option is monocytes themselves, which have shown to be recruited upon injury and exposure to PAMP, resulting in the differentiation to DCs or macrophages.[72,76] Monocytes can be cultured *in vitro* and have shown to respond to a range of PAMPs.[77] In summary, there is a range of both primary and cell line derived APC's that provide the opportunity to study APC activation *in vitro*, though their advantages and limitations need to be considered, as well as their distinct role during immune responses.[75]

Next to activation, adhesion and migration are essential for homing of immune cells to lymphoid tissues and the induction of adaptive immune responses. The capacity of a vaccine to induce adhesion and migration is therefore of importance for induction of protective immunity. Several adhesion and migration assays have been described. Often, adhesion is studied by APCs cultured in cell culture plates coated with adhesion molecules[78] or endothelial cells.[79] Migration assays frequently involves transwell systems, where the APC migrate into the lower chamber of the system as a result of a chemokine/cytokine gradient[79,80] or into a semi-solid matrix.[81] In the last decades modifications of these cell culture plate methods have become available, such as the Venaflux[82], which more accurately reflect the blood flow and endothelial cell barrier function.

Taken together, there is a variety of models that can be used to assess innate immune properties *in vitro*. However, these models have been used primarily for R&D purposes and to our knowledge have not been evaluated for vaccine lot release testing. Since these methods provide the possibility to study innate immune cell activation by immune modulatory agents, they might be valuable tools for monitoring of vaccine quality within the context of the Consistency Approach.

1.5 *In vitro* assays for testing of vaccine safety

Despite the inherent disadvantages discussed in the first section of this introduction, there are still several animal models in place primarily for safety testing of legacy vaccines. Since demonstration of safety is inherent to the principles of the Consistency Approach, the development and use of non-animal methods for this purpose will be important for successful implementation of this strategy. Fortunately, the mechanism of action of the majority of the

toxins and several contaminants in legacy vaccines has been elucidated and this information is used for developing non-animal methods (information is provided in Table 2[83-92]). Since the focus of this PhD study is on pertussis vaccines, we will address the mechanisms of toxicity of pertussis toxin (PTx) in acellular pertussis vaccines as an example and described the physicochemical, immunochemical or cellular alternatives under development.

PTx is an AB_5 exotoxin that binds to glycoproteins on the cell surface, upon which it is internalized and enters the cytosol. Inside an eukaryotic cell, PTx ADP-ribosylates the α-subunit of G_i proteins and consequently interferes with downstream cell signalling. To test for residual PTx in acellular pertussis vaccine, the mouse-based Histamine Sensitization test (HIST) is a regulatory requirement (described in more detail in Section 1.6). Several alternatives are under development, however the level of mechanistic understanding varies between the methods. One of the alternative approaches entails a combination of two methods, one consisting of a fetuin-ELISA[93,94] used to measures the binding properties of PTx, while the other method is based on an enzymatic HPLC assay that measures the enzymatic activity of A subunit.[95-97] The combination of these assays reflects properties essential for PTx toxicity[98], but do not cover the internalisation and translocation of the toxin from the cell membrane to the cytosol, aspect which are an inherent part of the pharmacological effects of the toxin. All these aspects are covered by three cell culture alternatives. The first cell culture method is the Chinese Hamster Ovary cell (CHO) test and is based on a PTx-induced clustered growth pattern.[99,100] Although the clustering relies on the A subunit[101], B oligomer binding and internalisation and translocation of the toxin are necessary under physiological conditions for the A subunit to execute its effect in the cytosol. Because of the inherent toxicity of the aluminium salts used as vaccine adjuvants for CHO cells, the test is not yet suitable for pre-formulated products.[102] Another hurdle is the visual reading of the cell clustering. Despite attempts of several research groups, automatic reading is not yet possible. The second cellular method is based on the intracellular effects of PTx on adenylate cyclase regulation, in terms of the measurement of changes in ATP levels.[103] Since toxin binding, internalisation and disturbance of the regulation of adenylate cyclase are intrinsic elements of these adverse outcome pathways, this method covers all cellular aspects relevant for the safety of aP vaccines. However, the assay is not sensitive to low levels of PTx, and therefore not suitable for quality control purposes.[103] The last alternative is the cAMP-PTx assay, which will be addressed in this thesis.

Table 2 Animal tests for the evaluation of vaccine safety and possible *in vitro* methods

Vaccine	Mechanism of toxin/ type of contamination	*In vivo* method	Description of possible *in vitro* alternative	Ref.
Tetanus	Tetanus toxin blocks the release of neurotransmitters through proteolytic cleavage of synaptobrevin and thereby interferes with neural signalling	Subcutaneous injection into guinea pigs	BINACLE assay: evaluatio of binding to ganglioside GT1b and synaptobrevin cleavage in a microtiter plate	83
Oral Polio	Vaccine virus can be neurovirulent	Intracerebral injection into non-human primates	Mutant analysis by PCR and restriction enzyme cleavage test (MAPREC) or deep sequencing or massively parallel sequencing	84-87
BCG	Vaccine bacterium can be virulent	Subcutaneous injection into guinea pigs	Deep sequencing or massive parallel sequencing	87-89
Vaccines in general	General toxicity	Intraperitoneal injection into mice and guinea pigs	Randomly primed PCR combined with/or massive parallel sequencing	90-92
Viral vaccines	Extranous agents	Injection of the vaccine into a specific animal species	Randomly primed PCR combined with/or massive parallel sequencing	90-92

1.6 Outline of this thesis

In first paragraph of this Introduction, we concluded that the animal models being used are highly disputed because of their doubtful relevance and the substantial number of animals that they require. There is an urgent need for new *in vitro* models; however these models have not been evaluated for vaccine quality control purposes. The primary aim of the studies described in this thesis was to develop, optimize and evaluate *in vitro* methods and bring animal-free lot release testing of human vaccines one step closer. *Bordetella pertussis* vaccines were used for this evaluation, because a) an extensive number of animals are used for safety and potency testing of this type of vaccine, b) the animal models for these vaccines are linked to severe pain and distress, c) results obtained from animal models are associated with high variability and poor reproducibility and d) the vaccines are attractive from a scientific point of view, as is discussed below.

A short description of the bacterium *B. pertussis* is given in Box 2.[104-111] There are two types of pertussis vaccines, the whole-cell and the acellular pertussis vaccine. The whole-cell pertussis (wP) vaccine consists of inactivated *B. pertussis* bacteria[112] and was introduced in the '40-'60 of the 20th century. Due to occasional reactivity associated with wP vaccination, safer acellular pertussis (aP) vaccines - containing the inactivated form of PTx, alone or in combination with other virulence proteins such as FHA, PRN, FIM2 or FIM3 - were developed and introduced in

most industrialised countries in the '90 of the previous century.[113] Yet, wP vaccines are still used in many national immunisation programs, mainly in Asia, Africa and Latin America.[114] Though both aP and wP vaccines are effective in preventing pertussis, wP vaccination is considered to result in superior protection and durability of immunity, at lower costs[115-117], and it is therefore likely that wP vaccines will be still widely utilized in the foreseeable future. This mere fact adds urgency to the need for *in vitro* alternatives to the animal models used. For potency testing of aP vaccines, a mouse serological model is required, while a mouse intracerebral challenge based model (Kendrick test) is used for wP vaccines.[9,118] Especially the Kendrick test is under debate, because of the disputable relevance of an intracerebral challenge in mice with a human respiratory pathogen[2], the high level of pain and distress involved, but also because of variability in test results within and between laboratories. An *in vivo* alternative has been described: the pertussis serological potency test (PSPT).[119-121] This test is based on the quantitative assessment of vaccine-induced IgG antibodies in mouse serum directed against inactivated virulent *B. pertussis* bacteria. Since antibodies contribute to protection against pertussis[122], it is likely that the *B. pertussis*-specific antibodies assessed in the PSPT provide a relevant parameter.

Box 2 *Bordetella pertussis*

Bordetella pertussis is a Gram-negative bacterium and causes the human respiratory disease whooping cough, also referred to as pertussis. Pertussis is a highly contagious disease and can be severe and occasionally lethal especially in infants. The bacterium expresses a range of virulent proteins, e.g. PTx, filamentous hemagglutinin (FHA), pertactin (PRN), fimbriae 2 (FIM2) and fimbriae 3 (FIM3). These proteins promote attachment, but also modulate the host immune response[104]. The expression of these virulence activated genes is controlled by the master regulatory system BvgA/BvgS/BvgR. Depending on the environmental stimuli, the transmembrane protein BvgS can phosphorylate itself and BvgA.[105] BvgA is a transcription activator and controls the expression of the virulent activated genes (vag) and bvgR of which the product[106], BvgR, represses the transcription of virulence repressed genes (vrg).[107] Modulating conditions such as temperatures below 26°C, nutrient depletion, or the presence of sulfate ($MgSO_4$) or nicotinic acid, result in an absence of phosphorylation of BvgASR, repression of vags and expression of vrgs.[108] This state is referred to as the Bvg^- phase as opposed to the Bvg^+ phase where BvgASR and virulence proteins are expressed. The Bvg phase and expression of virulence genes have been shown to affect lung colonization[109] and immune responses.[110, 111]

For safety evaluation of aP vaccines the HIST is performed to detect residual active PTx, while for safety evaluation of wP vaccines the mouse weight gain (MWG) test is used to detect LPS, PTx and additional non-specific toxicities. Since the MWG test aims to assess non-specific and therefore unknown contaminants, its mechanism cannot be specified, and this represents a challenge for the development of *in vitro* alternatives. The HIST is based on the empirical finding that PTx reduces the lethal dose of histamine for mice up to 300 fold[123], though there is a large diversity with regard to sensitivity of different mouse strains.[124] Although the exact mechanism of the HIST remains to be elucidated, it is clear that PTx affects the contractile properties of arteries[125-127] and it is likely that this phenomenon is involved in HIST as well.

In this thesis mostly cellular *in vitro* methods to determine the quality of wP vaccines and the safety of aP vaccines were developed and evaluated. To investigate the capacity of alternative methods to characterise wP vaccine quality, vaccines of various qualities were required. Since such vaccines are not commercially available, we used vaccine lots of varying quality prepared at experimental scale by deliberately manipulating the expression of virulence factors of *B. pertussis* bacteria using $MgSO_4$, as described in **Chapter 2**. Since wP vaccines have not been analysed in the context of the Consistency Approach before, the effect of the $MgSO_4$ manipulation on bacterial gene expression and vaccine protein composition was studied by microarray analysis and physicochemical and immunochemical methods, such as mass spectrometry and ELISA. In addition, the *in vivo* potencies of these vaccines were determined by the regulatory required Kendrick test. In **Chapter 3**, we studied the activation of human Toll-like receptors (hTLR) 2 and 4 induced by our wP vaccine, the effects of $MgSO_4$ suppression on the activation of these PRRs, as well as the roles of these PRRs in wP vaccine-mediated activation of APCs. In **Chapter 4**, four APC platforms (primary monocytes, moDC, MonoMac6 and MUTZ-3 cells) were evaluated for their ability to assess innate immune cell activation by our experimental wP vaccines. In addition, several characteristics that are considered to be important for quality control testing, i.e. relevance, sensitivity and specificity, were analysed for these APC platforms. In **Chapter 5**, the *in vivo* innate and adaptive immune mechanisms induced by our wP vaccines of various qualities were studied in more detail in the PSPT. In addition to conventional detection of *B. pertussis*-specific antibodies in this animal model, several other parameters were evaluated including a) cytokine levels in serum during the innate immune response, b) the magnitude of cytokines produced by T helper cells 28 days after immunization, and c) antibody specificity.

In **Chapter 6**, an alternative *in vitro* method to the HIST is described. This alternative method is based upon the effect of PTx on intracellular cAMP levels, using a rat vascular smooth muscle cell line. The read-out of this assay is deemed relevant, since changes in cAMP levels are considered responsible for the majority of PTx clinical effects. In **Chapter 7**, we constructed

four cell lines expressing a cAMP-sensitive reporter construct that enables simple detection of changes in intracellular cAMP levels. Sensitivity of the reporter cell lines for PTx activity was studied, together with their capacity to detect PTx in an aP vaccine matrix. In **Chapter 8** we assessed the global numbers of mice used for the HIST and discussed the potential impact of 3R alternatives on animal numbers used for aP vaccine testing by industry and national control laboratories. In **Chapter 9**, the results and implications of the work presented in the preceding chapters is summarized and discussed and future perspectives are described.

In conclusion, in this thesis we (1) evaluated physicochemical, immunochemical and cellular *in vitro* means for characterisation of wP vaccine quality, (2) investigated the effects of wP vaccine quality on *in vivo* innate and adaptive immune responses and (3) developed an *in vitro* method for aP safety quality testing. The ultimate goal of these methods is to reduce the number of experimental animals needed for quality control of vaccines and provide *in vitro* means in support of the Consistency Approach.

References

1. Plotkin, S., History of vaccination. Proc Natl Acad Sci U S A, 2014. 111(34): p. 12283-7.
2. van der Ark, A.A., *et al.*, Resurgence of pertussis calls for re-evaluation of pertussis animal models. Expert Rev Vaccines, 2012. 11(9): p. 1121-37.
3. Seok, J., *et al.*, Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A, 2013.
4. Netherlands Food and Consumer Product Safety Authority. Zo doende. 2013: Utrecht.
5. Hendriksen, C., *et al.*, Validation of alternative methods for the potency testing of vaccines. Altern Lab Anim, 1998. 26(6): p. 747-61.
6. European Pharmacopoeia, Clostridium septicum vaccine for veterinary use. 2013. 8th edition: p. 957-959.
7. European Pharmacopoeia, Clostridium novyi (type B) vaccine for veterinary use. 2013. 8th edition: p. 952-953.
8. European Pharmacopoeia, Clostridium perfringens vaccine for veterinary use. 2013. 8th edition: p. 955-957.
9. European Pharmacopoeia, Assay of pertussis vaccine (whole cell). 2013. 8th edition: p. 242.
10. European Pharmacopoeia, Measles vaccine (live). 2008. 8th edition: p. 874-875.
11. European Pharmacopoeia, Rubella vaccine (live). 2010. 8th edition: p. 900-901.
12. European Pharmacopoeia, Varicella vaccine (live). 2011. 8th edition: p. 913-914.
13. European Pharmacopoeia, Pneumococcal polysaccharide vaccine. 2016. 8th edition: p. 5673-5674.
14. Landys Chovel Cuervo, M. and N. Reyes Huerta, Validation of an *in vitro* potency test for the Cuban hepatitis B vaccine. Dev Biol (Basel), 2002. 111: p. 305-12.
15. Descamps, J., *et al.*, Release potency tests of hepatitis vaccines. Dev Biol Stand, 1999. 101: p. 289-94.
16. van de Waterbeemd, B., *et al.*, Gene-expression-based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnol Bioeng, 2009. 103(5): p. 900-8.
17. WHO, Good manufacturing practices for biological products. WHO Technical Report Series, 1992. 822.
18. De Mattia, F., *et al.*, The consistency approach for quality control of vaccines - a strategy to improve quality control and implement 3Rs. Biologicals, 2011. 39(1): p. 59-65.
19. Metz, B., *et al.*, Reduction of animal use in human vaccine quality control: opportunities and problems. Vaccine, 2002. 20(19-20): p. 2411-30.
20. Hendriksen, C.F., Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev Vaccines, 2009. 8(3): p. 313-22.
21. Streefland, M., *et al.*, A practical approach for exploration and modeling of the design space of a bacterial vaccine cultivation process. Biotechnol Bioeng, 2009. 104(3): p. 492-504.
22. Streefland, M., *et al.*, PAT for vaccines: the first stage of PAT implementation for development of a well-defined whole-cell vaccine against whooping cough disease. Vaccine, 2007. 25(16): p. 2994-3000.
23. Regnier, M., *et al.*, Structural perturbation of diphtheria toxoid upon adsorption to aluminium hydroxide adjuvant. Vaccine, 2012. 30(48): p. 6783-8.
24. Metz, B., *et al.*, Quality-control issues and approaches in vaccine development. Expert Rev Vaccines, 2009. 8(2): p. 227-38.
25. Zhu, D., *et al.*, Development of a Direct Alhydrogel Formulation Immunoassay (DAFIA). J Immunol Methods, 2009. 344(1): p. 73-8.

26. Peek, L.J., *et al.*, Effects of stabilizers on the destabilization of proteins upon adsorption to aluminum salt adjuvants. J Pharm Sci, 2007. 96(3): p. 547-57.
27. Jones, L.S., *et al.*, Effects of adsorption to aluminum salt adjuvants on the structure and stability of model protein antigens. J Biol Chem, 2005. 280(14): p. 13406-14.
28. Capelle, M.A., P. Brugger, and T. Arvinte, Spectroscopic characterization of antibodies adsorbed to aluminium adjuvants: correlation with antibody vaccine immunogenicity. Vaccine, 2005. 23(14): p. 1686-94.
29. Drake, D.R., *et al.*, *In vitro* Biomimetic Model of the Human Immune System for Predictive Vaccine Assessments. Disruptive science and technology, 2012. 1(1): p. 28-40.
30. Giese, C., *et al.*, Immunological substance testing on human lymphatic micro-organoids *in vitro*. J Biotechnol, 2010. 148(1): p. 38-45.
31. Moxon, E.R. and C.A. Siegrist, The next decade of vaccines: societal and scientific challenges. Lancet, 2011. 378(9788): p. 348-59.
32. Mekori, Y.A. and D.D. Metcalfe, Mast cells in innate immunity. Immunol Rev, 2000. 173: p. 131-40.
33. Hume, D.A., Macrophages as APC and the dendritic cell myth. J Immunol, 2008. 181(9): p. 5829-35.
34. Haanstra, K.G., *et al.*, Induction of experimental autoimmune encephalomyelitis with recombinant human myelin oligodendrocyte glycoprotein in incomplete Freund's adjuvant in three non-human primate species. J Neuroimmune Pharmacol, 2013. 8(5): p. 1251-64.
35. Huang, L.Y., *et al.*, Use of toll-like receptor assays to detect and identify microbial contaminants in biological products. J Clin Microbiol, 2009. 47(11): p. 3427-34.
36. Ziegler-Heitbrock, H.W., *et al.*, Differential expression of cytokines in human blood monocyte subpopulations. Blood, 1992. 79(2): p. 503-11.
37. Neustock, P., *et al.*, Cytokine production of the human monocytic cell line Mono Mac 6 in comparison to mature monocytes in peripheral blood mononuclear cells. Immunobiology, 1993. 188(3): p. 293-302.
38. Cross, A.K., *et al.*, Migration responses of human monocytic cell lines to alpha- and beta-chemokines. Cytokine, 1997. 9(7): p. 521-8.
39. Santegoets, S.J., *et al.*, Human dendritic cell line models for DC differentiation and clinical DC vaccination studies. J Leukoc Biol, 2008. 84(6): p. 1364-73.
40. Santegoets, S.J., *et al.*, A CD34(+) human cell line model of myeloid dendritic cell differentiation: evidence for a CD14(+)CD11b(+) Langerhans cell precursor. J Leukoc Biol, 2006. 80(6): p. 1337-44.
41. Masterson, A.J., *et al.*, MUTZ-3, a human cell line model for the cytokine-induced differentiation of dendritic cells from CD34+ precursors. Blood, 2002. 100(2): p. 701-3.
42. Berges, C., *et al.*, A cell line model for the differentiation of human dendritic cells. Biochem Biophys Res Commun, 2005. 333(3): p. 896-907.
43. Hulette, B.C., *et al.*, Cytokine induction of a human acute myelogenous leukemia cell line (KG-1) to a CD1a+ dendritic cell phenotype. Arch Dermatol Res, 2001. 293(3): p. 147-58.
44. Kiss, Z., *et al.*, Differential effects of various protein kinase C activators on protein phosphorylation in human acute myeloblastic leukemia cell line KG-1 and its phorbol ester-resistant subline KG-1a. Cancer Res, 1987. 47(5): p. 1302-7.
45. Koeffler, H.P., M. Bar-Eli, and M.C. Territo, Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation. Cancer Res, 1981. 41(3): p. 919-26.
46. St Louis, D.C., *et al.*, Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. J Immunol, 1999. 162(6): p. 3237-48.

47. Minta, J.O. and L. Pambrun, *In vitro* induction of cytologic and functional differentiation of the immature human monocytelike cell line U-937 with phorbol myristate acetate. Am J Pathol, 1985. 119(1): p. 111-26.
48. Nilsson, M., K. Nilsson, and K. Forsbeck, Increased endocytosis and formation of multivesicular bodies in phorbol ester-stimulated human monoblastic U-937 cells. Exp Cell Res, 1989. 181(2): p. 551-65.
49. Sundstrom, C. and K. Nilsson, Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer, 1976. 17(5): p. 565-77.
50. Rovera, G., D. Santoli, and C. Damsky, Human promyelocytic leukemia cells in culture differentiate into macrophage-like cells when treated with a phorbol diester. Proc Natl Acad Sci U S A, 1979. 76(6): p. 2779-83.
51. Ball, E.D., *et al.*, Gamma interferon induces monocytoid differentiation in the HL-60 cell line. J Clin Invest, 1984. 73(4): p. 1072-7.
52. Miyaura, C., *et al.*, 1 alpha,25-Dihydroxyvitamin D3 induces differentiation of human myeloid leukemia cells. Biochem Biophys Res Commun, 1981. 102(3): p. 937-43.
53. Sutherland, J.A., *et al.*, Differentiation of K562 leukemia cells along erythroid, macrophage, and megakaryocyte lineages. J Biol Response Mod, 1986. 5(3): p. 250-62.
54. Lozzio, B.B. and C.B. Lozzio, Properties of the K562 cell line derived from a patient with chronic myeloid leukemia. Int J Cancer, 1977. 19(1): p. 136.
55. Tetteroo, P.A., *et al.*, Megakaryoblastic differentiation of proerythroblastic K562 cell-line cells. Leuk Res, 1984. 8(2): p. 197-206.
56. Mallon, D.F., *et al.*, Monocyte-derived dendritic cells as a model for the study of HIV-1 infection: productive infection and phenotypic changes during culture in human serum. Immunol Cell Biol, 1999. 77(5): p. 442-50.
57. Vakkila, J. and M. Hurme, Both dendritic cells and monocytes induce autologous and allogeneic T cells receptive to interleukin 2. Scand J Immunol, 1990. 31(1): p. 75-83.
58. Short, K.R., *et al.*, A Novel Method Linking Antigen Presentation by Human Monocyte-Derived Macrophages to CD8(+) T Cell Polyfunctionality. Front Immunol, 2013. 4: p. 389.
59. Loudovaris, M., *et al.*, Differential effects of autologous serum on CD34(+) or monocyte-derived dendritic cells. J Hematother Stem Cell Res, 2001. 10(4): p. 569-78.
60. Dauer, M., *et al.*, Mature dendritic cells derived from human monocytes within 48 hours: a novel strategy for dendritic cell differentiation from blood precursors. J Immunol, 2003. 170(8): p. 4069-76.
61. Davison, G.M., N. Novitzky, and R. Abdulla, Monocyte derived dendritic cells have reduced expression of co-stimulatory molecules but are able to stimulate autologous T-cells in patients with MDS. Hematol Oncol Stem Cell Ther, 2013. 6(2): p. 49-57.
62. Balan, S., *et al.*, Human XCR1+ dendritic cells derived *in vitro* from CD34+ progenitors closely resemble blood dendritic cells, including their adjuvant responsiveness, contrary to monocyte-derived dendritic cells. J Immunol, 2014. 193(4): p. 1622-35.
63. Wei, S., *et al.*, Plasmacytoid dendritic cells induce CD8+ regulatory T cells in human ovarian carcinoma. Cancer Res, 2005. 65(12): p. 5020-6.
64. Monji, T., *et al.*, Competent dendritic cells derived from CD34+ progenitors express CMRF-44 antigen early in the differentiation pathway. Immunol Cell Biol, 2002. 80(3): p. 216-25.
65. Fedele, G., *et al.*, *Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PLoS One, 2010. 5(1): p. e8734.

66. Brummelman, J., *et al.*, *Bordetella pertussis* naturally occurring isolates with altered lipooligosaccharide structure fail to fully mature human dendritic cells. Infect Immun, 2015. 83(1): p. 227-38.
67. Matsuda, M., *et al.*, Establishment of a newly improved detection system for NF-kappaB activity. Immunol Lett, 2007. 109(2): p. 175-81.
68. Butler, M.O., *et al.*, Long-lived antitumor CD8+ lymphocytes for adoptive therapy generated using an artificial antigen-presenting cell. Clin Cancer Res, 2007. 13(6): p. 1857-67.
69. van Helden, S.F., F.N. van Leeuwen, and C.G. Figdor, Human and murine model cell lines for dendritic cell biology evaluated. Immunol Lett, 2008. 117(2): p. 191-7.
70. Hoonakker, M.E., *et al.*, *In vitro* innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle. Biologicals, 2015. 43(2): p. 100-9.
71. Trombetta, E.S. and I. Mellman, Cell biology of antigen processing *in vitro* and *in vivo*. Annu Rev Immunol, 2005. 23: p. 975-1028.
72. Sallusto, F. and A. Lanzavecchia, Monocytes join the dendritic cell family. Cell, 2010. 143(3): p. 339-40.
73. Vandebriel, R. and M.M. Hoefnagel, Dendritic cell-based *in vitro* assays for vaccine immunogenicity. Hum Vaccin Immunother, 2012. 8(9): p. 1323-5.
74. Adema, G.J., *et al.*, Migration of dendritic cell based cancer vaccines: *in vivo* veritas? Curr Opin Immunol, 2005. 17(2): p. 170-4.
75. Ginhoux, F. and S. Jung, Monocytes and macrophages: developmental pathways and tissue homeostasis. Nat Rev Immunol, 2014. 14(6): p. 392-404.
76. Auffray, C., *et al.*, Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science, 2007. 317(5838): p. 666-70.
77. Farina, C., *et al.*, Distinct responses of monocytes to Toll-like receptor ligands and inflammatory cytokines. Int Immunol, 2004. 16(6): p. 799-809.
78. Obermajer, N., *et al.*, An assay for functional dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) inhibitors of human dendritic cell adhesion. Anal Biochem, 2010. 406(2): p. 222-9.
79. de la Rosa, G., *et al.*, Migration of human blood dendritic cells across endothelial cell monolayers: adhesion molecules and chemokines involved in subset-specific transmigration. J Leukoc Biol, 2003. 73(5): p. 639-49.
80. Frasca, L., *et al.*, CD38 orchestrates migration, survival, and Th1 immune response of human mature dendritic cells. Blood, 2006. 107(6): p. 2392-9.
81. Randolph, G.J., *et al.*, The CD16(+) (FcgammaRIII(+)) subset of human monocytes preferentially becomes migratory dendritic cells in a model tissue setting. J Exp Med, 2002. 196(4): p. 517-27.
82. Munir, H., *et al.*, Analyzing the effects of stromal cells on the recruitment of leukocytes from flow. J Vis Exp, 2015(95): p. e52480.
83. Behrensdorf-Nicol, H.A., *et al.*, Binding and cleavage (BINACLE) assay for the functional *in vitro* detection of tetanus toxin: applicability as alternative method for the safety testing of tetanus toxoids during vaccine production. Vaccine, 2013. 31(52): p. 6247-53.
84. WHO, Recommendations to assure the quality, safety and efficacy of poliomyelitis vaccines (oral, live, attenuated). Replacement of Annex 1 of WHO Technical Report Series, No. 904, 2012.
85. Rumiantsev, A.A., *et al.*, [Use of molecular biological method (MAPREC-test) for evaluating residual neurovirulence and genetic stability of oral poliovirus vaccine type 3]. Vopr Virusol, 2001. 46(6): p. 11-5.
86. Bidzhieva, B., M. Laassri, and K. Chumakov, Allele-specific PCR for quantitative analysis of mutants in live viral vaccines. J Virol Methods, 2014. 201: p. 86-92.

87. Neverov, A. and K. Chumakov, Massively parallel sequencing for monitoring genetic consistency and quality control of live viral vaccines. Proc Natl Acad Sci U S A, 2010. 107(46): p. 20063-8.
88. Behr, M.A., *et al.*, Comparative genomics of BCG vaccines by whole-genome DNA microarray. Science, 1999. 284(5419): p. 1520-3.
89. Kouzaki, Y., *et al.*, A Simple and Rapid Identification Method for Mycobacterium bovis BCG with Loop-Mediated Isothermal Amplification. PLoS One, 2015. 10(7): p. e0133759.
90. Sheets, R.L., Opinion on adventitious agents testing for vaccines: why do we worry so much about adventitious agents in vaccines? Vaccine, 2013. 31(26): p. 2791-5.
91. Petricciani, J., *et al.*, Adventitious agents in viral vaccines: lessons learned from 4 case studies. Biologicals, 2014. 42(5): p. 223-36.
92. Bruckner, L., Viral safety and extraneous agents testing for veterinary vaccines: rationale for requirements, the European approach. Biologicals, 2010. 38(3): p. 338-9.
93. Isbrucker, R.A., A. Bliu, and F. Prior, Modified binding assay for improved sensitivity and specificity in the detection of residual pertussis toxin in vaccine preparations. Vaccine, 2010. 28(15): p. 2687-92.
94. Gomez, S.R., *et al.*, Development of a carbohydrate binding assay for the B-oligomer of pertussis toxin and toxoid. Anal Biochem, 2006. 356(2): p. 244-53.
95. Cyr, T., *et al.*, A quantitative analysis for the ADP-ribosylation activity of pertussis toxin: an enzymatic-HPLC coupled assay applicable to formulated whole cell and acellular pertussis vaccine products. Biologicals, 2001. 29(2): p. 81-95.
96. Yuen, C.T., *et al.*, Detection of residual pertussis toxin in vaccines using a modified ribosylation assay. Vaccine, 2002. 21(1-2): p. 44-52.
97. Yuen, C.T., *et al.*, An *in vitro* assay system as a potential replacement for the histamine sensitisation test for acellular pertussis based combination vaccines. Vaccine, 2010. 28(21): p. 3714-21.
98. Ochiai, M., *et al.*, Highly sensitive histamine-sensitization test for residual activity of pertussis toxin in acellular pertussis vaccine. Biologicals, 2007. 35(4): p. 259-64.
99. Fujiwara, H. and S. Iwasa, The quantitative assay of the clustering activity of the lymphocytosis-promoting factor (pertussis toxin) of *Bordetella pertussis* on Chinese hamster ovary (CHO) cells. J Biol Stand, 1989. 17(1): p. 53-64.
100. Gillenius, P., *et al.*, The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J Biol Stand, 1985. 13(1): p. 61-6.
101. Castro, M.G., U. McNamara, and N.H. Carbonetti, Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. Cell Microbiol, 2001. 3(1): p. 45-54.
102. Kataoka, M., *et al.*, Chinese hamster ovary (CHO) cell clustering does not correlate with *in vivo* histamine-sensitization when measuring residual activity of aldehyde-treated pertussis toxin (PT). Biologicals, 2002. 30(4): p. 297-302.
103. Isbrucker, R., *et al.*, Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. Biologicals, 2014. 42(2): p. 114-22.
104. de Gouw, D., *et al.*, Pertussis: a matter of immune modulation. FEMS Microbiol Rev, 2011. 35(3): p. 441-74.
105. Uhl, M.A. and J.F. Miller, Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. J Biol Chem, 1996. 271(52): p. 33176-80.
106. Merkel, T.J., *et al.*, Analysis of bvgR expression in *Bordetella pertussis*. J Bacteriol, 2003. 185(23): p. 6902-12.

107. Merkel, T.J. and S. Stibitz, Identification of a locus required for the regulation of bvg-repressed genes in *Bordetella pertussis*. J Bacteriol, 1995. 177(10): p. 2727-36.
108. Melton, A.R. and A.A. Weiss, Characterization of environmental regulators of *Bordetella pertussis*. Infect Immun, 1993. 61(3): p. 807-15.
109. Martinez de Tejada, G., *et al.*, Neither the Bvg- phase nor the vrg6 locus of *Bordetella pertussis* is required for respiratory infection in mice. Infect Immun, 1998. 66(6): p. 2762-8.
110. Bellalou, J. and E.H. Relyveld, Studies on culture conditions of *Bordetella pertussis* and relationship to immunogenicity of vaccines. Ann Microbiol (Paris), 1984. 135B(1): p. 101-10.
111. Hamstra, H.J., *et al.*, The purification and protective capacity of *Bordetella pertussis* outer membrane proteins. Vaccine, 1995. 13(8): p. 747-52.
112. Girard, D.Z., Which strategy for pertussis vaccination today? Paediatr Drugs, 2002. 4(5): p. 299-313.
113. Tan, T., *et al.*, Pertussis Across the Globe: Recent Epidemiologic Trends From 2000 to 2013. Pediatr Infect Dis J, 2015. 34(9): p. e222-32.
114. Libster, R. and K.M. Edwards, Re-emergence of pertussis: what are the solutions? Expert Rev Vaccines, 2012. 11(11): p. 1331-46.
115. Mills, K.H., *et al.*, Do we need a new vaccine to control the re-emergence of pertussis? Trends Microbiol, 2014. 22(2): p. 49-52.
116. Klein, N.P., *et al.*, Waning protection after fifth dose of acellular pertussis vaccine in children. N Engl J Med, 2012. 367(11): p. 1012-9.
117. Wendelboe, A.M., *et al.*, Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J, 2005. 24(5 Suppl): p. S58-61.
118. WHO, Recommendations for whole-cell pertussis vaccine. WHO Technical Report Series 2007. 941, Geneva, Switzerland: p. 301-33.
119. van der Ark, A., *et al.*, Development of pertussis serological potency test. Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. Biologicals, 1994. 22(3): p. 233-42.
120. van der Ark, A., *et al.*, The Pertussis Serological Potency Test. Collaborative study to evaluatereplacement of the Mouse Protection Test. Biologicals, 2000. 28(2): p. 105-18.
121. von Hunolstein, C., *et al.*, Evaluation of two serological methods for potency testing of whole cell pertussis vaccines. Pharmeuropa Bio, 2008. 2008(1): p. 7-18.
122. Mills, K.H., *et al.*, A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun, 1998. 66(2): p. 594-602.
123. Kind, L.S., The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bacteriol Rev, 1958. 22(3): p. 173-82.
124. van Straaten-van de Kappelle, I., *et al.*, Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biologicals, 1997. 25(1): p. 41-57.
125. van Meijeren, C.E., *et al.*, Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system? Eur J Pharmacol, 2004. 493(1-3): p. 139-50.
126. Petitcolin, M.A., *et al.*, Role of G(i)-proteins in norepinephrine-mediated vasoconstriction in rat tail artery smooth muscle. Biochem Pharmacol, 2001. 61(9): p. 1169-75.
127. Gurdal, H., *et al.*, Role of G alpha q or G alpha o proteins in alpha 1-adrenoceptor subtype-mediated responses in Fischer 344 rat aorta. Mol Pharmacol, 1997. 52(6): p. 1064-70.

2

Chapter 2

Proteome analysis is a powerful tool to evaluate antigen expression during upstream processing of whole-cell pertussis vaccines

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Submitted to Journal of Proteome Research

Abstract

Physicochemical and immunochemical assays were applied to substantiate the relation between upstream processing and the quality of whole-cell pertussis vaccines. *Bordetella pertussis* bacteria were cultured on a chemically defined medium using a continuous cultivation process in stirred tank reactors to obtain uniform protein expression. Continue culture favours the consistent production of proteins known as virulence factors. Magnesium sulphate was added during the steady state of the culture in order to diminish the expression of virulence proteins. Changes in gene expression and antigen composition were measured by microarrays, mass spectrometry and ELISA. Transcriptome and proteome data revealed high similarity between the biological triplicates demonstrating consistent cultivation of *B. pertussis*. The addition of magnesium sulphate resulted in an instant downregulation of the virulence genes in *B. pertussis*, but a gradual decrease of virulence proteins. The quantity of virulence proteins concurred highly with the potency of the corresponding whole-cell pertussis vaccines which were determined by the Kendrick test. In conclusion, proteome analysis provided detailed information on the composition and proportion of virulence proteins present in the whole-cell preparations of *B. pertussis*. Moreover, proteome analysis is a valuable method to monitor the production process of whole-cell biomass and predict the product quality of whole-cell pertussis vaccines.

Introduction

Pertussis is a widely spread and highly infectious disease, which can have a severe impact on infants and children.[1] The major pathogen causing pertussis is the Gram-negative bacterium *Bordetella pertussis*. Vaccination is the best strategy to protect against *B. pertussis*. The introduction of whole-cell pertussis (wP) vaccines in the 1950s rapidly reduced the morbidity and mortality caused by pertussis infections. wP vaccines were used worldwide until the 1990s when the acellular pertussis vaccines (aP) were released on the market. Many high income countries replaced wP vaccines for aP vaccines due to reduced societal acceptance of side effects initiated by wP vaccines. Nevertheless, wP vaccines are still given to about 75 percent of the global infant population. They are used in many countries in Latin America, Africa and Asia.[2] The replacement of wP vaccines has a major drawback. Despite the high vaccination coverage, a resurgence of pertussis has been observed in countries using aP vaccines.[3-6] Recent studies indicated that the duration of protection by aP vaccines is rather limited.[7,8] This stimulated the development of new pertussis vaccines, such as live-attenuated vaccines[9], improved whole-cell vaccines[9-11] and outer membrane vesicle vaccines.[12] However, these vaccine candidates are still at a pre-clinical stage. Meanwhile, countries considering a switch to aP vaccines are advised to postpone or to assure multiple immunizations with aP vaccine in their national immunization program.[13] Therefore, we expect that wP vaccines will remain an important cornerstone in the protection against pertussis.

The quality of wP vaccines is significantly determined by the protein antigens expressed by *B. pertussis*. Also, the presence of lipooligosaccharide (LPS) endotoxin, which is a major antigen of wP vaccines, has an important role in the generation of a protective immune response.[14,15] wP vaccines comprises hundreds of different proteins. The protein composition may vary significantly between production runs due to variations in cultivation and medium conditions. For example, a temperature decrease during fermentation or a nutrient depletion of the culture medium result in reduced expression of virulence proteins in *B. pertussis*.[16-17] Differences in the antigen composition affects the final potency of wP vaccines[18] as was measured in the mandatory intracerebral mouse protection test (ic-MPT).[19] Another approach to ensure the quality of vaccines is Process Analytical Technology (PAT). PAT provides a strategy leading to maximum control on product and process based upon scientific understanding. This approach is applicable for well-characterized products, such as subunit vaccines (e.g. aP vaccine), but was not directly suitable for complex and less-defined products (e.g. wP vaccine). Nevertheless, PAT is recently applied in pertussis fermentation processes.[20,21] However, additional analytical assays are needed to examine if a disturbance during fermentation affects product quality of wP vaccines. Microarrays and liquid chromatography–mass spectrometry (LC-MS) are valuable tools to investigate gene and protein expression during bacterial cultivation. These techniques are very sensitive and highly reproducible.

In this study, the application of using transcriptomics, proteomics and antigenicity is investigated to ensure the quality of wP vaccines. To that end, *B. pertussis* is cultured in triplicate in continuous stirred tank reactors (CSTR) on a chemically defined medium. During the continuous culture, fresh culture medium is continuously pumped into the bioreactor and consumed medium with *B. pertussis* is pumped out the reactor at the same rate. Many conditions were kept constant during continuous cultivation, e.g. culture volume, medium composition, pH value, temperature, dissolved oxygen, cell density.[22,23] Continuous culture is utilised to obtain uniform protein expression. Subsequently, the protein expression is disturbed by adding magnesium sulphate to the culture medium. This intervention caused a downregulation of virulence proteins in *B. pertussis*.[16] The bacteria shifts reversibly from virulent state to a-virulent state.[24] The regulator is a two-component system also called BvgAS system.[25-27] Nine pertussis samples were taken after addition of magnesium sulphate during the next 24 hours of bacterial cultivation. Gene and protein expressions are examined by microarrays and LC-MS, respectively. The antigenicity was determined by ELISA using monoclonal antibodies against particular pertussis antigens. Heat and formaldehyde-inactivated wP vaccines are prepared from different bacterial samples and their potency was assessed by measuring protective immune responses in the ic-MPT.[19] The hypothesis is that vaccine quality (i.e. potency) reduces after downregulation of virulence proteins. The study revealed that proteome analysis was the most powerful tool to demonstrate the relationship between culture conditions and the quality of wP vaccine.

Experimental Section

Bacterial cultivation

Bordetella pertussis strain BP509 (Intravacc, The Netherlands) was cultured three times in a chemically defined THIJS medium.[28,29] Briefly, a 3L-bioreactor with a working volume of 2L medium was inoculated with strain BP509. Dissolved oxygen (DO), pH, and temperature were controlled at 30%, 7.2 and 35°C, respectively. The batch phase was continued until an optical density (OD) of 1.0 ± 0.2 was reached. The chemostat culture was started by adding fresh THIJS medium and removing whole-cell biomass at flow rates of 0.1 lh^{-1} to control the growth rate at $\mu = 0.05\ h^{-1}$ (generation time of 13.9 hours(h)). Steady state was assumed after 3-5 reactor volumes. As a deliberate intervention (t=0 h), 2M $MgSO_4$ was added to the reactor to change immediately the final concentration to 50 mM $MgSO_4$. At the same time, the medium feed was replaced by THIJS medium containing 50 mM $MgSO_4$. Subsequently, the culture was monitored for at least 24h after the addition of $MgSO_4$.

Sample preparations

Biomass samples were taken from the continuous culture at distinct time points before and after the addition of $MgSO_4$ (at t=0h) for transcriptome analysis, proteome analysis, whole-cell ELISA and preparation of experimental wP vaccines.

Transcriptome analysis – Biomass samples of 3.6 ml were taken at t=-24, 0, 1, 2, 6, 10, and 24h. Transcriptome profile of the samples was fixed by prompt addition of 7.2 ml cold RNAse blocker (12.5 mM sodium citrate, 10 mM EDTA, 2.6 M ammonium sulphate, 0.1% (v/v) DEPC in ultrapure water and adjusted by 1 M sulphuric acid to a pH of 5.2) and incubated for 5 min on ice.[30-32] Then mixtures were centrifuged for 10 minutes at 4000 x g and +4°C. Supernatant was carefully removed by decantation. Pellets were stored at - 80°C.

Proteome analysis – Biomass samples of 3.6 ml were taken at t=0, 1, 2, 4, 6, 8, 10, 12 and 24 h. They were centrifuged for 10 minutes at 4000 x g and +4°C. Supernatant was carefully removed by decantation. Pellets were stored at - 80°C.

ELISA – Biomass samples of 10 ml were taken at t=0, 1, 2, 4, 8, 12 and 24h. They were centrifuged for 10 minutes at 4000 x g and +4°C. Pellets were resuspended in 150 mM saline to a density of 4 international opacity units (IOU)/ml. The bacterial suspensions were inactivated by heat (10 min at 56°C) and stored at 4°C prior to the ELISA.

Vaccine preparation – Biomass samples of 100 ml were taken to prepare heat and formaldehyde-inactivated pertussis vaccines (wP vaccines) for potency testing (t=0, 2, 6, 12 and 24 h). The

experimental vaccines were prepared according Thalen *et al.*[18] Briefly, whole cell biomass was centrifuged for 10 min at 3000 x g at 4°C. The pellet was suspended in 150 mM saline to a concentration of 200 IOU/mL, corresponding to an OD of 10. Formaldehyde was added to a final concentration of 16 mM, pH was adjusted to 7.7 by adding NaOH and the vaccine was heat-inactivated for 10 min at 56°C.

RNA extraction

The pellet was resuspended in 100 µl of 0.5 mg/ml lysozyme (Sigma-Aldrich) and dissolved in 1 ml Tris-EDTA buffer and incubated for 3 min at room temperature. Total RNA was extracted using SV total RNA isolation system (Promega, The Netherlands) according to the protocol of the manufacturer. Spectrometric analysis was used to determine final nucleic acid concentration and purity. RNA integrity was confirmed with the Bioanalyzer RNA6000 Nano assay (Agilent Technologies, The Netherlands), according to the protocol of the manufacturer.

Gene expression analysis

B. pertussis transcriptome analysis was performed as described previously.[17] Data from all genes were used to confirm reproducibility (principle component analysis, PCA) and to calculate gene expression statistics. P-values were calculated using one-way ANOVA statistical analysis and adjusted for multiple testing by calculating the False Discovery Rate (FDR). Genes with a false discovery rate of <10.0% and a maximal Fold Ratio of >2.0 were included in the final analysis (Table S-1). To assess the impact on virulence gene expression, a subset of 56 virulence genes was used (previously described as the virulence core regulon[17,21,33]).

Protein extraction

The pellet was resuspended in 100 µl of 0.5 mg/ml lysozyme (Sigma-Aldrich) dissolved in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated for 3 min at room temperature. One ml of Trizol (Invitrogen, The Netherlands) was added to suspension and incubated for 5 min at room temperature. Then, 200 µl chloroform (Merck, The Netherlands) was added, samples were vortexed intensively for 30 sec and centrifuged for 10 min at 13.000 x g. The organic phase containing the proteins was transferred to new tubes. The extractions of the aqueous phase were repeated by adding another 200 µl of chloroform, mixing and centrifugation. After the transfer of the organic phase to the 2-ml tubes, 750 µl of isopropanol was added to the samples. Sample were mixed by inversion and incubated for 20 min at 4°C. Sedimentation of the protein was performed by centrifuging for 10 min at 12.000 x g at 2-8°C. After removal of supernatant, protein pellets were washed three times with 0.5 ml ethanol. Samples were dried in 5 min using a vacuum centrifuge (Eppendorf) and stored at - 80°C.

Protein digestion

Proteins pellets were dissolved in a denaturation buffer containing 4 M guanidine-HCl and 50 mM triethyl ammonium bicarbonate, pH 8.5 (TEAB). The samples were diluted to final concentrations of 0.5 mg/ml protein, 1 M guanidine-HCl and 50 mM TEAB. Each protein sample (100 µg) was digested with 0.5 µg endoproteinase Lys-C (Roche) for 4 h at 37°C. Subsequently, 1 µg trypsin (Promega) was added and the digests were incubated overnight at 37°C. The protein digestion samples were stored at –20°C before dimethyl labelling.

Peptide labelling

Relative quantification of proteins in each protein sample was performed as described previously[34] using dimethyl labelling of peptides and a common reference as internal standard. Individual protein digests were incubated with native formaldehyde (CH_2O; Sigma-Aldrich) and $NaCNBH_3$ (Sigma-Aldrich) in final concentrations of 50 mM. A common reference was prepared by mixing thoroughly 50 µg of each unlabelled digests. The common reference was incubated with deuterium-labelled formaldehyde (CD_2O; Sigma-Aldrich) and $NaCNBH_3$ in final concentrations of 50 mM reagents. Each of the individual dimethylated $(C_1H_3)_2$ peptide samples were mixed with the deuterium dimethylated $(C_1D_2H_1)_2$ common reference in equal amounts and fortified with 5% formic acid and 5% DMSO in water. Analytes were purified by solid phase extraction (SPE) using an in-house made trapping column (Reprosil-Pur C18-QA d=5 µm, 20 mm length x 100 µm inner diameter). Peptides recovered from the SPE using 90% acetonitrile and 0.5% acetic acid and dried using a vacuum centrifuge (Eppendorf). Differentially labelled peptide mixtures were dissolved in 100 µl of 5% formic acid and 5% DMSO in water, containing 1 fmol/µl of angiotensin-III (Sigma-Aldrich) and oxytocin (Sigma-Aldrich) each. Samples were stored at -20°C prior to LC-MS analysis.

Fractionation of common reference

The dimethylated common reference was fractionated utilising Strong Cation eXchange (SCX) chromatography, as described previously.[35] The mixture was loaded onto a trapping column (Hypercarb™, 5-mm length × 200-µm internal diameter) at a flow rate of 5 µl/min with 0.1 M acetic acid. After sample loading, the trapping column was switched in series with an SCX column (PolySULFOETHYL Aspartamide, 12-cm length × 200-µm internal diameter) and the peptides were eluted to the SCX column by a plug injection of 50% acetonitrile in 0.1 M acetic acid at a column flow rate of 2 µl/min. Finally, a linear salt gradient was run to 500 mM KCl in 35% acetonitrile at a column flow rate of 2 µl/min. Fractions were collected at 1 minute intervals. The six most informative fractions were used for LC-MS/MS analysis.

Peptide identification by LC-MS/MS analysis

The common reference aliquots were analyzed by nano-scale reversed-phase liquid chromatography electrospray mass spectrometry using an LTQ-Orbitrap XL (Thermo Scientific, USA) and Agilent 1290 (Agilent Technologies, USA), essentially as previously described by Meiring et al.[35] Analytes were loaded on a trapping column (Reprosil-Pur C_{18}-AQ d=5 µm; 20-mm length x 100-µm inner diameter) with solvent A (0.1% (v/v) formic acid in water) in 10 min at 7 µl/min. The analytes were separated by reversed-phase chromatography on an analytical column (Reprosil-Pur C18-AQ d=3 µm; 36-cm length x 50-µm inner diameter) at a flow rate of 125 nl/min using a 2hr gradient (7.5% to 47.5% solvent B (0.1% (v/v) formic acid in acetonitrile)). A survey scan was performed in the Orbitrap with a resolution of 60,000 (FWHM) (m/z 350 – 1500) followed by collision-induced dissociation (CID) of the four most abundant precursor ions in the ion trap. The threshold value for these precursor ions was set at 104 counts. The normalized collision energy was set at 35% and the isolation width of 2.0 Da. Proteome Discoverer 1.4 software was used for processing the raw MS data. MS/MS scans were searched against the protein database of *B. pertussis* Tohama (Uniprot taxonomic identifier 257313A) with trypsin enzyme specificity and mass tolerance of precursor ions and fragment ions set to 5 ppm and 0.5Da, respectively. Asparagine deamidation and methionine oxidation were set as variable modifications and lysine dimethylation and N-terminal dimethylation as a fixed modification. Peptides were filtered to <1% FDR using the Percolator algorithm (Thermo Scientific). The peptide output list was used for relative quantification of differentially labelled peptide mixtures.

Relative peptide quantification by LC-MS

The relative abundances of differentially labelled peptides were determined by LC-MS analysis, essentially as described by Van de Waterbeemd *et al.*[34] Peptides appeared as mass spectral doublets representing the light-dimethylated peptides from the experimental sample and the heavy-dimethylated peptides from the common reference. Raw LC-MS spectra were deconvoluted using Xcalibur Xtract software (Thermo Scientific) and imported in MS-Xelerator software (MsMetrix, Then Netherlands). The MSX-quant module was used to calculate the peak area ratios between the light and heavy labelled peptides (Table S-1), as described by Van de Waterbeemd *et al.*[34] Peak areas ratios were determined for peptides present in the peptide output list based on their accurate masses (± 0.01 Da) and retention times (± 5 min).

Statistical analysis

For relative quantification of the proteins, the peak area ratios of the identified peptides were 2-log transformed. If the doublet ratio of a particular peptide was only two times determined in three biological replicates, the missing ratio was imputed as the average value of the other replicates. The peptide was removed from the peptide list, if only one doublet

ratio was determined in the three biological replicates. The log-transformed ratios of the peptides belonging to the same protein were averaged. These protein expression data were analysed across the different time-points using one-way ANOVA to identify proteins that were differentially expressed ($p<0.001$ and Fold ratio>2). The altered protein expression was visualised in a heat map using Genemaths XT (Applied Maths, Belgium). Enrichment analysis of proteins with particular biological function was carried out by using DAVID.[36,37]

Measurement of virulence proteins by ELISA

Levels of four virulence proteins present in heat-inactivated wP vaccines preparations were determined by a whole-cell ELISA using monoclonal antibodies against the specific virulence proteins: serotype 2 fimbrial subunit (Fim2), filamentous hemagglutinin (FHA), pertactin (Prn) and autotransporter (Vag8) . Therefore, 96-wells plates were coated with serial dilutions of the heat-inactivated wP vaccines preparations. The initial bacterial density of these preparations was 4 IOU/ml which was twofold serial diluted in the plate. Then, plates were incubated with IgG antibodies (home-made) targeted against a specific virulence protein: anti-Fim2 (136A6), anti-FHA (31E2), anti-Prn (PEM4) and anti-Vag8 (32C11).[38] Goat anti-mouse IgG-biotin (Amersham, U.K.) was used as a secondary antibody, followed by incubation with avidine-conjugated horseradish peroxidase. The antibody binding was visualised with a TMB-substrate (Sigma-Aldrich). The absorbance was recorded at 450 nm with a plate reader (ELx808, BioTek Instruments). Protein amounts were calculated using 4-parameter fitting analysis and expressed as a percentage of the concentration at time point t=0 h. P-values were calculated using repeated measures ANOVA for each protein followed by Tukey's multiple comparison test to analyse the individual time points.

Ethics Statement

The independent ethical committee for animal experimentations 'Dierexperimentele Commissie (DEC)' of the Institute for Translational Vaccinology (Intravacc), reviewed the animal experiments in this study according to the guidelines provided by the Dutch Animal Protection Act. The committee approved the animal experiment with identification number 'DPA201300054'.

Intracerebral challenge test

The lyophilised vaccine Kh96/1 was used as an in-house reference to determine the potency of experimental pertussis vaccines (wP). Kh96/1 contains *B. pertussis* strains 134 and 509 and was calibrated against the international standard IS 94/532. The potency of Kh96/1 was 59 IU/200 IOU. Mice were immunised to determine the protective immunogenicity of the wP vaccines as described by Kendrick *et al.*[19] Therefore, vaccines A (t=0 h), C (t=6 h), E (t=24 h) were diluted in PBS (10 mM sodium phosphate, pH7.2 and 150 mM NaCl in water) at

concentrations of 0.04, 0.2, 1 and 5 IOU/mL, and the reference vaccine (Kh96/1) was diluted in PBS at a concentration of 0.04, 0.2, 1 and 5 IOU/ml. Mice (rivmN:NIH) were divided in 16 groups of 16 animals (8 male: 8 female), 1 group of 12 mice (6 male: 6 female) and 1 group of 10 mice (5 male: 5 female). Animals were housed in cages (macrolon III including filter top). Each diluted vaccine (0.5 ml) was used to intraperitoneally immunize 16 mice (1 group). After 14 days, the mice received intracerebrally 10 µl of a challenge suspension (10.000 bacteria/ 10 µl of *B. pertussis* 18323 in a solution of 1% casamino acid in saline (Bilthoven Biologicals). As a positive control, one group of 12 non-immunised mice received also a challenge. As a negative control, a casamino acid solution was administered intracerebrally to one group of 10 non-immunised mice. The mice were monitored twice a day for clinical signs (convulsions) until 14 days after the challenge. Mice with disease symptoms were sacrificed. The number of living animals is counted every day. The potency of vaccines A, C and E were calculated based on the survival scores and the potency of the reference vaccine by using Combistat 5.0 (statistical analysis software).

Results

Continuous cultivation of *B. pertussis*

Three continuous cultivations of *B. pertussis* were performed with 2L of chemical-defined medium in 3L-bioreactors to obtain samples for transcriptome and proteome analyses (Figure 1). The fermentations started with a batch phase (20.1 ± 0.9 hours) and were stopped at an approximate optical density (OD) of 1.047 (±0.030). The growth rate during the continuous phase was controlled at 0.05 h^{-1} and steady state was reached when OD stabilized at 1.043 (±0.033). The limited variations in OD and air demand indicated that the cultivations were executed in a highly reproducible manner (Figure S1). Then, the steady state was perturbed by the addition of 50 mM $MgSO_4$ (t=0 h). The cultures showed an increased need for oxygen to maintain a dissolved oxygen level (DO) of 30%. Also, a minor increase in OD was observed after biomass sampling. Subsequently, the effect of the perturbation on the quality of wP vaccines was studied in detail.

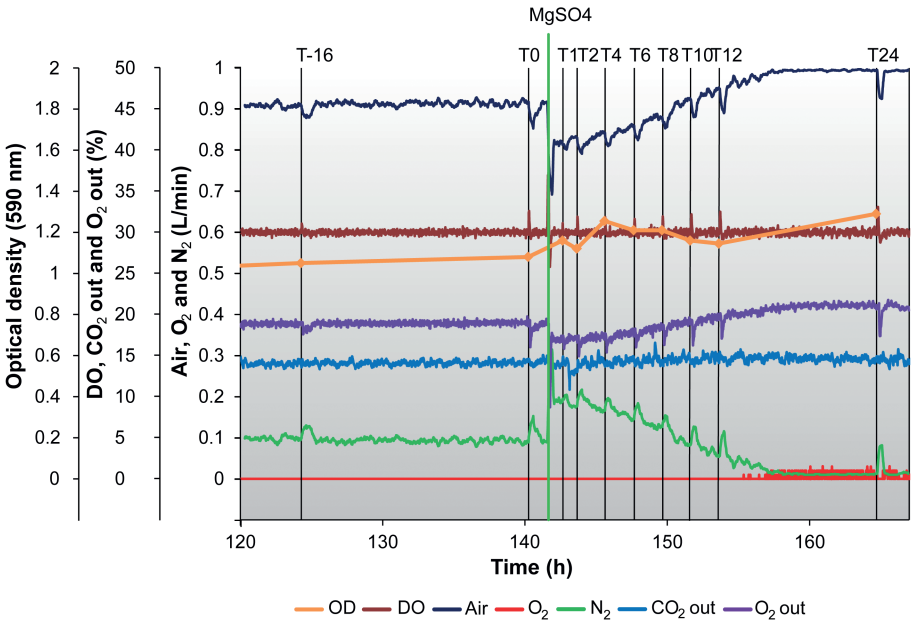


Figure 1 Continuous cultivation of *B. pertussis*. The bacteria were grown on a chemically defined medium for about 170h. $MgSO_4$ was added at t=0 h to reduce the gene expression of virulence proteins[19]. Biomass samples were collected at ten different time points (as depicted from t=-24 h until t=24 h) for vaccine preparation and biochemical analysis, e.g. transcriptome and proteome analyses. During cultivation, the optical density (OD), dissolved oxygen (DO) and gas inflows (air, O_2, N_2) and gas outflows (CO_2 out and O_2 out) were monitored.

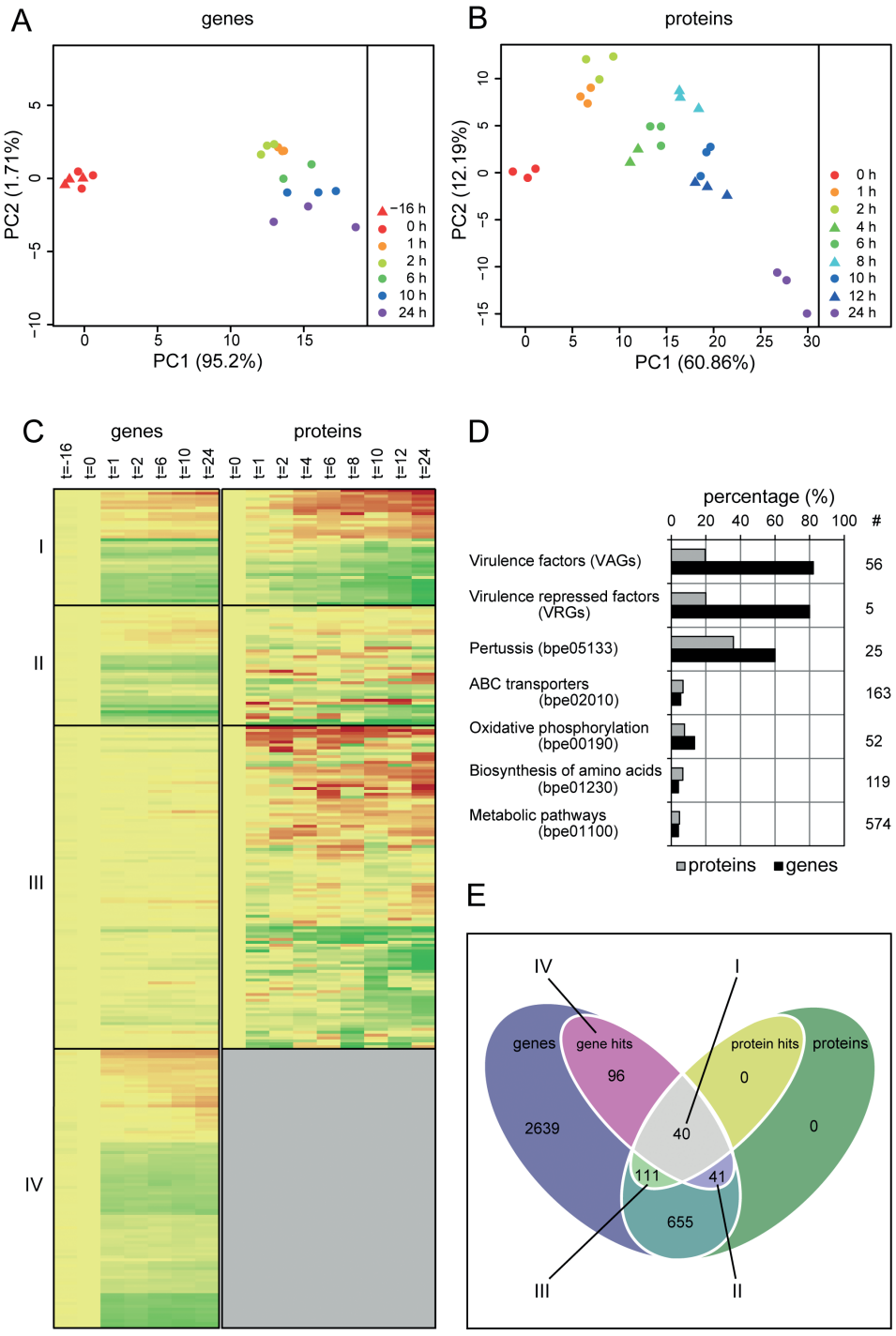

A
genes
PC2 (1.71%)
PC1 (95.2%)
−16 h
0 h
1 h
2 h
6 h
10 h
24 h
B
proteins
PC2 (12.19%)
PC1 (60.86%)
0 h
1 h
2 h
4 h
6 h
8 h
10 h
12 h
24 h
C
genes
proteins
t=-16
t=0
t=1
t=2
t=6
t=10
t=24
t=0
t=1
t=2
t=4
t=6
t=8
t=10
t=12
t=24
I
II
III
IV
D
percentage (%)
0
20
40
60
80
100
#
Virulence factors (VAGs)
56
Virulence repressed factors (VRGs)
5
Pertussis (bpe05133)
25
ABC transporters (bpe02010)
163
Oxidative phosphorylation (bpe00190)
52
Biosynthesis of amino acids (bpe01230)
119
Metabolic pathways (bpe01100)
574
proteins
genes
E
IV
I
genes
gene hits
protein hits
proteins
96
0
2639
40
0
111
41
655
III
II

Figure 2 Differential gene and protein expression of *B. pertussis* (left). A steady state chemostat of *B. pertussis* was deliberately disturbed by adding $MgSO_4$ to the culture medium, which reduced the expression of virulence proteins. (A) Principal Component analysis of overall gene expression variance and (B) Principal Component analysis of overall protein expression variance. (C) Significantly regulated genes and proteins were clustered in 4 groups: (I) both genes and proteins hits, (II) only gene hits, (III) only protein hits, and (IV) gene hits but the corresponding protein was not detected by LC-MS. (D) Functional analysis of differentially expressed genes (black) and proteins (grey). Seven significantly regulated pathways were identified of which five pathways were described by the KEGG pathway database (entries are given between brackets). Furthermore, the number (#) of genes or proteins involved in a particular pathway is given in the figure. (E) A representative overview of gene and protein expression determined by microarray and LC-MS analysis, respectively (differentially regulated groups are marked by Roman numerals).

Altered gene expression by adding magnesium sulphate

RNA isolates from different time points (t=-24, 0, 1, 2, 6, 10 and 24 h) were analysed with whole-genome microarrays of *B. pertussis* to assess downregulation of virulence genes. Microarrays revealed that approximately 84% of the 3582 genes were expressed during the cultivation of *B. pertussis*. Uniform gene expression was observed in the 24 hours before the intervention with $MgSO_4$. However, 177 genes were differentially regulated upon the addition of $MgSO_4$ to the culture medium, of which 66 genes were upregulated and 111 genes downregulated (Figure 2). It was reported in the literature that 56 genes encode for virulence proteins.[21,33] Out of these 56 virulence genes, 47 were significantly downregulated in this study. The remaining nine virulence genes did not show significant downregulation, although they had the expected downwards trend (Figure S2). Principal Component analysis was used to determine the largest sources of variance in the data (Figure 2A). Principal Component 1 (PC1) accounts for 95.2% of total gene expression variance and coincides with the induction of the a-virulent state by $MgSO_4$. PC2 accounts for only 1.7% and is most likely caused by a time effect after disturbing the steady state with $MgSO_4$. The remaining principal components had minor contributions to the total variance (<1%). This indicates that the three continuous cultivations were highly reproducible and that downregulation of virulence genes is the predominant source of variation in the dataset.

Differential protein expression in *B. pertussis*

The addition of $MgSO_4$ to the culture medium affected the gene expression of *B. pertussis*. Next, differences in protein expression were determined by LC-MS after the deliberate addition of $MgSO_4$. We expect the deliberate intervention will lead to different kinetics of gene and protein expression, due to a slower turnover of proteins compared to mRNA. Therefore,

biomass samples were taken from the cultivations on nine time points after addition of $MgSO_4$ (t=0, 1, 2, 4, 6, 8, 10, 12 and 24 h). LC-MS/MS analysis provided a number of 7691 unique peptides, which corresponded to 1221 *B. pertussis* proteins. In conclusion, 34% of all potentially expressed proteins were actually detected and identified. The relative quantities of identified proteins were determined based on the three most abundant peptides from each protein.[39] These quantities of protein expressed by *B. pertussis* were visualised in Figure 3. Quantitative proteome analysis showed large differences in the concentrations of individual proteins.

Furthermore, the proteome analysis revealed that 151 proteins were differentially expressed after addition of $MgSO_4$ to the medium (Figure 2C). Eighty-five proteins were significantly upregulated and 66 proteins were downregulated during 24 hours of cultivation. Principal Component analysis was used to determine the main variances in the dataset (Figure 2B). Principal component 1 (PC1) explained 61% of the total variances in data. PC1 appeared to be a time effect, indicating the transfer from virulent state to a-virulent state. PC2 accounted for 12% of the variances and implied also to be a time effect. PC3 explained 8% variances of the data and is caused by an unknown technical issue. Here, a set of proteins was differentially

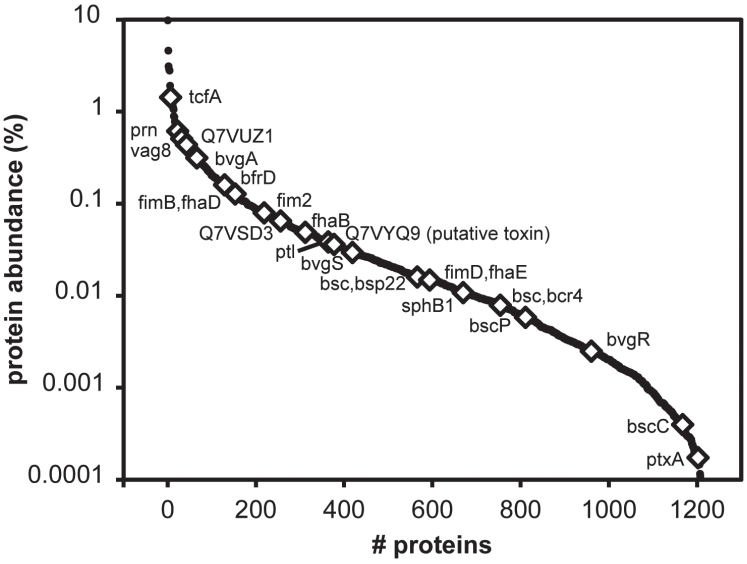


Figure 3 The relative concentration of 1221 *B. pertussis* proteins determined in a common reference. This reference was prepared by mixing equal concentrations of proteins from all *B. pertussis* samples taken after $MgSO_4$ addition to the culture medium (t = 0, 1, 2, 3, 4, 6, 8, 10, 12 and 24 h). Among all identified proteins, virulence proteins are depicted as diamonds.

expressed at time points t=2 h and t=8 h (data not shown). The next principal components had a minor contribution to the variances in protein expression (<5%).

Functional classification of highly associated proteins was performed using DAVID bioinformatics.[36] The classification revealed alterations in cellular processes of *B. pertussis* by the deliberate intervention. According to annotation, differentially expressed proteins are involved in protein translation, citrate cycle (TCA), glycolysis, amino acid synthesis and virulence. Twenty-one proteins were identified as virulence proteins.[21] Twelve virulence proteins were significantly downregulated ($p<0.001$). In addition, six other virulence proteins showed the expected downward trend ($p<0.1$). However, three virulence proteins, i.e. BvgR, FimD, and PtxA did not manifest in a reduced expression after adding $MgSO_4$ (Figure S3). In conclusion, LC-MS analysis can be used to analyse the protein expression of *B. pertussis* during upstream processing.

Comparison between gene and protein expression

The protein content of *B. pertussis* culture samples was compared with the corresponding gene expression data. A summary of the results is given in a Venn diagram (Figure 2E), depicting the overlap in identified genes and proteins as well as those that were significant regulated (i.e. gene hits/protein hits). The comparison showed that for all proteins gene expression data were available, but not vice versa. Cluster I shows consistent results for forty proteins with respect to gene and protein expression. If upregulation of RNA was observed, the protein concentration rose quickly too (cluster I, Figure 2C). Although RNA repression is generally recognised as a fast process, this does not one-to-one apply to protein repression (cluster I). The protein concentration decreased at a much slower rate, mainly associated with the washout of the bacteria during continuous cultivation. Forty-one genes showed a significant difference in expression after the addition of $MgSO_4$, but the corresponding protein expression was variable (cluster II). Many of these proteins were involved in lactate, pyruvate and acetate metabolism. In contrast, 111 proteins were differentially regulated, but their genes did not show significant changes in expression (cluster III). In many cases, gene expression had the same trend as the majority of the 111 proteins, but they were not significantly regulated ($p>0.001$). Functional analysis here revealed enrichment for amino acid transport and metabolism (Figure 2D). No corresponding proteins were found for 96 differentially expressed genes (cluster IV). In conclusion, combined analyses of gene and protein expression gave a detailed picture on the performance of bacterial cultivations of *B. pertussis*.

Virulence proteins in whole-cell pertussis suspensions affect potency

The relative amounts of virulence proteins present in whole-cell pertussis suspensions measured by LC-MS analysis showed a gradual decrease of virulence proteins following

addition of $MgSO_4$ (Figure 4A). The abundance was depicted for ten virulence proteins (TcfA, Vag8, Prn, BP2924, BvgA, Fim2, FhaB, BfrD, BP0500 and BvgS). Each virulence protein had its own clearance rate (Figure S3). However, all these virulence proteins declined significantly within four hours after downregulation of the BvgAS-regulated virulence genes. Moreover, the expression of four virulence proteins (Fim2, FHA, Prn and Vag8) in *B. pertussis* was assessed by using distinct monoclonal antibodies in a whole-cell ELISA (Figure 4B). After addition of $MgSO_4$ to the culture medium the concentration of the proteins decreased significantly. FHA showed a rapid decline to less than 30% of the start concentration within one hour ($p<0.001$). Perhaps FHA declined more quickly than the washout rate, because it is a secreted antigen.[40] Prn decreased with 50% within four hours ($p<0.001$). Contrarily, Fim2 and the Vag8 (except for t=24 h) were not significantly different from the theoretical washout of the bacteria during continuous cultivations (2 l of medium and an efflux of 0.1 lh^{-1}). The decrease of FHA and Prn was also significantly different until t=12 h if compared to Vag8, Fim2 and the washout ($p<0.001$). The expected washout was calculated assuming that protein production stopped immediately after induction with $MgSO_4$ without considering protein secretion or degradation. The decrease rate of the sum of all virulence proteins was almost similar when measured by LC-MS and ELISA. Both assays correlated well ($p<0.001$) in a Pearson correlation analysis ($R=0.977$).

The potencies of three experimental wP vaccines were determined in the ic-MPT.[19] Individual groups of mice were immunized with in one of the five concentrations of vaccine A (t=0 h), vaccine C (t=6 h), vaccine E (t=24 h) or a reference vaccine. Survival scores of mice that received vaccine A, C, or E were compared to the survival scores of a reference vaccine (Figure S4). The potencies of these vaccines were calculated based on the survival scores (Figure 4C). Potencies of vaccine A, C and E were 7 IU/ml (95% confidence interval of 2 – 27 IU/ml,), 5 IU/ml (2 – 10 IU/ml) and 1 IU/ml (0 – 3 IU/ml), respectively. These values were also reported by Hoonakker *et al.*[29] The potencies of vaccine A and C were above the minimum requirements provided by the European Pharmacopoeia and WHO (4 IU/mL and 95% interval with a lower limit of 2 IU/mL).[41,42] However, the potency of vaccine E was below the specifications. Furthermore, the potencies showed a gradual decrease which corresponded well with the total amount of virulence proteins present in corresponding wP vaccines products ($R=0.994$). However, this downward trend was not significant ($p=0.071$) due to the large confidence intervals of the estimated potencies. The wide spread of data supports the urgency of replacing the potency test with comprehensive quantitative methods, such as LC-MS. The observed descending potencies are probably caused by the reduced amounts of virulence proteins.

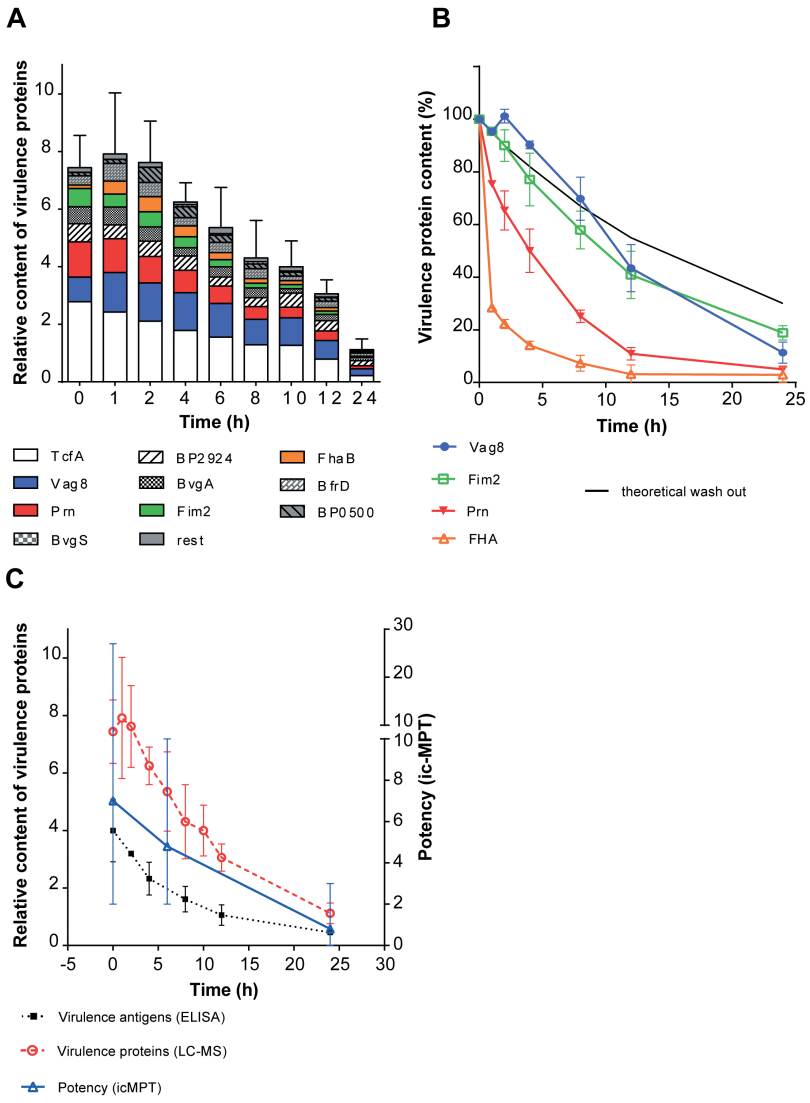


Figure 4 The gradual decrease of virulence proteins in wP vaccines were measured by (A) LC-MS and (B) ELISA. $MgSO_4$ was added to the culture medium during chemostat cultivations of *B. pertussis* strain 509. The addition resulted in a decreased expression of the virulence proteins, because $MgSO_4$ downregulates the BvgAS-regulated virulence genes. (C) Both assays correlated significantly ($p< 0.001$) in a Pearson correlation analysis ($R = 0.977$). Moreover, the reduction of virulence proteins corresponded well with the decreasing potency of corresponding WCV. However, the concentration of virulence proteins and potency were not significantly related due to the poor reliability of the ic-MPT (large 95% confidence intervals).

Discussion

Consistent protein expression is a prerequisite for producing whole-cell pertussis (wP) vaccines with a high quality. wP vaccines consist of hundreds of different proteins. This antigen composition in wP vaccine is influenced by several factors in the upstream process, e.g. the selected *B. pertussis* strain, medium composition and culture conditions.[21,28] For example, the cultivation temperature, the presence of particular vitamins, iron concentration, nutrient limitation and in culture medium modulate the expression of many virulence proteins in *B. pertussis*.[17,25,43,44] In turn, the final antigen composition in wP vaccines determines considerably the protective immune response against *B. pertussis*.[45] In the present study, a deliberate intervention in the upstream process of wP vaccines was introduced to evaluate the effect on vaccine quality. Magnesium sulphate was added during a continuous fermentation of *B. pertussis* and downregulated the expression of virulence proteins. Gene and protein expression of *B. pertussis* was monitored by using gene arrays, mass spectrometry and ELISA. Significant differences in gene and protein expression were detected using these analytical techniques. Finally, the data was compared with potencies of the corresponding wP vaccines. A downward trend of the potency was observed for these wP vaccines as a result of the disturbance by adding magnesium sulphate.

Recently, Process Analytical Technology (PAT) is applied to monitor the upstream processing of wP vaccines.[20,21] These online measurements promote timely release of the intermediate products. Deviations observed by PAT during the production process have to be translated to changes in the potency of wP vaccines. Ideally, PAT data correlate with the potency. In practice, the limited reliability of the potency test (ic-MPT) precludes to find that relationship. Moreover, a deviation in the production process will not always result in a different vaccine quality.[46] Therefore, additional methods are needed in order to study the relationship between culture conditions and vaccine quality.

Microarrays and LC-MS are comprehensive techniques used in this study to reveal the effect of a disturbance in the upstream process of wP vaccines. The expression of almost all pertussis genes (3,582 genes) could be analysed on the microarray. The transcriptome dataset revealed that approximately 84% of all genes were expressed by *B. pertussis*. In contrast, only 1,221 proteins were identified by LC-MS in the protein preparations of *B. pertussis*. The number of proteins counts for 34% of the whole proteome. The mismatch between proteome and transcriptome can partly be explained by low concentrations of particular proteins and the limited dynamic range of a mass spectrometer (under-sampling). However, LC-MS analysis determines the definite protein compositions in intermediate and final products of a wP vaccines. This is not the case for microarray analysis, because gene expression does not

correspond to the actual protein composition of *B. pertussis* bacteria (Figure 2). In general, the overlap between transcriptome and proteome is rather poor in bacteria.[47] Multiple factors are involved causing this mismatch, e.g. mRNA turnover, translational regulation, protein turnover.[47-49] Gene and protein expression have a different evolvement and progression. Nevertheless, this study showed that upregulation of gene expression generally results in a rapid reproduction of proteins. An increased gene expression gave almost simultaneously an increment of the corresponding proteins. On the other hand, downregulated expression of genes did not result in a quick reduction of proteins, e.g. the decline of virulence proteins (Figure 4A). In conclusion, the inhibition of gene expression by adding magnesium sulphate does not lead to an immediate absence of virulence proteins.

Furthermore, ELISAs were performed to monitor confirm the expression of particular virulence proteins (FHA, Fim2, Prn and Vag8) during bacterial cultivation (Figure 4B). The ELISA results were compared with the proteome dataset acquired with LC-MS (Figure 4A). The ELISA showed significant correlation ($p<0.001$) with the total amount of virulence proteins measured by LC-MS ($R=0.977$). Probably, this correlation depends on the selected virulence proteins and specificity of the monoclonal antibodies used. The potency of wP vaccines is considerably determined by the content of virulence proteins present in wP vaccines (Figure 4C), but also other proteins from *B. pertussis* are decisive for the final vaccine quality. ELISA can determine the concentration of an single antigen. It can be used to measure the virulence status of *B. pertussis*. Moreover, ELISA is not a comprehensive assay to evaluate the expression of hundreds of different proteins present in wP vaccines. The whole protein composition contribute jointly to the efficacy of wP vaccines.

The current study demonstrated that continuous cultivation of *B. pertussis* in a chemically defined medium contributes to highly consistent gene and protein expression, because nutrient consumption and growth rates were highly controlled. Therefore, chemostat bioreactor cultures are very useful to study changes in gene and protein expression after a disturbance of the culture conditions, e.g. by altering in the medium composition, nutrient depletion, culture temperature and shortage of dissolved oxygen. Each particular disturbance might have a different effect on the protein expression of *B. pertussis*. The deliberate intervention by adding magnesium sulphate downregulated the expression of many virulence proteins. These virulence proteins are essential in the pathogenesis of *B. pertussis* and are needed for the colonization of *B. pertussis* in animals and humans.[50-52] More importantly, virulence proteins are associated with the generation of protective immunity.[53] Antibody responses against virulence proteins may prevent colonization, enhance complement-mediated killing or phagocytosis of *B. pertussis*.[54,55] Therefore, it is expected that a certain amount of virulence proteins have to be present in wP vaccines in order to induce effective protection in humans against pertussis.

The present study showed there is gradual loss of product quality when the BvgAS system is turned off. Most likely, the potency of wP vaccines is not related to the expression of a single immune-dominant virulence protein, but to the sum of all virulence proteins present in wP vaccines. The present study showed that a decrease in total expression of virulence proteins coincides with a gradually reduced potency (Figure 4C), which assessed in the mandatory ic-MPT.[41,42] A fundamental problem of potency testing in animals is the large variance in the obtained data points. Indeed, the potencies of the wP vaccines under test had large 95% confidence intervals (Figure 4C). Therefore, the ic-MPT is of limited use to demonstrate significant differences in quality between wP vaccines. In contrast, proteome analysis is more precise, highly reproducible and may have predictive value for the effectiveness of whole-cell pertussis vaccines. Proteome analysis is highly suited to demonstrate consistent production of wP vaccines. In conclusion, proteome analysis proved to be a powerful tool for monitoring upstream processing of whole-cell pertussis vaccines.

Acknowledgements

We thank Corine Kruiswijk for the support of this study and Thijs Gerritzen for critical reading of the manuscript. This work was partially conducted as part of “FASTVAC: A generic framework for FAST production and evaluation of emergency VACcines”, a project funded by the Health Programme 2008-2013 of the European Union (Project No 20091106).

Supplementary information

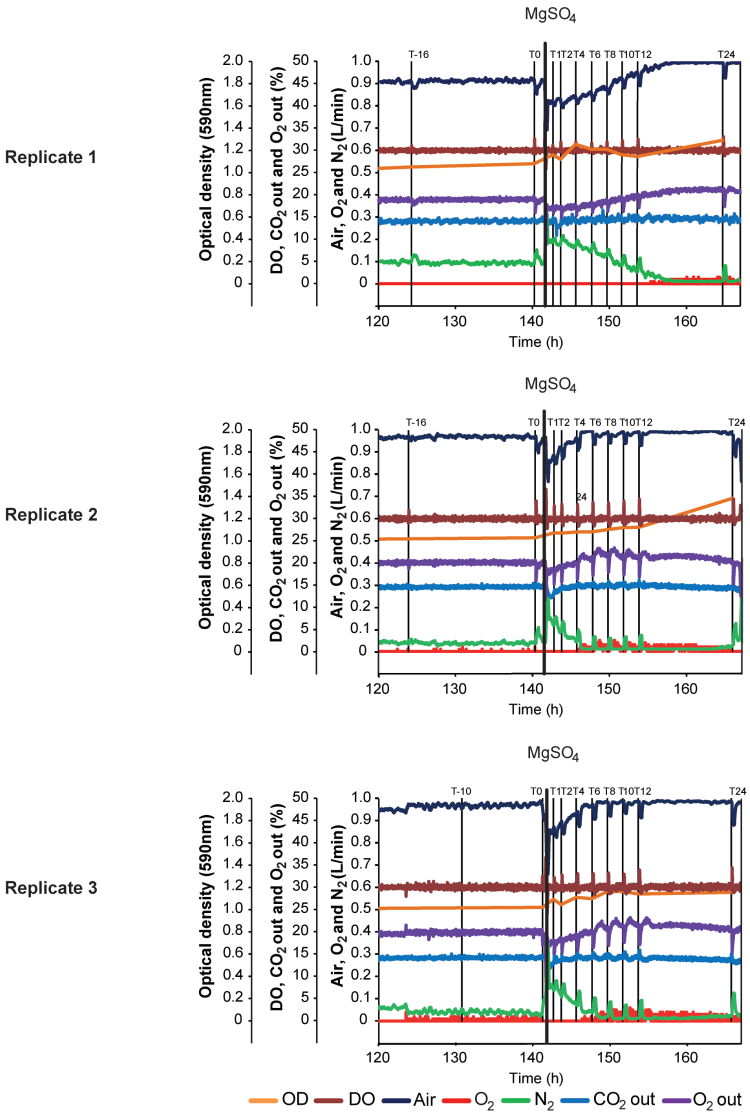
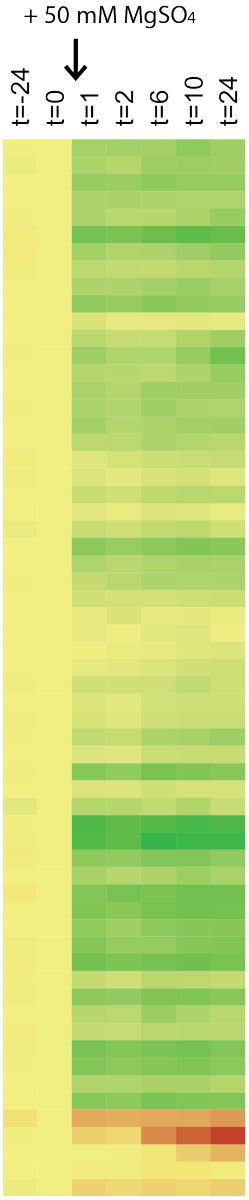
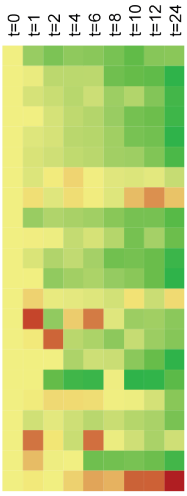


Figure S1 Three continuous cultivations of *B. pertussis* were performed in a 3L-bioreactor. The bacteria were grown on a chemically defined medium for about 170h. $MgSO_4$ was added at t=0h to reduce gene expression of virulence proteins. The optical densities (OD) were determined at 590 nm. The air, oxygen (O_2) and nitrogen (N_2) supply was monitored during cultivation. Furthermore, the percentage of dissolved oxygen (DO) in the medium, carbon dioxide (CO_2 out) and oxygen (O_2 out) in the gas outlet were measured.



Gene-ID	Remark	Set	Gene
BP0216		VAG	autotransporter subtilisin-like protease, sphB1
BP0500		VAG	hypothetical protein,
BP0760		VAG	bifunctional hemolysin-adenylate cyclase precursor, cyaA,cya
BP0856		VAG	probable TonB-dependent receptor for iron transport, bfrD
BP1054		VAG	pertactin precursor, prn
BP1119		VAG	serotype 2 fimbrial subunit precursor, fim2
BP1201		VAG	tracheal colonization factor precursor, tcfA
BP1251		VAG	putative toxin,
BP1252		VAG	putative exported protein,
BP1876		VAG	regulatory protein BvgR, bvgR
BP1877	*	VAG	virulence sensor protein, bvgS
BP1878		VAG	virulence factors transcription regulator, bvgA
BP1879		VAG	filamentous hemagglutinin/adhesin, fhaB
BP1880		VAG	fimbrial protein (pseudogene), fimA
BP1881		VAG	chaperone protein, fimB,fhaD
BP1882		VAG	outer membrane usher protein precursor, fimC,fhaA
BP1883		VAG	fimbrial adhesin, fimD,fhaE
BP2183		VAG	conserved hypothetical protein,
BP2226		VAG	putative anti-sigma factor antagonist,
BP2227	*	VAG	putative anti-sigma factor,
BP2228		VAG	alanine racemase, catabolic, alr,dadB
BP2231	*	VAG	putative exported protein,
BP2233		VAG	hypothetical protein,
BP2234		VAG	putative RNA polymerase sigma factor, brpL
BP2235		VAG	putative type III secretion protein, bscC
BP2243		VAG	hypothetical protein, bscP
BP2244		VAG	putative type III secretion protein, bscO
BP2246	*	VAG	putative type III secretion protein, bscL
BP2248	*	VAG	putative type III secretion protein, bscJ
BP2250	*	VAG	hypothetical protein, bcr4
BP2251	*	VAG	putative regulatory protein, bcrH2
BP2252		VAG	putative outer protein B, bopB
BP2254	*	VAG	putative regulatory protein, bcrH1
BP2255		VAG	hypothetical protein,
BP2256		VAG	putative secreted protein, bsp22
BP2257		VAG	putative outer protein N, bopN
BP2261		VAG	putative type III secretion pore protein, bcrD
BP2262	*	VAG	putative type III secretion protein, bscD
BP2264		VAG	putative type III secretion protein, bscF
BP2315		VAG	autotransporter, vag8
BP2924		VAG	putative exported protein,
BP2925		VAG	conserved hypothetical protein,
BP2927		VAG	putative integral membrane protein,
BP3783		VAG	pertussis toxin subunit 1 precursor, ptxA
BP3784		VAG	pertussis toxin subunit 2 precursor, ptxB
BP3785		VAG	pertussis toxin subunit 4 precursor, ptxD
BP3786		VAG	pertussis toxin subunit 5 precursor, ptxE
BP3787		VAG	pertussis toxin subunit 3 precursor, ptxC
BP3788		VAG	pertussis toxin transport protein, ptlA
BP3789		VAG	pertussis toxin transport protein, ptlB
BP3790		VAG	putative bacterial secretion system protein,
BP3792		VAG	putative bacterial secretion system protein,
BP3793		VAG	putative bacterial secretion system protein,
BP3794		VAG	putative bacterial secretion system protein,
BP3795		VAG	putative bacterial secretion system protein,
BP3796		VAG	putative bacterial secretion system protein,
BP1311		VRG	putative membrane protein,
BP1618		VRG	putative N-terminal region of capsular polysaccharide biosynthesis protein
BP2020		VRG	hypothetical protein,
BP2413		VRG	putative lipoprotein,
BP2496		VRG	hypothetical protein,

Figure S2 Differential expression of virulence genes (VAG) and virulence-repressed genes (VRG). Differential expression of virulence genes (VAG) and virulence-repressed genes (VRG). The virulence genes were downregulated (green) by $MgSO_4$, whereas the virulence-repressed genes were upregulated (red). * virulence gene with non-significant downward trend.



Gene-ID	Set	Gene
BP0216	VAG	autotransporter subtilisin-like protease, sphB1
BP0500	VAG	hypothetical protein,
BP0856	VAG	probable TonB-dependent receptor for iron transport, bfrD
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BP1119	VAG	serotype 2 fimbrial subunit precursor, fim2
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BP1251	VAG	putative toxin,
BP1876	VAG	regulatory protein BvgR, bvgR
BP1877	VAG	virulence sensor protein, bvgS
BP1878	VAG	virulence factors transcription regulator, bvgA
BP1879	VAG	filamentous hemagglutinin/adhesin, fhaB
BP1881	VAG	chaperone protein, fimB,fhaD
BP1883	VAG	fimbrial adhesin, fimD,fhaE
BP2235	VAG	putative type III secretion protein, bscC
BP2243	VAG	hypothetical protein, bscP
BP2250	VAG	hypothetical protein, bcr4
BP2256	VAG	putative secreted protein, bsp22
BP2315	VAG	autotransporter, vag8
BP2924	VAG	putative exported protein,
BP3783	VAG	pertussis toxin subunit 1 precursor, ptxA
BP3794	VAG	putative bacterial secretion system protein,
BP1618	VRG	putative N-terminal region of capsular polysaccharide biosynthesis protein (partial)

Figure S3 Differential expression of virulence genes (VAG) and virulence-repressed genes (VRG). Differential expression of virulence genes (VAG) and virulence-repressed genes (VRG). The virulence genes were downregulated (green) by $MgSO_4$, whereas the virulence-repressed genes were upregulated (red). * virulence gene with non-significant downward trend.

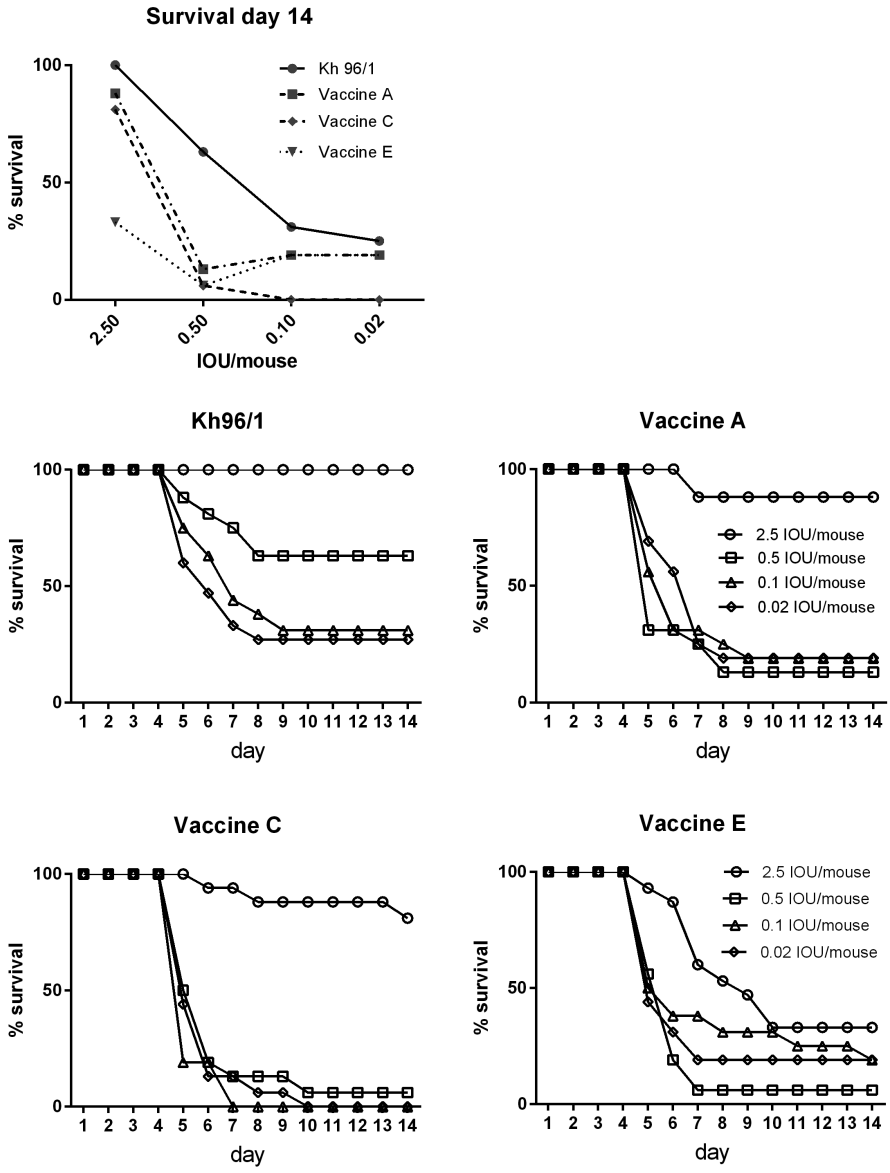

Figure S4 Survival scores of mice that had received different amounts vaccine A, C, or E were compared to the survival scores of a reference vaccine to calculate the potencies. Mice were immunized with vaccine A (t=0 h), vaccine C (t=6 h), vaccine E (t=24 h) or a reference vaccine receiving different amounts of vaccine (0.02, 0.1, 0.5 or 2.5 IOU). Survival scores of mice that received vaccine A, C, or E were compared to the survival scores of a reference vaccine to calculate the potencies.

References

1. Melvin, J. A.; Scheller, E. V.; Miller, J. F.; Cotter, P. A., *Bordetella pertussis* pathogenesis: current and future challenges. Nat Rev Microbiol 2014, 12, (4), 274-88.
2. Libster, R.; Edwards, K. M., Re-emergence of pertussis: what are the solutions? Expert Rev Vaccines 2012, 11, (11), 1331-46.
3. Pillsbury, A.; Quinn, H. E.; McIntyre, P. B., Australian vaccine preventable disease epidemiological review series: pertussis, 2006-2012. Commun Dis Intell Q Rep 2014, 38, (3), E179-94.
4. Theofiles, A. G.; Cunningham, S. A.; Chia, N.; Jeraldo, P. R.; Quest, D. J.; Mandrekar, J. N.; Patel, R., Pertussis outbreak, southeastern Minnesota, 2012. Mayo Clin Proc 2014, 89, (10), 1378-88.
5. Witt, M. A.; Katz, P. H.; Witt, D. J., Unexpectedly limited durability of immunity following acellular pertussis vaccination in preadolescents in a North American outbreak. Clin Infect Dis 2012, 54, (12), 1730-5.
6. Amirthalingam, G.; Gupta, S.; Campbell, H., Pertussis immunisation and control in England and Wales, 1957 to 2012: a historical review. Euro Surveill 2013, 18, (38).
7. Gambhir, M.; Clark, T. A.; Cauchemez, S.; Tartof, S. Y.; Swerdlow, D. L.; Ferguson, N. M., A change in vaccine efficacy and duration of protection explains recent rises in pertussis incidence in the United States. PLoS Comput Biol 2015, 11, (4), e1004138.
8. Klein, N. P.; Bartlett, J.; Rowhani-Rahbar, A.; Fireman, B.; Baxter, R., Waning protection after fifth dose of acellular pertussis vaccine in children. N Engl J Med 2012, 367, (11), 1012-9.
9. Locht, C.; Mielcarek, N., Live attenuated vaccines against pertussis. Expert Rev Vaccines 2014, 13, (9), 1147-58.
10. Dias, W. O.; van der Ark, A. A.; Sakauchi, M. A.; Kubrusly, F. S.; Prestes, A. F.; Borges, M. M.; Furuyama, N.; Horton, D. S.; Quintilio, W.; Antoniazi, M.; Kuipers, B.; van der Zeijst, B. A.; Raw, I., An improved whole cell pertussis vaccine with reduced content of endotoxin. Hum Vaccin Immunother 2013, 9, (2), 339-48.
11. Sebo, P.; Osicka, R.; Masin, J., Adenylate cyclase toxin-hemolysin relevance for pertussis vaccines. Expert Rev Vaccines 2014, 13, (10), 1215-27.
12. Asensio, C. J.; Gaillard, M. E.; Moreno, G.; Bottero, D.; Zurita, E.; Rumbo, M.; van der Ley, P.; van der Ark, A.; Hozbor, D., Outer membrane vesicles obtained from *Bordetella pertussis* Tohama expressing the lipid A deacylase PagL as a novel acellular vaccine candidate. Vaccine 2011, 29, (8), 1649-56.
13. World Health, O., Pertussis vaccines. Wkly Epidemiol Rec 2014, 21, (89), 230-233.
14. Robbins, J. B.; Schneerson, R.; Kubler-Kielb, J.; Keith, J. M.; Trollfors, B.; Vinogradov, E.; Shiloach, J., Toward a new vaccine for pertussis. Proc Natl Acad Sci U S A 2014, 111, (9), 3213-6.
15. Kubler-Kielb, J.; Vinogradov, E.; Lagergard, T.; Ginzberg, A.; King, J. D.; Preston, A.; Maskell, D. J.; Pozsgay, V.; Keith, J. M.; Robbins, J. B.; Schneerson, R., Oligosaccharide conjugates of *Bordetella pertussis* and bronchiseptica induce bactericidal antibodies, an addition to pertussis vaccine. Proc Natl Acad Sci U S A 2011, 108, (10), 4087-92.
16. Melton, A. R.; Weiss, A. A., Environmental regulation of expression of virulence determinants in *Bordetella pertussis*. J Bacteriol 1989, 171, (11), 6206-12.
17. van de Waterbeemd, B.; Streefland, M.; Pennings, J.; van der Pol, L.; Beuvery, C.; Tramper, J.; Martens, D., Gene-expression-based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnol Bioeng 2009, 103, (5), 900-8.

18. Thalen, M.; van der Ark, A.; van den Ijssel, J.; van Straaten, I.; Jansen, D.; Beuvery, C.; Martens, D.; Tramper, J., Improving the cellular pertussis vaccine: increased potency and consistency. Vaccine 2008, 26, (5), 653-63.
19. Kendrick, P. L.; Eldering, G.; Dixon, M. K.; Misner, J., Mouse Protection Tests in the Study of Pertussis Vaccine: A Comparative Series Using the Intracerebral Route for Challenge. Am J Public Health Nations Health 1947, 37, (7), 803-10.
20. Soons, Z. I. T. A.; Streefland, M.; Van Straten, G.; Van Boxtel, A. J. B., Assessment of near infrared and "software sensor" for biomass monitoring and control. Chemometrics and Intelligent Laboratory Systems 2008, 94, 166-74.
21. Streefland, M.; van de Waterbeemd, B.; Happe, H.; van der Pol, L. A.; Beuvery, E. C.; Tramper, J.; Martens, D. E., PAT for vaccines: the first stage of PAT implementation for development of a well-defined whole-cell vaccine against whooping cough disease. Vaccine 2007, 25, (16), 2994-3000.
22. Herbert, D.; Elsworth, R.; Telling, R. C., The continuous culture of bacteria; a theoretical and experimental study. J Gen Microbiol 1956, 14, (3), 601-22.
23. Robinson, A.; Gorringe, A. R.; Irons, L. I.; Keevil, C. W., Antigenic modulation of *Bordetella pertussis* in continuous culture. FEMS Microbiology Letters 1983, 19, (1), 105-109.
24. Weiss, A. A.; Falkow, S., Genetic analysis of phase change in *Bordetella pertussis*. Infect Immun 1984, 43, (1), 263-9.
25. Decker, K. B.; James, T. D.; Stibitz, S.; Hinton, D. M., The *Bordetella pertussis* model of exquisite gene control by the global transcription factor BvgA. Microbiology 2012, 158, (Pt 7), 1665-76.
26. Merkel, T. J.; Barros, C.; Stibitz, S., Characterization of the bvgR locus of *Bordetella pertussis*. J Bacteriol 1998, 180, (7), 1682-90.
27. Stibitz, S.; Yang, M. S., Subcellular localization and immunological detection of proteins encoded by the vir locus of *Bordetella pertussis*. J Bacteriol 1991, 173, (14), 4288-96.
28. Thalen, M.; van den, I. J.; Jiskoot, W.; Zomer, B.; Roholl, P.; de Gooijer, C.; Beuvery, C.; Tramper, J., Rational medium design for *Bordetella pertussis*: basic metabolism. J Biotechnol 1999, 75, (2-3), 147-59.
29. Hoonakker, M. E.; Verhagen, L. M.; Hendriksen, C. F.; van Els, C. A.; Vandebriel, R. J.; Sloots, A.; Han, W. G., *In vitro* innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle. Biologicals 2015, 43, (2), 100-9.
30. Ramalho, A. S.; Beck, S.; Farinha, C. M.; Clarke, L. A.; Heda, G. D.; Steiner, B.; Sanz, J.; Gallati, S.; Amaral, M. D.; Harris, A.; Tzetis, M., Methods for RNA extraction, cDNA preparation and analysis of CFTR transcripts. J Cyst Fibros 2004, 3 Suppl 2, 11-5.
31. Mutter, G. L.; Zahrieh, D.; Liu, C.; Neuberg, D.; Finkelstein, D.; Baker, H. E.; Warrington, J. A., Comparison of frozen and RNALater solid tissue storage methods for use in RNA expression microarrays. BMC Genomics 2004, 5, 88.
32. Kasahara, T.; Miyazaki, T.; Nitta, H.; Ono, A.; Miyagishima, T.; Nagao, T.; Urushidani, T., Evaluation of methods for duration of preservation of RNA quality in rat liver used for transcriptome analysis. J Toxicol Sci 2006, 31, (5), 509-19.
33. Cummings, C. A.; Bootsma, H. J.; Relman, D. A.; Miller, J. F., Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. J Bacteriol 2006, 188, (5), 1775-85.
34. van de Waterbeemd, B.; Mommen, G. P.; Pennings, J. L.; Eppink, M. H.; Wijffels, R. H.; van der Pol, L. A.; de Jong, A. P., Quantitative proteomics reveals distinct differences in the protein content of outer membrane vesicle vaccines. J Proteome Res 2013, 12, (4), 1898-908.

35. Meiring, H. D.; Heeft, E. v. d.; Hove, G. J. t.; Jong, A. d., Nanoscale LC-MS(n): technical design and applications to peptide and protein analysis. J Sep Sci 2002, 25, 557-568.
36. Huang da, W.; Sherman, B. T.; Lempicki, R. A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 2009, 4, (1), 44-57.
37. Huang da, W.; Sherman, B. T.; Lempicki, R. A., Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res 2009, 37, (1), 1-13.
38. Poolman, J. T.; Kuipers, B.; Vogel, M. L.; Hamstra, H. J.; Nagel, J., Description of a hybridoma bank towards *Bordetella pertussis* toxin and surface antigens. Microb Pathog 1990, 8, (6), 377-82.
39. Silva, J. C.; Gorenstein, M. V.; Li, G. Z.; Vissers, J. P.; Geromanos, S. J., Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Mol Cell Proteomics 2006, 5, (1), 144-56.
40. Alonso, S.; Reveneau, N.; Pethe, K.; Locht, C., Eighty-kilodalton N-terminal moiety of *Bordetella pertussis* filamentous hemagglutinin: adherence, immunogenicity, and protective role. Infect Immun 2002, 70, (8), 4142-7.
41. WHO, Recommendations for whole-cell pertussis vaccine. WHO Technical Report Series 2007, Annex 6.
42. European Pharmacopoeia, Assay of pertussis vaccine (whole cell). 2014, 8.0, 242.
43. Thalen, M.; Venema, M.; van den, I. J.; Berwald, L.; Beuvery, C.; Martens, D.; Tramper, J., Effect of relevant culture parameters on Pertussis Toxin expression by *Bordetella pertussis*. Biologicals 2006, 34, (3), 213-20.
44. Vidakovics, M. L.; Paba, J.; Lamberti, Y.; Ricart, C. A.; de Sousa, M. V.; Rodriguez, M. E., Profiling the *Bordetella pertussis* proteome during iron starvation. J Proteome Res 2007, 6, (7), 2518-28.
45. Raeven, R. H.; van der Maas, L.; Tilstra, W.; Uittenbogaard, J. P.; Bindels, T. H.; Kuipers, B.; van der Ark, A.; Pennings, J. L.; van Riet, E.; Jiskoot, W.; Kersten, G. F.; Metz, B., Immunoproteomic Profiling of *Bordetella pertussis* Outer Membrane Vesicle Vaccine Reveals Broad and Balanced Humoral Immunogenicity. J Proteome Res 2015, 14, (7), 2929-42.
46. Streefland, M.; van de Waterbeemd, B.; Kint, J.; van der Pol, L. A.; Beuvery, E. C.; Tramper, J.; Martens, D. E., Evaluation of a critical process parameter: oxygen limitation during cultivation has a fully reversible effect on gene expression of *Bordetella pertussis*. Biotechnol Bioeng 2009, 102, (1), 161-7.
47. Basler, M.; Linhartova, I.; Halada, P.; Novotna, J.; Bezouskova, S.; Osicka, R.; Weiser, J.; Vohradsky, J.; Sebo, P., The iron-regulated transcriptome and proteome of Neisseria meningitidis serogroup C. Proteomics 2006, 6, (23), 6194-206.
48. Hegde, P. S.; White, I. R.; Debouck, C., Interplay of transcriptomics and proteomics. Curr Opin Biotechnol 2003, 14, (6), 647-51.
49. Kozak, M., Regulation of translation via mRNA structure in prokaryotes and eukaryotes. Gene 2005, 361, 13-37.
50. Kinnear, S. M.; Boucher, P. E.; Stibitz, S.; Carbonetti, N. H., Analysis of BvgA activation of the pertactin gene promoter in *Bordetella pertussis*. J Bacteriol 1999, 181, (17), 5234-41.
51. Akerley, B. J.; Cotter, P. A.; Miller, J. F., Ectopic expression of the flagellar regulon alters development of the *Bordetella*-host interaction. Cell 1995, 80, (4), 611-20.
52. Cotter, P. A.; Miller, J. F., BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. Infect Immun 1994, 62, (8), 3381-90.
53. van den Berg, B. M.; Beekhuizen, H.; Mooi, F. R.; van Furth, R., Role of antibodies against *Bordetella pertussis* virulence factors in adherence of *Bordetella pertussis* and *Bordetella parapertussis* to human bronchial epithelial cells. Infect Immun 1999, 67, (3), 1050-5.

54. Aase, A.; Herstad, T. K.; Merino, S.; Bolstad, M.; Sandbu, S.; Bakke, H.; Aaberge, I. S., Immunization of teenagers with a fifth dose of reduced DTaP-IPV induces high levels of pertussis antibodies with a significant increase in opsonophagocytic activity. Clin Vaccine Immunol 2011, 18, (8), 1269-74.
55. Ganguly, T.; Johnson, J. B.; Kock, N. D.; Parks, G. D.; Deora, R., The *Bordetella pertussis* Bps polysaccharide enhances lung colonization by conferring protection from complement-mediated killing. Cell Microbiol 2014, 16, (7), 1105-18.

3

Chapter 3

Vaccine-Mediated Activation of Human TLR4 Is Affected by Modulation of Culture Conditions during Whole-Cell Pertussis Vaccine Preparation

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PLoS One 2016 Aug; 11(8): e0161428

Abstract

The potency of whole-cell pertussis (wP) vaccines is still determined by an intracerebral mouse protection test. To allow development of suitable *in vitro* alternatives to this test, insight into relevant parameters to monitor the consistency of vaccine quality is essential. To this end, a panel of experimental wP vaccines of varying quality was prepared by sulfate-mediated suppression of the BvgASR master virulence regulatory system of *Bordetella pertussis* during cultivation. This system regulates the transcription of a range of virulence proteins, many of which are considered important for the induction of effective host immunity. The protein compositions and *in vivo* potencies of the vaccines were BvgASR dependent, with the vaccine containing the highest amount of virulence proteins having the highest *in vivo* potency. Here, the capacities of these vaccines to stimulate human Toll-like receptors (hTLR) 2 and 4 and the role these receptors play in wP vaccine-mediated activation of antigen-presenting cells *in vitro* were studied. Prolonged BvgASR suppression was associated with a decreased capacity of vaccines to activate hTLR4. In contrast, no significant differences in hTLR2 activation were observed. Similarly, vaccine-induced activation of MonoMac-6 and monocyte-derived dendritic cells was strongest with the highest potency vaccine. Blocking of TLR2 and TLR4 showed that differences in antigen-presenting cell activation could be largely attributed to vaccine-dependent variation in hTLR4 signalling. Interestingly, this BvgASR-dependent decrease in hTLR4 activation coincided with a reduction in GlcN-modified lipopolysaccharides in these vaccines. Accordingly, expression of the *lgmA-C* genes, required for this glucosamine modification was significantly reduced in bacteria exposed to sulfate. Together, these findings demonstrate that the BvgASR status of bacteria during wP vaccine preparation is critical for their hTLR4 activation capacity and suggest that including such parameters to assess consistency of newly produced vaccines could bring *in vitro* testing of vaccine quality a step closer.

Introduction

Bordetella pertussis is a Gram-negative pathogen that causes whooping cough in humans. As pertussis disease in children can be severe, development of whole-cell pertussis (wP) vaccines started soon after it was known how to cultivate the bacterium under laboratory conditions. The introduction of these vaccines on a large scale resulted in the control of epidemic pertussis disease.[1,2] Although today wP vaccines have been replaced by acellular pertussis vaccines in most industrialized countries due to concerns regarding their reactogenicity, wP vaccines are still used in many countries in Latin America, Africa and Asia.[1] Furthermore, recent evidence points towards a higher efficacy of vaccination schemes including a first dose of wP compared to schedules solely using aP vaccines.[3-6] Along with lower costs of production[7], this will likely make these the pertussis vaccine of choice in many regions for the years to come. For lot release of wP vaccines, the use of the intracerebral challenge test, also known as the Kendrick test, is a regulatory requirement at this moment.[8,9] As it is questionable whether this mouse model appropriately reflects human pertussis disease, the results using these animal tests are highly variable within and among laboratories[10] and there is concern with respect to animal welfare[11], novel *in vitro* alternatives to assess the quality of newly produced wP vaccine lots are urgently needed.

For the quality of wP vaccines, the bacterial cultivation process is considered crucial as growth conditions are known to affect gene expression in *B. pertussis*.[12,13] Expression of most virulence genes, whose products are involved in pathogenesis, is controlled by a master regulatory system encoded by the BvgASR locus.[14] This regulatory system enables the bacterium to adapt to environmental changes. In response to conditions such as temperatures below 26°C or the presence of sulfate ($MgSO_4$) or nicotinic acid, expression of most virulence genes is suppressed.[15] This state is referred to as the Bvg^- phase as opposed to the Bvg^+ phase in which the majority of virulence proteins are expressed. Using mutants locked in either the Bvg^- or Bvg^+ phase, it has been shown that bacteria in the Bvg^- phase are unable to survive *in vivo* and that the Bvg^+ phase is required to cause respiratory infection in mice.[16] Importantly, the presence of many of the virulence proteins expressed in the Bvg^+ phase in wP vaccines has also been associated with the induction of protective immune responses. It has been shown that the amount of virulence proteins in a vaccine based on outer membrane vesicles correlated with protection.[17] In another study, immunization with inactivated *B. pertussis* bacteria harvested during the logarithmic growth phase (considered to contain high amounts of virulence proteins) induced an immune response with higher protective capacity compared with inactivated bacteria harvested after logarithmic growth (considered to contain lower amounts of virulence proteins).[18] In addition, other investigators were able to confirm that a decreased availability of nutrients in *B. pertussis* cultures after the logarithmic growth phase is associated with a

lower expression of many virulence genes in a BvgASR-dependent manner.[12, 13] Taken together, these results strongly suggest that the composition (i.e. quality) and hence protective capacity (i.e. potency) of a wP vaccine can be influenced by the culture conditions used within the wP manufacturing process. By sensing differences in the culture conditions, the BvgASR system most likely plays an important role in controlling these vaccine characteristics. Although some of these reports directly link differences in wP vaccine composition to the potency of these batches in the intracerebral challenge model[18], not much is known about the immunological consequences of vaccination with wP vaccines produced from *B. pertussis* bacteria cultured under BvgASR-modulating conditions.

The potency of a vaccine depends on the type of adaptive immune response that is initiated and directed by antigen-presenting cells (APC).[19] This requires proper activation of APC through recognition of conserved microbial structures by pathogen recognition receptors (PRR), including the Toll-like receptors (TLR). Although *B. pertussis* is known to produce ligands for both TLR2 and TLR4[20,21], TLR4 signalling in particular was found to affect the development of immune responses against *B. pertussis*, whereas TLR2 was not.[22] In addition, TLR4 was shown to be essential for protection against *B. pertussis* in mice.[23] The canonical ligand for TLR4 is lipopolysaccharide (LPS), a well-known component of wP vaccines. It has been shown that *B. pertussis* can substitute the phosphate groups of the lipid A moiety of its LPS with glucosamine (GlcN), a modification that leads to enhanced hTLR4 signalling and the secretion of pro-inflammatory cytokines.[24] The genes *lgmA*, *lgmB* and *lgmC* have recently been identified to encode the enzymes required for this GlcN modification of *B. pertussis* LPS.[24-26] Importantly, the expression of *lgmA* and *lgmB* was found to be regulated by BvgASR master regulatory system.[27] Since culture conditions (such as nutrient availability) can affect the BvgASR system, they might also affect LPS structure during cultivation of *B. pertussis* bacteria. As LPS is an important contributor to the wP vaccine-induced activation of APC, we hypothesised that culture condition-induced changes in Bvg phase could affect APC activation and thereby influence the induction of adaptive immune responses and vaccine potency.

In this study, we investigated the capacity of several *in vitro* methods to assess wP vaccine quality. To properly address the potential of these assays, we used a set of experimental wP vaccines of varying quality that were produced by deliberate addition of sulfate to the bioreactor cultures in order to modulate the BvgASR system. *B. pertussis* bacteria (vaccine strain 509) were harvested just before sulfate addition and at several time points afterwards, resulting in vaccine products that contain varying amounts of virulence proteins. Based on protein composition and *in vivo* potency testing (Metz *et al.*, submitted for publication), the qualities of these wP vaccines were considered to range from good to poor (vaccine A_{ref} - E). Previously, we showed that these vaccines differed in their capacity to induce activation

of monocyte-derived dendritic cells (moDC) and MonoMac-6 (MM6) cells *in vitro* and demonstrated that these cellular platforms had considerable potential as *in vitro* alternatives to animal testing for the quality control of wP vaccines.[28] Here, we investigated the relative contribution of TLR2 and TLR4 to the activation of these cells and showed that prolonged cultivation in the presence of sulfate not only triggered alterations in the expression of known virulence proteins but also in the expression of genes associated with LPS modification, leading to variations in LPS structure. These modifications were associated with the ability of the wP vaccines to induce human TLR4 but not TLR2 signalling and therefore likely influenced activation of human APC. Taken together, these findings demonstrate the necessity to monitor vaccine quality after production, and more importantly, they provide a scientific basis to the use of cell-based assays to assess aspects of the immunological potency of wP vaccines *in vitro*.

Materials and Methods

Production of experimental wP vaccines of different quality

In this study, experimental wP vaccine batches were used that were based on cultivation of *B. pertussis* strain 509 (Intravacc, Bilthoven, The Netherlands). This clinical isolate was collected in 1963 and was used for the production of wP vaccine for the national vaccination program of the Netherlands until 2005. The wP vaccines were produced as described in detail elsewhere (Metz *et al.* submitted for publication). Briefly, all cultures were grown in chemically defined THIJS medium[29, 30] using a 3L bioreactor equipped with a Rushton stirrer (Applikon, Schiedam, The Netherlands) at a constant temperature of 35°C. After obtaining a steady-state culture, deliberate down-regulation of virulence genes (t=0) was initiated by adding $MgSO_4$ to the medium at final concentrations of 50 mM. Samples were taken just before the addition of $MgSO_4$ (0 hours (h)) and 2, 6, 12 and 24h after this addition, inactivated with formaldehyde (16 mM) and heating (56°C) for 10 min. The resulting wP vaccines batches are referred to as vaccine A_{ref} (t=0) (reference vaccine), vaccine B (t=2), vaccine C (t=6), vaccine D (t=12) and vaccine E (t=24). Three separate cultivation runs were performed. Unless mentioned otherwise, corresponding vaccine preparations derived from the different runs were pooled and used for the stimulation of different cell lines or monocyte derived dendritic cells (moDC) at indicated OD_{590nm}.

Reagents

Ultrapure LPS from *E. coli* K12 (LPS-EC), ultrapure LPS from Rhodobacter sphaeroides (LPS-RS), PAM3CSK4 (PAM), HEK-Blue selection antibiotics, Normocin, Zeocin and QUANTI-Blue were all purchased from InvivoGen Europe (Toulouse, France). The α-TLR2 blocking antibody was obtained from R&D systems. Recombinant human GM-CSF was purchased from PeproTech (Rocky Hill, NJ, USA) and recombinant human IL-4 was purchased from Sanquin (Amsterdam, The Netherlands). The IL-6 ELISA kit was purchased from Sanquin, the IL-12p40 ELISA kit was purchased from Diaclone and the IL-8 ELISA kit was obtained from R&D systems. Dulbecco's modified Eagle's medium (DMEM) and Iscove's modified Dulbecco's medium (IMDM) were purchased from Gibco, FCS was obtained from Thermo scientific (Waltham, MA). Fatty acid standards C14:0, C14:0-3OH, NaOH, HCl and tert-butyl methyl ether were purchased from Sigma (Zwijndrecht, The Netherlands). Methanol and n-Hexane were from JT Baker and n-Hexane from Biosolve.

ELISA for the specific detection of virulence antigens

In contrast to the ELISA that was performed directly after vaccine preparation (Metz *et al.*, submitted for publication), here specific *B. pertussis* antigens (FHA, PRN, FIM2, FIM3, Vag8, PT, LPS) in vaccines A_{ref} - E were quantified by ELISA approximately 1.5 years after production to

verify virulence protein content. Immulon-2HB (Thermo Scientific) were coated with 100 µL of the vaccines of separate runs (vaccine A_{ref} -E) diluted to a final OD_{590nm} of 0.2. Plates were sealed and incubated overnight at room temperature. The next day, triplicate wells coated with each vaccine run were incubated with antibodies specific for six *B. pertussis* virulence proteins FHA (Mab 29E7), PRN (Mab Pem4), FIM2 (Mab 118E10), FIM3 (Mab 81H1), PT (Mab P8), Vag8 (Mab 14B4) and an antibody specific for band A LPS (Mab 88F3) or band B LPS (Mab BL-8) for 1h at 37°C. Binding of the antibodies was detected using an HRP-conjugated goat anti-mouse IgG in PBS containing 0.5% skim milk followed by incubation with peroxidase substrate (0.1 mg/mL TMB with 0.012% H_2O_2 in 0.11 M sodium acetate buffer [pH 5.5]) for 10 minutes. The reaction was stopped by addition of 100 µl 2 M H_2SO_4 to each well. The absorbance at 450nm was then measured using an ELISA reader (Bio-Tek). Heat-inactivated whole bacteria of the 509 strain that were either treated or not treated with formaldehyde were used to determine the effect of formaldehyde treatment on antibody recognition of target proteins.

Cell lines and culture conditions

HEK-Blue cells stably transfected with human TLR4, MD-2 and CD14 (HB-hTLR4) or stably transfected with human TLR2 and CD14 (HB-hTLR2) were purchased from InvivoGen. As a control, the HEK-Blue Null1-cell line (HB-Null1) was used to determine the effect of endogenously expressed receptors. These cell lines express a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of NF-κB-inducible promoter. HEK-293 cells stably transfected with murine TLR4, MD-2 and CD14 (HEK-mTLR4) were purchased from InvivoGen. To study the combinational effect of PRR, the human monocytic cell line MM6 was used.[31] All HEK cell lines were grown in DMEM and the MM6 cell line was grown in IMDM. Both media were supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 300 ng/mL L-glutamine. In addition, media were supplemented with 1x HEK-Blue selection antibiotics and Normocin (HB-hTLR4 and HB-hTLR2), Zeocin and Normocin (HB-Null1), Blasticidin, HygroGold and Normocin (HEK-mTLR4) or 20 µM β-mercaptoethanol (MM6). All cells were cultivated at 37°C in a humidified atmosphere of 5% CO_2.

Generation and culture of moDC

For generation of moDC, peripheral blood from healthy donors was used. Donor blood was kindly provided by the internal blood donor system of the National Institute for Public Health and the Environment (RIVM) in the Netherlands. This study was conducted according to the principles expressed in the Declaration of Helsinki. All donors provided written informed consent for the collection of samples and subsequent analysis. The blood samples were processed anonymously. Peripheral blood mononuclear cells were isolated by density centrifugation on Lymphoprep (Nycomed) at 1000xg for 30 minutes. Cells were washed, harvested, and resuspended in PBS supplemented with 0.5% BSA and 2 mM EDTA. The cells were incubated

with anti-$CD14^+$ microbeads and $CD14^+$ monocytes were isolated by magnetic sorting using MACS columns (Miltenyi Biotech). $CD14^+$ positive cells were then cultured in 24-wells plates at 4×10^5 cells/mL in IMDM supplemented with penicillin (100 U/mL), streptomycin (100 µg/mL), L-glutamine (300 ng/mL), 1% heat-inactivated FCS, human GM-CSF (500 U/mL), and human IL-4 (800 U/mL) for a total of 6 days.

Stimulation of cell lines and moDC with wP vaccines and use of hTLR2 and hTLR4 blocking agents

HB-hTLR2, HB-hTLR4 and HB-Null1 cells were seeded in 96-well plates at 50000, 25000 and 50000 cells/well (96 well), respectively, and cultivated overnight. The HB-hTLR4, HB-hTLR2 and HB-Null1 cells were subsequently stimulated overnight with wP vaccines A_{ref} - E at the indicated OD_{590nm}. The activation of these cell lines was measured by mixing 20 µL of cell supernatant with 180 µL of QUANTI-Blue substrate™ followed by incubation at 37°C for 2h. The absorption at 649nm was measured using a microplate reader (Bio-Tek). MM6 cells were plated at 1.5×10^5 cells/well (96-well plates), just prior to addition of wP vaccine A_{ref} - E at indicated OD_{590nm} or the control stimulants described below. Activation of MM6 cells was assessed by measuring IL-6 and IL-12p40 secretion in culture supernatant using ELISA and the absorbance was measured at 450nm. As controls, HB-hTLR4, HB-hTLR2 and HB-Null1 and MM6 cells were stimulated with LPS-EC or PAM at indicated concentrations. Blocking of TLR4, TLR2 or both on HB-hTLR4 or MM6 cells was performed by incubating cells with LPS-RS (1 µg/mL), α-TLR2 antibody (0.5 µg/mL) or α-TLR2 antibody (0.5 µg/mL) and LPS-RS (1 µg/mL) for 3h. Subsequently, both cell lines were exposed to wP vaccines and control stimulants overnight and activation was measured by IL-6 and IL-12p40 secretion in the supernatant. In order to block TLR4 or TLR2 signalling or both on moDC, these cells were pre-incubated with LPS-RS (1 µg/mL), or α-TLR2 (0.5 µg/mL), or both TLR2 and TLR4 antagonists for 3h. Subsequently, the moDC were stimulated with wP vaccines A_{ref}, C, E, LPS or PAM at the indicated concentration for two days. After stimulation, the presence of IL-12p40 in the supernatants of these cells was then measured using ELISA.

Mass Spectrometry (MS) analysis of LPS

The LPS from 250 µl of vaccine preparations of *B. pertussis* were extracted with hot phenol/water as described elsewhere.[32] LPS was purified further for mass spectrometry by using ZipTipC4 micropipette tips (Merck Millipore Ltd, Tullagreen, Carrigtwohill, Co. Cork, Ireland). Electrospray ionization mass spectrometry (ESI-MS) was performed on an LCQ Classic quadrupole ion trap mass spectrometer (Finnigan, San Jose, CA) in the negative-ion mode. Typically, from 5 to 10 µl of LPS in 50% (v/v) 2-propanol, 0.07 mM triethylammonium acetate pH 8.5 were infused into the mass spectrometer by static nanoelectrospray using gold-coated, pulled glass capillaries.[33,34] The spray voltage was set to -2 kV and the capillary temperature to 200 ˚C. Under these ionization conditions, no appreciable fragmentation of LPS was produced.

Composition proposals for LPS molecular ions were based on the chemical structure of the LPS from *B. pertussis* reported previously.[35]

Microarray analysis

mRNA expression profiles of the *B. pertussis* bacteria harvested at different time points after sulfate addition were analysed using full genome *B. pertussis* DNA-microarrays according to the procedure described in detail elsewhere (Metz *et al.* submitted for publication). The data processing steps were done with the free statistical software R (http://www.r-project.org, R Foundation for Statistical Computing, Vienna, Austria), using an in-house developed script.[12] P-values for expression changes at any of the time points were calculated using a one-way ANOVA statistical analysis. The resulting p-values were then adjusted for multiple testing by calculating the false discovery rate (FDR). Maximal fold ratio (FR) values were expressed as the maximal/minimal normalized signal value between any of the time points. A p-value of 0.01 (FDR of 10%) was used to select genes whose gene-expression showed a statistically significant difference. To further select for biologically relevant effects, only statistically significant genes with a maximal FR above 1.25 were included in the final analysis.

Gas chromatography of fatty acids in wP vaccines

The amount of fatty acids and the fatty acid composition in lipids within the wP vaccines was analysed using a modified gas chromatography method as described elsewhere.[36] The fatty acid methyl-esters were analysed based on their retention times compared to retentions times of the commercial standards. For quantification of hydroxy-fatty acids C14:0-3OH and C12:0-2OH were used as calibration standard and internal standard, respectively. For non-hydroxy-fatty acids C14:0 was used as standard and C15:0 was employed as internal standard.

Statistical analysis

Data are presented as the mean ± the standard deviation of three independent determinations, unless mentioned otherwise. Unless mentioned otherwise, significant differences were analysed between the reference vaccine (A_{ref}) and the other vaccines (B - E) using a Student's t-test and considered significant when $p<0.05$.

Results

Modulation of the BvgASR system affects protein composition, but not LPS quantity of wP vaccines

The relative amounts of six important virulence proteins in vaccine A_{ref} - E, harvested 0 – 24 hours after the addition of sulfate, were determined using monoclonal antibodies to verify if the sulfate-induced suppression of the BvgASR system during the production process had resulted in differences in wP vaccine protein composition and thus quality (Figure 1A). PRN, FIM3 and Vag8 proteins were readily detectable in reference vaccine A_{ref}, whereas FIM2 levels were clearly lower. In contrast, FHA and PT protein levels were at the lower limit of detection in all runs of each vaccine tested. Importantly, PRN, FIM3 and Vag8 protein levels decreased steadily over time and this decrease proved significant starting two (PRN), six (FIM3) and twelve hours (Vag8) after the addition of sulfate. Similarly, FIM2 protein levels were also significantly decreased six hours after the addition of sulfate. The levels of FIM2 and PRN were minimal and close to zero after 24 hours. These data clearly confirm that sulfate-mediated suppression of virulence during *B. pertussis* cultivation had resulted in the production of experimental wP vaccines of different protein compositions. In general, the levels of virulence proteins present in the wP vaccines coincided with the gradually decreasing potencies of these vaccines as determined in the *in vivo* Kendrick test (potency of vaccine A_{ref} was 7.0 IU/mL (95% interval 2 IU/mL and 27 IU/mL), the potency of vaccine C was 4.8 IU/mL (95% interval 2 IU and 10 IU) and the potency of vaccine E was 0.8 IU/mL (95% interval 0 IU/mL and 3 IU/mL) (Metz *et al*., submitted for publication)). According to the acceptance criteria of the European Pharmacopoeia which specify a potency of at least 4 IU/mL and 95% interval with a lower limit of 2 IU/mL, the potencies of vaccine A and C were sufficient, whereas the potency of vaccine E was insufficient. Therefore, based on assessment of protein content and *in vivo* potency testing, we considered the qualities of vaccines A_{ref} - E to range from good to poor.

The amount of LPS, another virulence factor of *B. pertussis*[15,17], is not known to be regulated by the BvgASR system. In line with this, LPS was detected in all vaccines using an antibody specific for *B. pertussis* band A LPS and no significant differences were observed between vaccines A_{ref} - E regarding their LPS content (Figure 1B). In addition, quantification of LPS in vaccine A_{ref} - E by gas chromatography of LPS-specific fatty acids (non-hydroxy and hydroxy) revealed that there were no pronounced differences among the vaccines (Figure 1C). These results demonstrate that sulfate-mediated modulation of the BvgASR system did not affect the quantity of LPS in these wP vaccines.

Since formaldehyde treatment of the vaccines could have affected epitope recognition by the antibodies used in the ELISA, its effect on antibody recognition of virulence proteins was

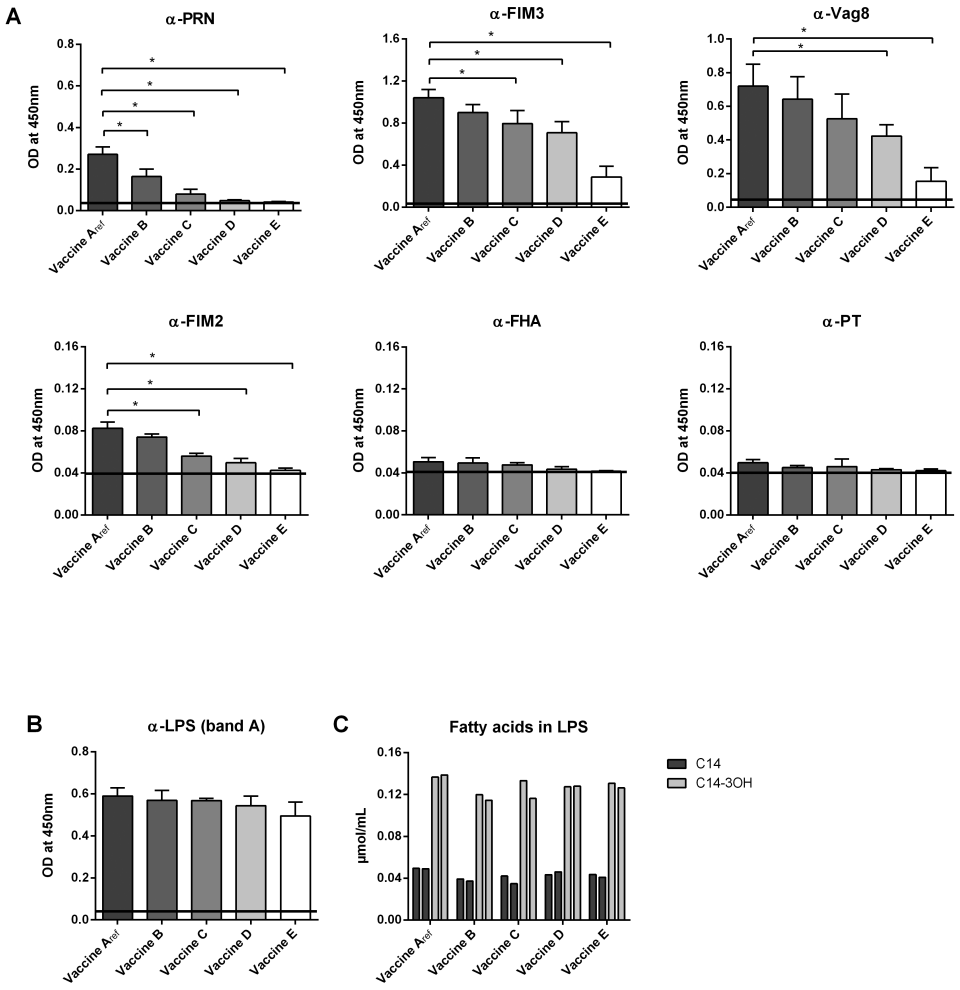

Figure 1 Bvg status of *B. pertussis* bacteria at time of harvest affects protein composition of the resulting wP vaccines. Amounts of proteins (A) and LPS (B) present in wP vaccines A_{ref}, B, C, D, E (harvested 0, 2, 6, 12, 24h after the addition of sulfate, respectively) derived from three individual *B. pertussis* culture runs were measured by ELISA using specific monoclonal antibodies directed against individual proteins or LPS. (C) Fatty acid composition of vaccines A_{ref}, B, C, D, and E analysed using a modified gas chromatography method (in duplicate). The black lines indicate the background levels measured in PBS only by ELISA. * = $p < 0.05$.

evaluated using *B. pertussis* strain 509 treated with or without formaldehyde (Figure S1). Formaldehyde treatment had no effect on epitope recognition by FIM3- and Vag8-specific antibodies, while the detected levels of FIM2, FHA and PT were slightly lower. Surprisingly, detected PRN levels were higher after formaldehyde treatment. These results show that although formaldehyde treatment can affect the availability of epitopes for some of the antibodies, it does not seem to be responsible for the absence of an FHA- and PT-specific signal in vaccines A_{ref} - E (Figure 1A). It is therefore likely that both proteins are absent or present at very low concentrations in the vaccine preparations rather than not detected.

Quality of wP vaccines affects human TLR4 but not TLR2 signalling

It is well established that *B. pertussis* can activate TLR4 and TLR2. However, while there is no evidence that *B. pertussis* can actively modulate TLR2 activation, it has been reported that *B. pertussis* is able to modify the structure of its LPS in a BvgASR dependent manner, thereby influencing host TLR4 signalling.[26,27] Therefore, the capacity of wP vaccines A_{ref} - E to activate human TLR4 (hTLR4) and TLR2 (hTLR2) was tested using HB-hTLR4 and HB-hTLR2 reporter cell lines. The vaccines induced a dose dependent production of SEAP through both hTLR4 and hTLR2 (Figure 2A). Responses were hTLR4 and hTLR2 specific since SEAP activity was not induced in the HB-Null1 control cells (Figure 2B). Importantly, vaccine E induced a consistently lower hTLR4 response than vaccines A_{ref}, B, C and D for all shown dilutions (Figure 2A). The difference between vaccine A_{ref} and E proved significant for each of the four vaccine concentrations shown in three independent experiments (Figure 2C and Figure S2A). Significant differences in hTLR4 responses were not observed when the cells were stimulated with wP vaccines at higher or lower ODs. In contrast, no consistent differences in hTLR2 activation were found between vaccines A_{ref} - E (Figure 2A, C), although there was some variation at indicated ODs (Figure S2B). Together, these results demonstrate that the Bvg status of the bacteria at the time of harvest influenced the capacity of the vaccines to induce hTLR4 signalling, while leaving hTLR2 signalling capacity largely unaffected.

wP vaccines of varying quality differ in their capacity to activate APC, primarily in a human TLR4-dependent manner

In order to investigate if wP vaccines A_{ref} - E also differed in their capabilities to activate innate immune cells expressing several different TLRs, vaccine-induced activation of MM6 cells and moDC was studied, as well as the contribution of hTLR2 and hTLR4 signalling to the activation of these cell types. MM6 is a human monocytic cell line that expresses both TLR2 and TLR4[38,39] and responded to purified agonists for these receptors as well as our wP vaccines (Figure 3A).[28] Vaccine E induced consistently lower IL-6 and IL-12p40 secretion by MM6 cells for all indicated ODs compared with vaccine A_{ref} (Figure 3A), while vaccines B, C and D induced secretion of intermediate amounts of these cytokines. Importantly, these differences between vaccine

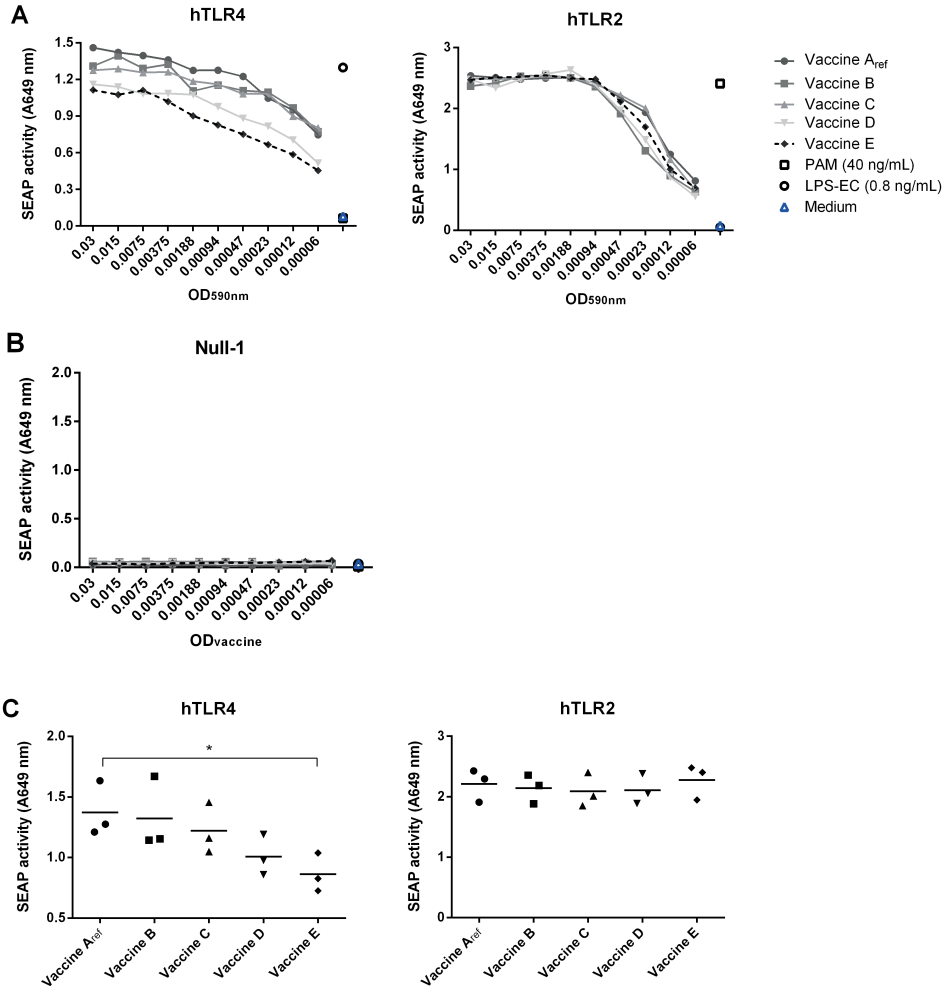

Figure 2 Activation of hTLR4- and hTLR2-mediated signalling by wP vaccines A_{ref} - E. HB-hTLR2, HB-hTLR4 and HB-Null-1 cells were stimulated overnight with wP vaccines A_{ref}, B, C, D, E, LPS-EC (0.8 ng/mL) or PAM (40 ng/mL). Shown is the SEAP activity in supernatants of HB-hTLR2, HB-hTLR4 (A) and HB-Null-1 (B) cells in response to 2-fold serial dilutions of the vaccines (representative responses are shown from one out of three independent experiments). (C) Shown is the SEAP activity of HB-hTLR2 and HB-hTLR4 cells in response to vaccines A_{ref}, B, C, D and E at an OD_{590nm} of 0.00094. Each dot represents one value of three individually performed cell culture experiments. * = $p < 0.05$.

A_{ref} and E were significant at indicated ODs in three independent experiments (Figure 3B and Figure S2C, D). This indicates that also activation of MM6 cells by wP vaccines was affected by the bacterial Bvg status at the time of harvest during vaccine production.

Since the vaccines derived from *B. pertussis* bacteria harvested after the addition of sulfate (vaccine B-E) displayed a clear trend towards lower hTLR4 activation than our reference vaccine derived from this bacterial culture before sulfate was added (vaccine A_{ref}), we wanted to gain more insight into the relative contribution of hTLR4- and hTLR2-mediated signalling to overall vaccine-induced activation of APC. To study this, MM6 cells were pre-incubated with a constant concentration of the TLR4 antagonist LPS-RS, a TLR2 blocking antibody or

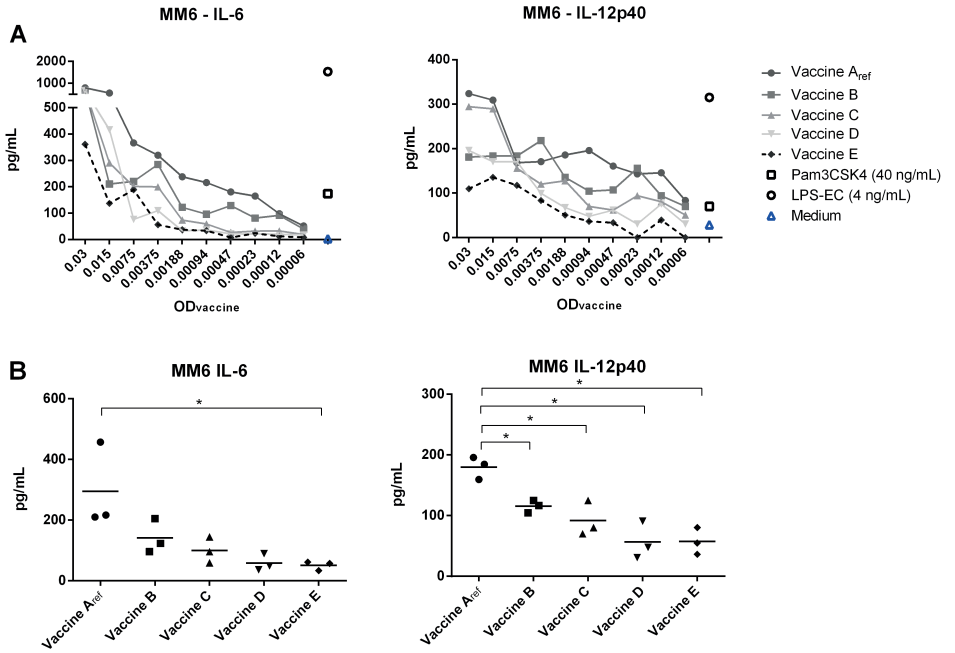

Figure 3 Secretion of IL-6 and IL-12p40 by MM6 cells stimulated with wP vaccines A_{ref} - E. MM6 cells were stimulated overnight with vaccines A_{ref}, B, C, D, E, LPS-EC (4 ng/mL) or PAM (40 ng/mL). Subsequently, IL-6 and IL-12p40 secretion was measured in culture supernatants. (A) Shown is the response of MM6 cells to 2-fold serial dilutions of vaccine A_{ref}, B, C, D and E (representative responses are shown from one out of three independent experiments). (B) Response of MM6 cells to vaccines A_{ref}, B, C, D and E at an OD_{590nm} of 0.00094. Each dot represents one value of three individually performed cell culture experiments. * = $p < 0.05$.

both. Blocking of hTLR2 on MM6 cells significantly decreased the IL-6 and IL-12p40 secretion in response to vaccine C and E (Figure 4A). However, hTLR2 blocking had a minor effect on secretion responses to vaccine A_{ref}, while hTLR4 blocking significantly decreased the MM6 cell response to all wP vaccines tested. When both hTLR2 and hTLR4 were blocked, the MM6 cell response to the wP vaccines became marginal. These data suggest that hTLR4-mediated signalling was the main contributor to MM6 activation by vaccine A_{ref} with no or a limited role for hTLR2. The relative contribution of hTLR4 to activation of these cells by vaccines of lower quality (C and E) deceased gradually, while the relative contribution of hTLR2 increased and was significant for vaccine C and E.

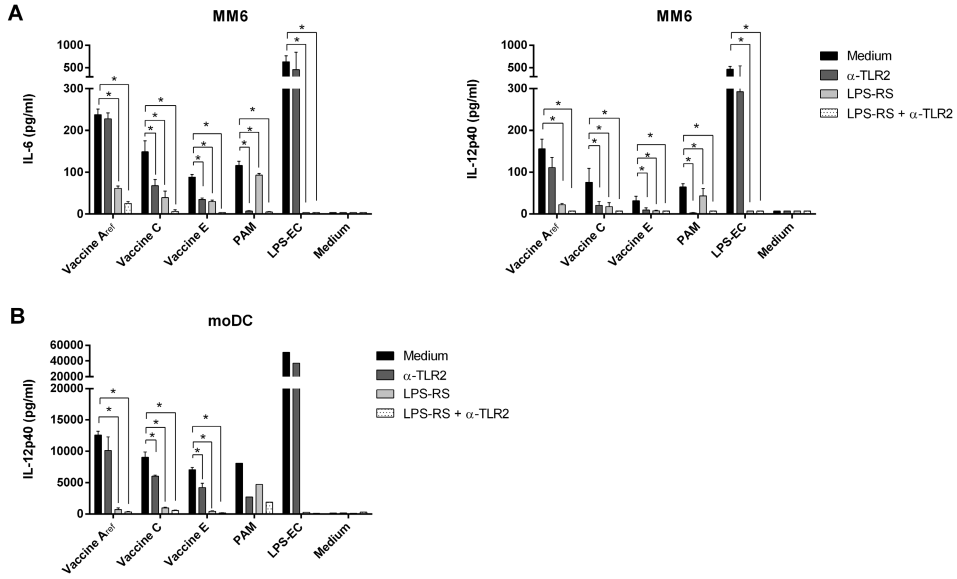

Figure 4 Activation of MM6 cells and moDC by wP vaccines is primarily mediated by hTLR4 signalling. MM6 cells and moDC were pre-treated for 3h with the TLR4 antagonist LPS-RS (1 µg/mL), a blocking antibody against human TLR2 (0.5 µg/mL) or a combination of both. Subsequently, MM6 cells were stimulated with wP vaccine A_{ref}, C, E (OD_{590nm} of 0.00094), PAM (100 ng/mL), LPS-EC (4 ng/mL) or medium overnight. Similarly, moDC were stimulated for 2 days with vaccine A_{ref}, C and E (OD_{590nm} of 0.00047), LPS-EC (100 ng/mL), PAM (1 µg/mL) or only medium. (A) Secretion of IL-6 and IL-12p40 by MM6 cells measured in culture supernatants (one experiment out of two experiments with similar results is shown). (B) Secretion of IL-12p40 by moDC measured in culture supernatants (one experiment out of two experiments with similar results is shown). * = $p < 0.05$.

Since dendritic cells (DC) are professional APC that direct adaptive immune responses, the response of moDC to vaccines A_{ref}, C, and E was investigated next. MoDC were pre-incubated with LPS-RS, α-TLR2 blocking antibody or both antagonists and stimulated with LPS, PAM and the wP vaccines A_{ref}, C and E. Within the performed experiments, the α-TLR2 antibody largely, but not completely inhibited the response to the TLR2 ligand PAM, most likely caused by incomplete inhibition of all hTLR2 receptors (Figure 4B). In a similar way as observed with MM6 cells, blocking of hTLR2 on moDC significantly decreased IL-12p40 secretion in response to vaccine C and E, but not to vaccine A_{ref}, while hTLR4 blocking significantly reduced the response to all wP vaccines tested. Simultaneous blockade of hTLR2 and hTLR4 in both wP stimulated MM6 and moDC resulted in a response close to the lower limit of detection of the used ELISAs, suggesting only TLR2 and TLR4 ligands rather than ligands for other PRR play a role in wP activation of APC. In general, the relative contribution of hTLR4 signalling to the activation of both cell types was higher compared with hTLR2 signalling. In addition, this effect was clearly more pronounced when vaccine A_{ref} was used as a stimulant and could explain the differences in APC activation induced by these wP vaccines of varying quality.

wP vaccines produced under Bvg-modulating conditions contain structurally different LPS molecules

Since the different hTLR4 signalling capacities of vaccines A_{ref} - E cannot be explained by differences in LPS quantity, we hypothesised that this might be the result of variations in the structure of the LPS molecules present in vaccines A_{ref} - E. To investigate this, LPS from the vaccine preparations was isolated and analysed by negative-ion ESI-MS. Mass spectrometry analysis showed that all vaccines mainly contained penta-acylated LPS carrying a branched dodecasaccharide chain (m/z 1351, Figure 5) of the same composition as that reported previously for the main species (band A LPS) of LPS from *B. pertussis*.[35] Similarly, minor LPS species were present in all vaccine preparations corresponding to tetra-acylated LPS lacking a 3-hydroxy-tetradecanoic acid (m/z 1276) or 3-hydroxy-decanoic acid (m/z 1294) as well as tetra-acylated and penta-acylated LPS species that lost a heptose (m/z 1212 and 1287, respectively), a phosphoethanolamine (m/z 1235 and 1310, respectively) or a pyrophosphoethanolamine group (m/z 1208 and 1283, respectively) from the dodecasaccharide core (Figure 5). However, when comparing the LPS spectra derived from vaccines A_{ref} - E, the peak corresponding to LPS carrying a GlcN substitution of lipid A phosphate (m/z 1405) gradually decreased from vaccine A_{ref} toward vaccine E (Figure 5). Since the wP vaccines did not differ in LPS amount (Figure 1B, C), it is likely that the presence of the GlcN modification on the lipid A of the LPS molecules in vaccine A_{ref} and the gradual decrease of this modification on the lipid A from vaccines B - E is responsible for the observed different capacities of the vaccines to induce hTLR4 signalling and APC activation.

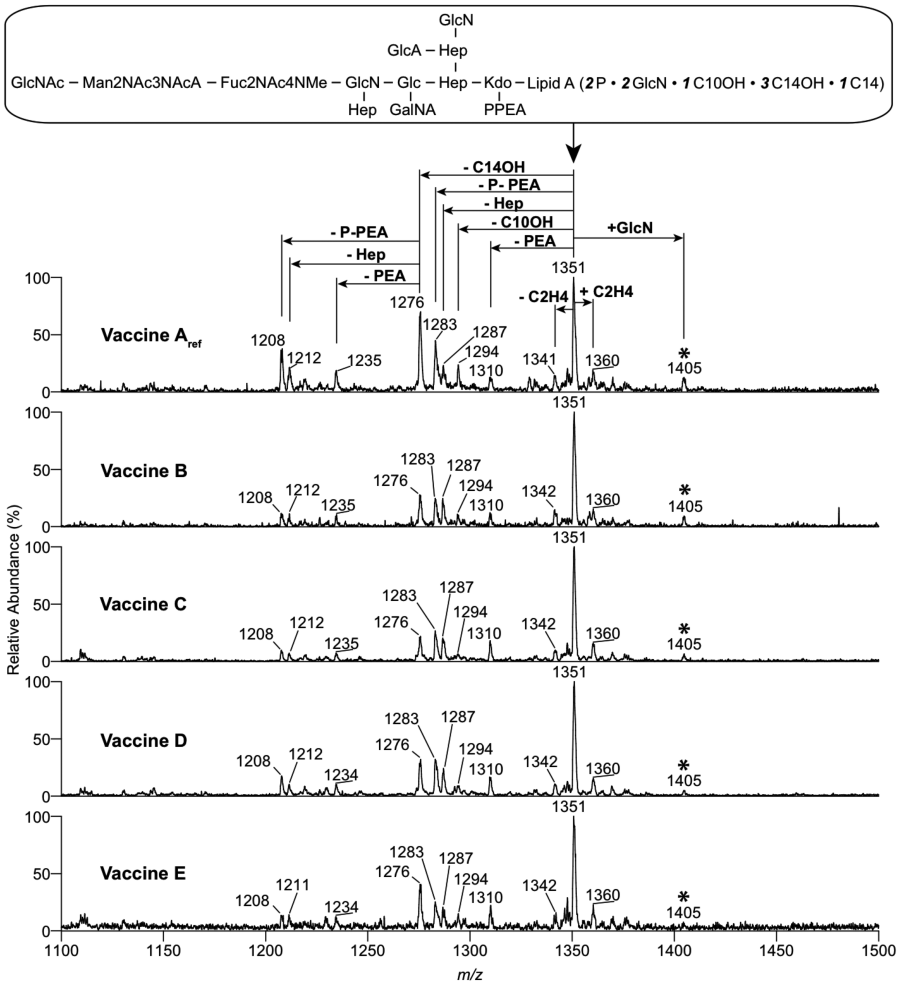

Figure 5 Negative-ion ESI mass spectra of LPS isolated from wP vaccine preparations A_{ref} - E. The triply charged (M-3H)3- molecular ion regions of the mass spectra of LPS from vaccine preparations A_{ref}, B, C, D and E are shown. The box on top of the mass spectra contains a simplified representation of the chemical structure of the LPS from *B. pertussis* reported previously.[35] This structure has been assigned to the ion of m/z 1351. The ions highlighted by an asterisk correspond to LPS with a glucosamine substitution of the lipid A phosphate. Abbreviations: Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; Glc, glucose; GlcN, glucosamine; GlcNAc, N-acetyl glucosamine; Fuc2NAc4NMe, 2-acetamido-4-N-methyl-2,4,6-deoxy-galactose; GalNA, galactosaminuronic acid; GlcA, glucuronic acid; Man2NAc3NAcA, 2-acetamido-3-acetamido-2,3-dideoxy-mannuronic acid; PPEA, pyrophosphoethanolamine; P, phosphate; C14OH, 3-hydroxy-tetradecanoic acid; C14, tetradecanoic acid; C10OH, 3-hydroxy-decanoic acid.

Previously, it has been shown that GlcN modification of *B. pertussis* lipid A specifically affects human TLR4 signalling, while this modification has no effect on murine TLR4 (mTLR4) signaling.[24] Therefore, the capacity of vaccines A_{ref}, C and E to bind and activate murine TLR4 was tested using a HEK-mTLR4 reporter cell line. All vaccines induced a dose dependent production of IL-8, but no consistent differences between the mTLR4 responses induced by vaccine A_{ref}, C and E were observed (Figure S3). Additional evidence for the presence of GlcN modifications comes from an experiment in which the vaccines were incubated with the antibiotic polymyxin B. GlcN modification of LPS is a known mechanism used by Bordetella bacteria to confer resistance to neutralisation with the antibiotic polymyxin B.[40] Incubating the wP vaccines with this antibiotic before stimulating the MM6 cells, demonstrated that vaccine A_{ref} was resistant to polymyxin B neutralisation, while vaccine E was neutralised and no longer induced MM6 cell activation (Figure S4). Both the absence of a difference in mTLR4 activation and the association between the GlcN modification and the resistance to polymyxin B, provide further evidence that the variations in GlcN substitution of the LPS molecules present in these vaccines are a main determinant for the observed differences in activation of hTLR4-expressing cells.

Prolonged culturing of *B. pertussis* in the presence of sulfate is associated with differential expression of genes encoding LPS modifying enzymes

Because of the differences found in the LPS structures present in vaccines A_{ref} - E and the clear differential hTLR4 signalling capacities of these wP vaccines, we wondered whether differential expression of genes involved in LPS modification would form the basis for these findings. Therefore, the gene expression profiles of a panel of 35 *B. pertussis* genes, known to be associated with synthesis or modification of LPS were analysed. This data set was derived from a whole-genome microarray experiment performed with RNA samples isolated from a sample of the bacterial culture just before the production of vaccines A_{ref} – E (Metz *et al.*, submitted for publication). The expression profiles revealed that addition of sulfate did not alter the expression of most of these genes (Figure 6). However, the gene expression profiles revealed that sulfate addition induced significantly enhanced expression of four genes that are part of the wlb gene cluster (wlbA, wlbB, wlbC and wlbI). This locus is associated with the addition of a trisaccharide moiety to the core structure of *B. pertussis* LPS, producing an LPS form known as band A LPS.[41-43] To investigate if this had any effect on the ratio of band A and B LPS in vaccines A_{ref} - E, we determined the relative amounts of these LPS species using band A or band B specific antibodies (Figure S6). Interestingly, band B LPS proved to be nearly undetectable in all vaccines, whereas clear differences in band A LPS amounts among the vaccines were not observed. As significant changes in the presence of the band A-specific trisaccharide were also not found by MS analysis of the LPS molecules present in vaccines A_{ref} - E (Figure 4), we cannot present any evidence for structural alterations of the LPS in the different vaccines, caused by sulfate-induced changes in the expression of the four wlb cluster genes.

t = -24	t = 0	t = 1	t = 2	t = 6	t = 10	t = 24	ORF	Gene	Regulated	Reference	Function
1.01	1.00	0.96	0.98	1.22	0.97	1.02	BP1431	lpxA		(52)	
1.06	1.00	1.11	1.25	1.42	1.13	1.16	BP1429	lpxD			
1.08	1.00	1.09	1.18	1.20	1.17	1.23	BP1432	lpxB			
0.98	1.00	1.21	1.21	1.37	1.18	1.33	BP1905	lpxH			Lipid A
1.16	1.00	1.10	1.18	1.18	1.29	1.09	BP3017	lpxC			
1.02	1.00	1.10	1.13	1.07	1.14	0.98	BP2766	lpxK		(53)	
0.92	1.00	1.08	1.03	1.12	1.18	1.01	BP3072	lpxL1		(20)	
1.01	1.00	1.20	1.09	1.20	1.22	1.07	BP3073	lpxL2		(20)	
0.96	1.00	0.83	0.97	1.09	1.01	1.00	BP0094	waaC			
1.22	1.00	1.06	0.91	1.36	1.28	1.03	BP0095	waaA		(54)	Inner core
1.02	1.00	1.12	1.08	1.40	1.13	1.27	BP2322	waaF		(55)	
0.93	1.00	1.05	1.08	1.09	1.00	1.10	BP2349	kdkA		(56, 57)	
1.12	1.00	1.03	1.19	0.97	0.97	0.90	BP0835	lpxE			
1.18	1.00	0.44	0.39	0.34	0.35	0.41	BP0399	lgmA	yes	(22)	GlcN substitution of lipid A
0.89	1.00	0.55	0.66	0.55	0.51	0.57	BP0398	lgmB	yes	(22)	
0.88	1.00	0.66	0.55	0.55	0.58	0.70	BP0397	lgmC	yes	(21)	
1.25	1.00	1.07	1.10	1.06	1.18	0.96	BP0396	lgmD		(21)	
1.00	1.00	1.16	1.21	1.04	1.08	1.02	BP2328			(58)	
1.01	1.00	1.19	1.20	1.08	1.11	1.02	BP2329			(58)	Inner core extension
1.12	1.00	1.02	1.01	1.22	1.27	1.04	BP2330			(58)	
1.10	1.00	1.14	1.30	1.22	1.20	1.15	BP2331			(58)	
0.90	1.00	0.96	0.98	0.97	0.92	0.99	BP0082	wlbL		(34)	
0.89	1.00	1.14	1.17	0.92	1.06	0.95	BP0083	wlbJ		(34)	
0.90	1.00	0.91	0.93	0.93	1.21	1.18	BP0085	wlbI	yes	(34)	
1.12	1.00	1.04	1.09	1.34	1.38	1.37	BP0086	wlbH		(34)	
1.14	1.00	1.10	1.01	1.20	1.23	1.08	BP0087	wlbG		(34)	
1.01	1.00	1.18	1.14	1.12	1.18	1.24	BP0088	wlbF		(34)	Trisaccharide
1.14	1.00	1.16	1.13	1.19	1.53	1.38	BP0089	wlbE		(34)	
0.94	1.00	0.94	0.98	0.96	1.07	1.18	BP0090	wlbD		(34)	
0.89	1.00	1.28	1.15	1.50	1.67	1.73	BP0091	wlbC	yes	(34)	
0.93	1.00	1.14	1.28	1.38	1.72	1.51	BP0092	wlbB	yes	(34)	
0.92	1.00	1.67	1.48	2.32	2.30	2.50	BP0093	wlbA	yes	(34)	
1.01	1.00	1.00	1.15	0.97	1.02	0.94	BP3329	imp			Membrane transport
1.06	1.00	0.99	0.94	0.95	0.96	1.05	BP2043	lptE			
0.90	1.00	1.03	0.93	1.10	0.98	0.94	BP2321	msbA			

Figure 6 Expression of genes associated with LPS synthesis and modification in *B. pertussis* in response to sulfate exposure. Relative gene expression of a panel of 35 genes associated with LPS synthesis or modification in *B. pertussis*.[25-27,41,44-50] Each row represents the relative transcript abundance of a single gene in *B. pertussis* bacteria harvested before and at different time points after sulfate addition to the growth medium. Column names (t=-24 – t=24) correspond to the time points after the addition of sulfate at which bacteria were harvested (0 = vaccine A_{ref}, 2h = vaccine B, 6h = vaccine C, 12h = vaccine D, 24h = E). The colour scale indicates gene regulation ranging from strong downregulation (dark green), to no regulation (yellow), and strong upregulation (dark red). Genes for which the expression changed significantly throughout the production process are marked with an asterisk ($p < 0.05$).

Most importantly, the expression of the *lgmA*, *lgmB* and *lgmC* genes, known to be responsible for GlcN modification of lipid A in *B. pertussis*[12], was significantly reduced following growth in sulfate-containing medium. This strongly suggests that changes in the gene expression of these enzymes are responsible for the gradual decrease in GlcN modification of lipid A that was observed in vaccines B - E (Figure 5).

Discussion

Differences in the culture conditions used during wP vaccine production can be sensed by the BvgASR regulatory system of *Bordetella pertussis* [4,15] and can thereby influence the expression of proteins involved in virulence. Several studies have linked the quality of wP vaccines to the presence of these virulence-associated proteins.[16,17,51] Recently, we have shown that moDC and MM6 cells represent suitable platforms to measure differences in the innate immune activation capacities of experimental wP vaccines of varying quality (A_{ref} - E)[28], that were prepared by deliberate manipulation of the bacterial BvgASR system during the production process (Metz *et al.*, submitted for publication). In the present study, we showed that the differences in APC activation are largely caused by the distinct capacities of these vaccines to activate hTLR4. Additionally, we present evidence strongly suggesting that the observed differences in hTLR4 activation induced by our wP vaccines are directly linked to the amount of GlcN-modified LPS molecules present in these vaccines, which in turn is dependent on BvgASR-controlled expression of the *lgmA*, *lgmB* and *lgmC* genes. These findings demonstrate that wP vaccine quality is connected to their capacity to activate human TLR4 and suggest that this association could serve as a useful parameter for *in vitro* assessment of wP vaccine quality.

Sulfate-mediated repression of the BvgASR system is known to impair the expression of *B. pertussis* virulence factors[52] and this method was therefore used within this study to mimic potential problems that could occur during vaccine production, such as nutrient limitation at the end of bacterial cultivation[12]. This way, a panel of five experimental wP vaccines of varying quality (A_{ref} - E) was produced by culturing *B. pertussis* bacteria either in the absence or presence of sulfate for different time spans. The protein composition of these vaccines was analysed in detail using ELISA and mass spectrometry immediately after vaccine production and this clearly confirmed that suppression of BvgASR-controlled gene expression in *B. pertussis* had resulted in vaccine products of different protein contents. These differences were also reflected in the potency of these vaccines, as determined for vaccines A_{ref}, C and E in the intracerebral challenge test (Metz *et al.*, submitted for publication). Using vaccines A_{ref} - E, in this study the effect of culture condition-induced changes in Bvg phase on various vaccine characteristics was investigated. To verify previous results on the protein composition of these vaccines and to show that the vaccines were still qualitatively different, we quantified the amount of several virulence antigens in these products by ELISA. The analysed antigens are considered to be important for the induction of protective antibody and T cell responses (FHA, PRN, FIM2, FIM3, PT and Vag8).[53-56] Although the results in this study confirmed the earlier data, showing that wP vaccines A_{ref} - E differ in antigen content, there are some slight differences between the results in both studies. The ELISA analysis described in this study, detected relatively lower concentrations of FIM2 and FHA in the vaccines compared to those

measured previously by Metz *et al.*, using both ELISA and mass spectrometry. This discrepancy between both analyses might be due to the use of different antibodies or the inherently higher sensitivity of a method such as mass spectrometry. In addition, the structure and stability of FIM2 and FHA may have changed since the first ELISA quantification, as the first ELISA was performed on heat inactivated, but not formaldehyde treated bacteria (Metz *et al.*, submitted for publication), whereas the products analysed in the current ELISA were 1.5 years older and were both treated with formaldehyde and heat inactivated. Nevertheless, overall our data showed that wP vaccines (A_{ref} - E) contained decreasing amounts of the virulence proteins FIM2, FIM3, Vag8 and PRN (Figure 1A), similar as described in Metz *et al.*, thereby confirming the previous conclusion that vaccines (A_{ref} - E) are qualitatively different, ranging from good (A_{ref}) to poor (E).

Importantly, adaptive immune responses not only depend on a vaccine's antigen composition, but also on the vaccines capacity to induce activation of APC, since these cells initiate and direct the adaptive immune response.[19] Activation of APC depends on the recognition of pathogen-associated molecular patterns by PRR. It is well known that wP vaccines induce APC activation through TLR2 and TLR4[20] leading to NF-κB dependent secretion of cytokines. The contribution of TLR4 and TLR2 to wP vaccine-induced responses has been demonstrated *in vivo* in mice, where TLR4 contributed to the early innate immune response as well as to subsequent antibody and T cell responses, while TLR2 did not.[22] In our *in vitro* assays, we observed that the modulation of the Bvg system by sulfate had resulted in a gradual decrease in MM6 and HB-hTLR4 activation capacities when comparing responses to vaccines A_{ref} - E (Figure 2 and 3). In addition, the differences in activation of MM6 cells and moDC between the vaccines mainly disappeared after blocking of TLR4 on the surface of these cells (Figure 4). In contrast, we found no indication that the hTLR2-activating capacities of vaccines A_{ref} - E had been affected by sulfate addition during vaccine production (Figure 2), although activation of hTLR2 contributed to the innate immune cell response induced by wP vaccines C and E, but did not significant contribute to the response to vaccine A (Figure 4). A recent report identified several lipoproteins in *B. pertussis* and two of them were shown to be specific TLR2 ligands.[21] Thus far, there is no evidence for Bvg-dependent regulation of lipoprotein expression in *B. pertussis*, which is in line with our TLR2-activation results. Together, these results indicate that TLR4-mediated signalling was primarily responsible for the observed differences in responses of MM6 cells and moDC to vaccines A_{ref} - E, whereas activation of TLR2 did not substantially contribute to these differences.

The major role of TLR4 signalling in MM6 cells and moDC activation by our wP vaccines was not unexpected, since mTLR4 is known to be involved in immune responses and protection against *B. pertussis* in mice[22,23] and LPS of *B. pertussis* is known to induce activation of hTLR4

in vitro.[57] However, the observed differences in hTLR4 activation capacities of vaccines A_{ref} - E were somewhat surprising, as no variation in LPS quantity between these vaccines was detected (Figure 1B, C). This implied that not LPS quantity, but LPS structure could be the reason for the different hTLR4-activating abilities of vaccine A_{ref} - E. Several studies have shown that *B. pertussis* can modify its lipid A by substituting the phosphate groups with GlcN in a BvgASR-controlled manner, resulting in enhanced hTLR4 activation.[24] In line with these studies, we demonstrated that LPS molecules derived from our good quality reference vaccine (A_{ref}), produced from Bvg^+ phase bacteria, were substituted with GlcN to the highest degree, while the LPS molecules isolated from vaccines B - E, produced from bacteria cultured in the presence of sulfate, displayed a gradual decrease in GlcN substitution (Figure 5). In contrast to previous studies, these changes in GlcN modifications were not the result of an introduced mutation in the bvgS or *lgmB* genes[24,27], but induced by culturing *B. pertussis* in the presence of sulfate. We confirmed that sulfate induced changes in the expression of *lgmA*, *lgmB* and *lgmC* genes (Figure 6), known to be responsible for GlcN modification of lipid A in *B. pertussis*[26]. Though the LPS molecules substituted with GlcN represented a minor LPS species, this was the only species for which the amount consistently corresponded with the culture time in the presence of sulfate. Moreover, Shah *et al.* showed that GlcN-modification of LPS had a very strong effect on hTLR4 activation.[24] Since LPS quantity did not differ between our wP vaccines, it is likely that Bvg^+ phase-dependent GlcN modification was responsible for the differences in hTLR4 signalling and APC activation induced by these vaccines, although we cannot completely exclude that other minor LPS species played a role in this as well.

Another remarkable characteristic of GlcN-modified LPS is that it confers resistance to neutralisation by the antibiotic polymyxin B, as demonstrated for *B. pertussis*[40] and *B. bronchiseptica* LPS.[58] Similar LPS modifications have been found in *E. coli* and *S. typhimurium*.[59] Interestingly, activation of MM6 cells by vaccine A_{ref}, proved to be unaffected or even enhanced by polymyxin B addition, whereas vaccine E, derived from bacteria cultured in the presence of sulfate for 24 hours, was neutralised by polymyxin B and no longer induced hTLR4 signalling (Figure S4). This observation suggests that polymyxin B specifically bound to LPS molecules without GlcN modification, within vaccine E leading to reduced activation of hTLR4, while it might have promoted the possibility of minor GlcN modified LPS species in vaccine A_{ref} to activate hTLR4. These data provide indirect evidence for the presence of GlcN-modified lipid A in wP vaccines A_{ref} and its absence in vaccine E.

The wlb gene cluster is responsible for the addition of a trisaccharide moiety to the core structure of *B. pertussis* LPS, thereby allowing the formation of band A LPS. In contrast, band B LPS does not carry a trisaccharide on its core structure[41,43]. The expression of the wlb cluster has been shown to influence the colonization of *B. pertussis* in the lungs and trachea of mice[42].

Surprisingly, the expression of four of the genes of the wlb cluster, wlbA, wlbB, wlbC and wlbH, was significantly upregulated by bacterial growth in the presence of sulfate. Nevertheless, this did not alter the relative amounts of band A or B LPS in vaccines A_{ref} - E (Figure S5). This apparent discrepancy may be attributed to the relatively high amount of band A LPS already present in wP vaccine A_{ref}. In addition, it may be possible that the trisaccharide moiety is synthesized in excess, but finally not attached to the LPS molecules.

A recent study demonstrated that the *lgmA*, *lgmB* and *lgmC* genes encode the enzymes that are required for the modification of *B. pertussis* lipid A with GlcN, while the enzyme encoded by the *lgmD* gene in the same cluster proved not essential.[26] Here, we showed that the *lgmA*, *lgmB* and *lgmC* genes were the major *B. pertussis* genes involved in LPS synthesis or modification of which the expression was significantly reduced by the addition of sulfate (Figure 6). This confirms that the transcription of these genes is indeed controlled by the BvgASR system[27] and provides an explanation for the steadily lower amounts of GlcN-modified LPS that were detected in those vaccines that were produced from bacteria cultured in the presence of sulfate for prolonged periods of time. Changes in *lgmA* and *lgmB* expression have also been described after culturing *B. pertussis* in the presence of only 5 mM sulfate, suggesting that the expression of both genes can also shift in response to minor environmental changes.[54]

Our results demonstrate that suppression of the BvgASR system of *B. pertussis* bacteria in the process of wP production leads to a time-dependent gradual reduction in the amount of virulence proteins present in the resulting vaccines. However, this not only affects protein composition, but can also lead to specific structural alterations of the LPS molecules in the wP vaccines, leading to differences in hTLR4 signalling capacity. This study highlights the need for monitoring of the production process of whole-cell pertussis vaccines and provides examples of *in vitro* cell-based and physico-chemical tools, such as HB-hTLR4, MM6 and moDC cell systems, and mass spectrometry of LPS structure, that can be used for this purpose. Nevertheless, comprehensive validation experiments will be necessary to implement these tools. Furthermore, these data provide a scientific explanation for our recent proof-of-principle study showing that moDC and MM6 cells represent suitable platforms for *in vitro* monitoring of the consistency of the quality of wP vaccine production[28], thereby paving the road toward the development of suitable *in vitro* methods to assess the quality of these vaccines in the future.

Acknowledgements

We are grateful to Johan van der Gun (Bilthoven Biologicals), Cécile van Els, Rob Vandebriel, Arnoud Akkermans, Hendrik-Jan Hamstra (RIVM) and Gideon Kersten (Intravacc) for helpful advice during the study. We thank Bas van de Waterbeemd (Intravacc) for preparing the experimental wP vaccines, and Jeroen Pennings (RIVM) for advice on the statistical analyses.

Supplementary information

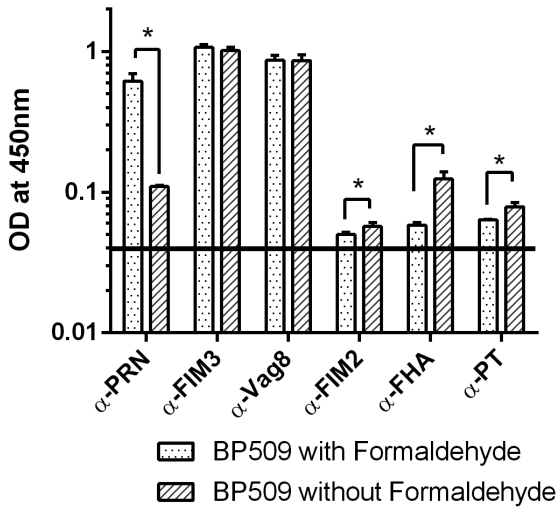

Figure S1 Effect of formaldehyde treatment on detection of *B. pertussis* proteins by monoclonal antibodies. Amounts of proteins present in whole bacteria of strain 509 either treated with formaldehyde (50 mM) or not, were measured in an ELISA, using specific monoclonal antibodies directed against individual proteins. * = $p < 0.05$.

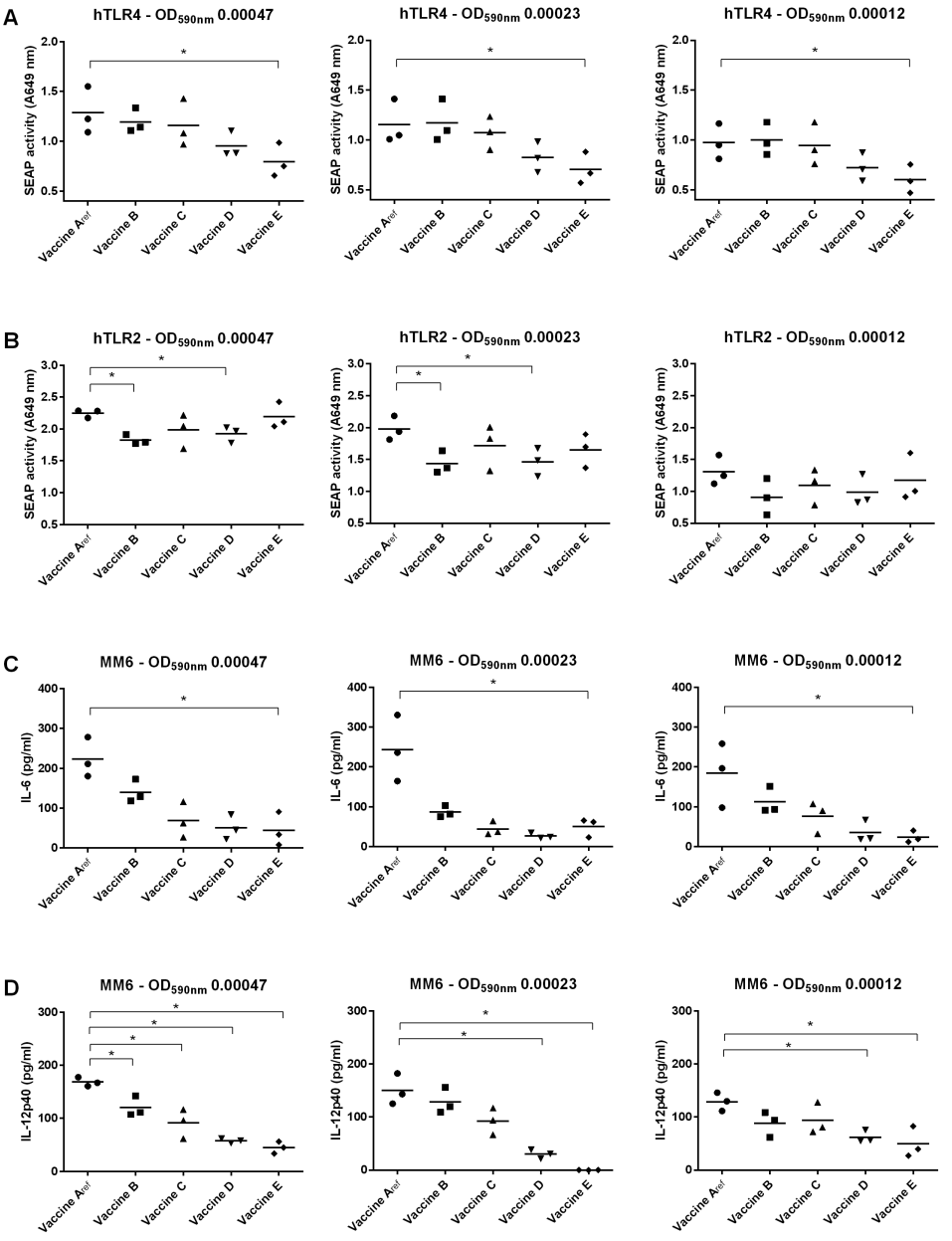
A
hTLR4 - OD590nm 0.00047
hTLR4 - OD590nm 0.00023
hTLR4 - OD590nm 0.00012
SEAP activity (A649 nm)
B
hTLR2 - OD590nm 0.00047
hTLR2 - OD590nm 0.00023
hTLR2 - OD590nm 0.00012
SEAP activity (A649 nm)
C
MM6 - OD590nm 0.00047
MM6 - OD590nm 0.00023
MM6 - OD590nm 0.00012
IL-6 (pg/ml)
D
MM6 - OD590nm 0.00047
MM6 - OD590nm 0.00023
MM6 - OD590nm 0.00012
IL-12p40 (pg/ml)
Vaccine Aref
Vaccine B
Vaccine C
Vaccine D
Vaccine E

Figure S2 Activation of hTLR4- and hTLR2-mediated signalling by wP vaccines A_{ref} - E at OD_{590nm} 0.00047-0.00012. HB-hTLR2, HB-hTLR4 and MM6 cells were stimulated overnight with wP vaccines A_{ref}, B, C. D, E at an OD_{590nm} of 0.00047, 0.00023, 0.00012. Vaccine-induced SEAP secretion (HB-hTLR2 and HB-hTLR4) or IL-6 and Il-12 secretion (MM6 cells) was measured in culture supernatants. Each dot represents one value of three individually performed cell culture experiments. * = $p < 0.05$.

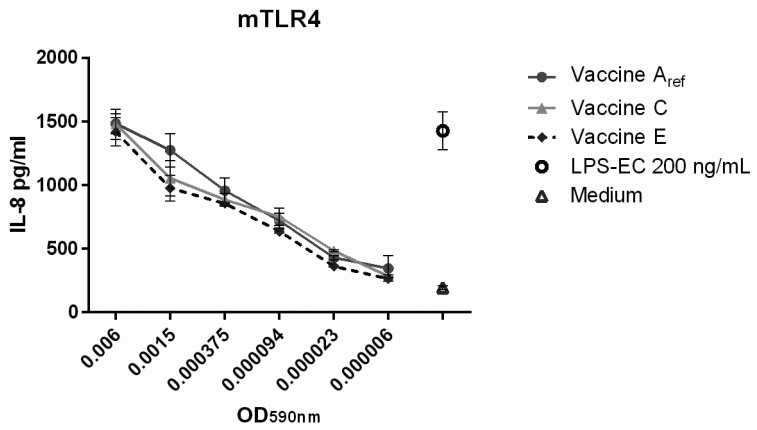

Figure S3 Activation of mTLR4 signalling by wP vaccines A_{ref}, C and E. HEK-mTLR4 cells were stimulated overnight with wP vaccines A_{ref}, C, E, or LPS-EC (200 ng/mL). NF-κB activity was measured by the secretion of IL-8 in the supernatants in response to 4-fold serial dilutions of the vaccines (representative responses are shown from one out of three independent experiments).

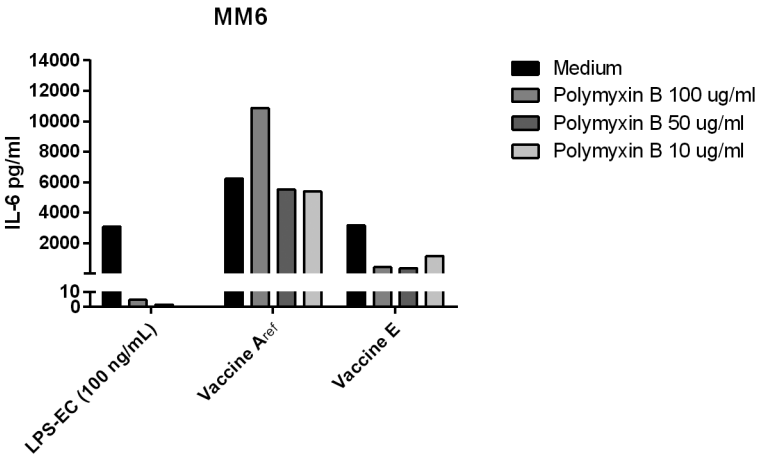

Figure S4 Neutralisation of *B. pertussis* LPS in vaccine A_{ref} and E by polymyxin B. Vaccines A_{ref} and E (OD_{590nm} 0.06) and LPS-EC (100 ng/mL) were pre-treated with varying concentrations of polymyxin B for 2h at 37°C. These solutions were used to stimulate the MM6 cells overnight. Activation of the MM6 cells was determined by assessing the amounts of IL-6 secreted into the supernatant using an ELISA (responses from one experiment out of two independent experiments with similar results are shown).

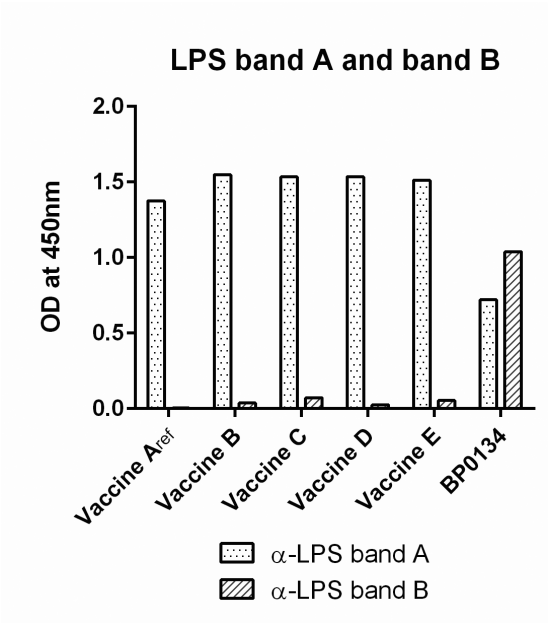

Figure S5 Effect of the BvgASR status of *B. pertussis* bacteria on band A and band B LPS within the wP vaccines. Amounts of band-A and band-B LPS in the pooled vaccine preparations A_{ref}, B, C, D, E measured by ELISA (OD_{590nm} 0.2), using specific monoclonal antibodies against band-A and band-B. Inactivated bacteria of *B. pertussis* strain 0134 (OD_{590nm} 0.28) served as positive controls.

References

1. Libster R, Edwards KM. Re-emergence of pertussis: what are the solutions? Expert review of vaccines. 2012;11(11):1331-46.
2. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other Bordetella subspecies. Clinical microbiology reviews. 2005;18(2):326-82.
3. Sheridan SL, Ware RS, Grimwood K, Lambert SB. Number and order of whole cell pertussis vaccines in infancy and disease protection. Jama. 2012;308(5):454-6.
4. Witt MA, Arias L, Katz PH, Truong ET, Witt DJ. Reduced risk of pertussis among persons ever vaccinated with whole cell pertussis vaccine compared to recipients of acellular pertussis vaccines in a large US cohort. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013;56(9):1248-54.
5. WHO. Pertussis vaccines. Wkly Epidemiol Rec. 2014;21(89):230-3.
6. Liko J, Robison SG, Cieslak PR. Priming with whole-cell versus acellular pertussis vaccine. The New England journal of medicine. 2013;368(6):581-2.
7. Xing D, Das RG, O'Neill T, Corbel M, Dellepiane N, Milstien J. Laboratory testing of whole cell pertussis vaccine: a WHO proficiency study using the Kendrick test. Vaccine. 2001;20(3-4):342-51.
8. WHO. Recommendations for whole-cell pertussis vaccine. WHO Technical Report Series 2007;941, Geneva, Switzerland:301-33.
9. European Pharmacopoeia, Assay of pertussis vaccine (whole cell). 2013;8th edition:242.
10. van Straaten-van de Kappelle I, van der Gun JW, Marsman FR, Hendriksen CF, van de Donk HJ. Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biologicals : journal of the International Association of Biological Standardization. 1997;25(1):41-57.
11. Hendriksen CF, Steen B. Refinement of vaccine potency testing with the use of humane endpoints. ILAR journal / National Research Council, Institute of Laboratory Animal Resources. 2000;41(2):105-13.
12. van de Waterbeemd B, Streefland M, Pennings J, van der Pol L, Beuvery C, Tramper J, *et al.* Gene-expression-based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnology and bioengineering. 2009;103(5):900-8.
13. Nakamura MM, Liew SY, Cummings CA, Brinig MM, Dieterich C, Relman DA. Growth phase- and nutrient limitation-associated transcript abundance regulation in *Bordetella pertussis*. Infection and immunity. 2006;74(10):5537-48.
14. Decker KB, James TD, Stibitz S, Hinton DM. The *Bordetella pertussis* model of exquisite gene control by the global transcription factor BvgA. Microbiology. 2012;158(Pt 7):1665-76.
15. de Gouw D, Diavatopoulos DA, Bootsma HJ, Hermans PW, Mooi FR. Pertussis: a matter of immune modulation. FEMS microbiology reviews. 2011;35(3):441-74.
16. Martinez de Tejada G, Cotter PA, Heininger U, Camilli A, Akerley BJ, Mekalanos JJ, *et al.* Neither the Bvg- phase nor the vrg6 locus of *Bordetella pertussis* is required for respiratory infection in mice. Infection and immunity. 1998;66(6):2762-8.
17. Hamstra HJ, Kuipers B, Schijf-Evers D, Loggen HG, Poolman JT. The purification and protective capacity of *Bordetella pertussis* outer membrane proteins. Vaccine. 1995;13(8):747-52.
18. Bellalou J, Relyveld EH. Studies on culture conditions of *Bordetella pertussis* and relationship to immunogenicity of vaccines. Annales de microbiologie. 1984;135B(1):101-10.
19. Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nature immunology.

2004;5(10):987-95.
20. Fedele G, Spensieri F, Palazzo R, Nasso M, Cheung GY, Coote JG, *et al. Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PloS one. 2010;5(1):e8734.
21. Dunne A, Mielke LA, Allen AC, Sutton CE, Higgs R, Cunningham CC, *et al.* A novel TLR2 agonist from *Bordetella pertussis* is a potent adjuvant that promotes protective immunity with an acellular pertussis vaccine. Mucosal immunology. 2014.
22. Fransen F, Stenger RM, Poelen MC, van Dijken HH, Kuipers B, Boog CJ, *et al.* Differential effect of TLR2 and TLR4 on the immune response after immunization with a vaccine against Neisseria meningitidis or *Bordetella pertussis*. PloS one. 2010;5(12):e15692.
23. Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. J Immunol. 2006;177(11):7980-9.
24. Marr N, Hajjar AM, Shah NR, Novikov A, Yam CS, Caroff M, *et al.* Substitution of the *Bordetella pertussis* lipid A phosphate groups with glucosamine is required for robust NF-kappaB activation and release of proinflammatory cytokines in cells expressing human but not murine Toll-like receptor 4-MD-2-CD14. Infection and immunity. 2010;78(5):2060-9. Epub 2010/02/24.
25. Geurtsen J, Angevaare E, Janssen M, Hamstra HJ, ten Hove J, de Haan A, *et al.* A novel secondary acyl chain in the lipopolysaccharide of *Bordetella pertussis* required for efficient infection of human macrophages. The Journal of biological chemistry. 2007;282(52):37875-84.
26. Shah NR, Albitar-Nehme S, Kim E, Marr N, Novikov A, Caroff M, *et al.* Minor modifications to the phosphate groups and the C3' acyl chain length of lipid A in two *Bordetella pertussis* strains, BP338 and 18-323, independently affect Toll-like receptor 4 protein activation. The Journal of biological chemistry. 2013;288(17):11751-60.
27. Marr N, Tirsoaga A, Blanot D, Fernandez R, Caroff M. Glucosamine found as a substituent of both phosphate groups in Bordetella lipid A backbones: role of a BvgAS-activated ArnT ortholog. Journal of bacteriology. 2008;190(12):4281-90. Epub 2008/04/22.
28. Hoonakker ME, Verhagen LM, Hendriksen CF, van Els CA, Vandebriel RJ, Sloots A, *et al. In vitro* innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle. Biologicals : journal of the International Association of Biological Standardization. 2015;43(2):100-9.
29. Thalen M, van den IJ, Jiskoot W, Zomer B, Roholl P, de Gooijer C, *et al.* Rational medium design for *Bordetella pertussis*: basic metabolism. Journal of biotechnology. 1999;75(2-3):147-59.
30. Thalen M, Venema M, van den IJ, Berwald L, Beuvery C, Martens D, *et al.* Effect of relevant culture parameters on Pertussis Toxin expression by *Bordetella pertussis*. Biologicals : journal of the International Association of Biological Standardization. 2006;34(3):213-20.
31. Ziegler-Heitbrock HW, Thiel E, Futterer A, Herzog V, Wirtz A, Riethmuller G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. International journal of cancer Journal international du cancer. 1988;41(3):456-61. Epub 1988/03/15.
32. Westphal O, Jann K. Bacterial lipopolysaccharides. Extraction with phenol-water and further applications of the procedure. Methods in Carbohydrate Chemistry. 1965:83-91.
33. Wilm MS, Mann M. Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? Int J Mass Spectrom Ion Proc. 1994;136 (2-3):167F.
34. Kondakov A, Lindner B. Structural characterization of complex bacterial glycolipids by Fourier transform mass spectrometry. European journal of mass spectrometry. 2005;11(5):535-46.
35. Caroff M, Brisson J, Martin A, Karibian D. Structure of the *Bordetella pertussis* 1414 endotoxin. FEBS letters. 2000;477(1-2):8-14

36. Baart GJ, Willemsen M, Khatami E, de Haan A, Zomer B, Beuvery EC, *et al.* Modeling Neisseria meningitidis B metabolism at different specific growth rates. Biotechnology and bioengineering. 2008;101(5):1022-35.
37. Higgs R, Higgins SC, Ross PJ, Mills KH. Immunity to the respiratory pathogen *Bordetella pertussis*. Mucosal immunology. 2012;5(5):485-500.
38. Flo TH, Halaas O, Torp S, Ryan L, Lien E, Dybdahl B, *et al.* Differential expression of Toll-like receptor 2 in human cells. Journal of leukocyte biology. 2001;69(3):474-81.
39. LeBouder E, Rey-Nores JE, Raby AC, Affolter M, Vidal K, Thornton CA, *et al.* Modulation of neonatal microbial recognition: TLR-mediated innate immune responses are specifically and differentially modulated by human milk. J Immunol. 2006;176(6):3742-52.
40. Shah NR, Hancock RE, Fernandez RC. *Bordetella pertussis* Lipid A Glucosamine Modification Confers Resistance to Cationic Antimicrobial Peptides and Increases Resistance to Outer Membrane Perturbation. Antimicrobial agents and chemotherapy. 2014;58(8):4931-4.
41. Allen AG, Thomas RM, Cadisch JT, Maskell DJ. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wlb from *Bordetella pertussis*, Bordetella parapertussis and Bordetella bronchiseptica. Molecular microbiology. 1998;29(1):27-38.
42. Harvill ET, Preston A, Cotter PA, Allen AG, Maskell DJ, Miller JF. Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. Infection and immunity. 2000;68(12):6720-8.
43. Preston A, Thomas R, Maskell DJ. Mutational analysis of the *Bordetella pertussis* wlb LPS biosynthesis locus. Microbial pathogenesis. 2002;33(3):91-5.
44. Robins LI, Williams AH, Raetz CR. Structural basis for the sugar nucleotide and acyl-chain selectivity of Leptospira interrogans LpxA. Biochemistry. 2009;48(26):6191-201.
45. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, Harris DE, *et al.* Comparative analysis of the genome sequences of *Bordetella pertussis*, Bordetella parapertussis and Bordetella bronchiseptica. Nature genetics. 2003;35(1):32-40.
46. Allen A, Maskell D. The identification, cloning and mutagenesis of a genetic locus required for lipopolysaccharide biosynthesis in *Bordetella pertussis*. Molecular microbiology. 1996;19(1):37-52.
47. Allen AG, Isobe T, Maskell DJ. Identification and cloning of waaF (rfaF) from *Bordetella pertussis* and use to generate mutants of Bordetella spp. with deep rough lipopolysaccharide. Journal of bacteriology. 1998;180(1):35-40.
48. White KA, Kaltashov IA, Cotter RJ, Raetz CR. A mono-functional 3-deoxy-D-manno-octulosonic acid (Kdo) transferase and a Kdo kinase in extracts of Haemophilus influenzae. The Journal of biological chemistry. 1997;272(26):16555-63.
49. White KA, Lin S, Cotter RJ, Raetz CR. A Haemophilus influenzae gene that encodes a membrane bound 3-deoxy-D-manno-octulosonic acid (Kdo) kinase. Possible involvement of kdo phosphorylation in bacterial virulence. The Journal of biological chemistry. 1999;274(44):31391-400.
50. Geurtsen J, Dzieciatkowska M, Steeghs L, Hamstra HJ, Boleij J, Broen K, *et al.* Identification of a novel lipopolysaccharide core biosynthesis gene cluster in *Bordetella pertussis*, and influence of core structure and lipid A glucosamine substitution on endotoxic activity. Infection and immunity. 2009;77(7):2602-11. Epub 2009/04/15.

51. Merkel TJ, Stibitz S, Keith JM, Leef M, Shahin R. Contribution of regulation by the bvg locus to respiratory infection of mice by *Bordetella pertussis*. Infection and immunity. 1998;66(9):4367-73.
52. Weiss AA, Falkow S. Genetic analysis of phase change in *Bordetella pertussis*. Infection and immunity. 1984;43(1):263-9.
53. Robinson A, Gorringe AR, Funnell SG, Fernandez M. Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. Vaccine. 1989;7(4):321-4.
54. de Gouw D, Hermans PW, Bootsma HJ, Zomer A, Heuvelman K, Diavatopoulos DA, *et al.* Differentially Expressed Genes in *Bordetella pertussis* Strains Belonging to a Lineage Which Recently Spread Globally. PloS one. 2014;9(1):e84523.
55. Mills KH, Ryan M, Ryan E, Mahon BP. A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infection and immunity. 1998;66(2):594-602.
56. Roberts M, Tite JP, Fairweather NF, Dougan G, Charles IG. Recombinant P.69/pertactin: immunogenicity and protection of mice against *Bordetella pertussis* infection. Vaccine. 1992;10(1):43-8.
57. Fedele G, Nasso M, Spensieri F, Palazzo R, Frasca L, Watanabe M, *et al.* Lipopolysaccharides from *Bordetella pertussis* and Bordetella parapertussis differently modulate human dendritic cell functions resulting in divergent prevalence of Th17-polarized responses. J Immunol. 2008;181(1):208-16. Epub 2008/06/21.
58. Weyrich LS, Feaga HA, Park J, Muse SJ, Safi CY, Rolin OY, *et al.* Resident microbiota affect *Bordetella pertussis* infectious dose and host specificity. The Journal of infectious diseases. 2014;209(6):913-21.
59. Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR. An inner membrane enzyme in Salmonella and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A: induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. The Journal of biological chemistry. 2001;276(46):43122-31.

4

Chapter 4

In vitro innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle

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Biologicals 2015 Mar; 43(2): 100-109

Abstract

Lot release testing of vaccines is primarily based on animal models that are costly, time-consuming and sometimes of questionable relevance. In order to reduce animal use, functional *in vitro* assays are being explored as an alternative approach for the current lot release testing paradigm. In this study, we present an evaluation of APC platforms assessing innate immune activation by whole cell *Bordetella pertussis* (wP) vaccines. Primary monocytes, monocyte-derived DC (moDC) and human monocyte/DC cell lines (MonoMac6 and MUTZ-3) were compared for their capacity to respond to wP vaccines of varying quality. To produce such vaccines, the production process of wP was manipulated, resulting in wP vaccines covering a range of *in vivo* potencies. The responses of MUTZ-3 cells and primary monocytes to these vaccines were marginal and these models were therefore considered inappropriate. Importantly, moDC and MonoMac6 cells responded to the wP vaccines and discriminated between vaccines of varying quality, although slight variations in the responses to wP vaccines of similar quality were also observed. This study provides a proof of principle for the use of *in vitro* APC platforms as part of a new strategy to assess wP vaccine lot consistency, though careful standardization of assay conditions is necessary.

Introduction

Before vaccines can be marketed, both safety and efficacy have to be demonstrated in preclinical and clinical studies. Preclinical studies often require the use of laboratory animals. In addition, animals are also required for vaccine lot release testing, particularly in the case of established vaccines, that is vaccines that are produced by inactivation of whole micro-organisms or the toxins thereof, such as vaccines against *Corynebacterium diphtheriae* and *Clostridium tetani* and *Bordetella pertussis* (*B. pertussis*). Generally, up to 100 animals are used for each lot potency test, in total accounting for approximately 5-10% of the annual experimental animal use in the Netherlands[1]. Though production conditions have improved significantly since the introduction of these type of vaccines in immunisation programs, regulatory authorities feel the urge to maintain animal-based potency tests for lot release testing of established vaccines, because these products are poorly defined and because of the unknown effect of the adjuvant-antigen interaction on the immune response. Nevertheless, the relevance of some of the animal models is questionable, since animal models are not always susceptible to the pathogen against which the vaccine is directed[2] and do not always reflect human pathology and immunity.[3] The potency test for whole cell pertussis (wP) vaccine is illustrative. As mice are not susceptible to pathology upon natural infection with *B. pertussis*, the protection induced by wP vaccination is determined by intracerebral challenge with the pathogen (the Kendrick test). Unprotected mice die due to meningitis or secondary symptoms, thus survival is the measure for vaccine efficacy.[2] Since *B. pertussis* is a pathogen that affects the respiratory tract rather than the neural system, it is not likely that the pathology and immunology induced by the intracerebral challenge reflect the human situation. Although acellular pertussis vaccines have replaced the wP vaccines in most western countries, wP vaccines are widely used e.g. in Latin America and Asia[4] and therefore the production of this vaccine and the associated lot release test is continued.

In general, the use of alternative methods to replace existing animal models is strongly recommended and a range of physico-chemical techniques is now available that can be used to characterise key aspects of vaccines[5,6]. However, these techniques do not assess vaccine-induced immune responses. For an effective immune response, pathogen-induced activation of antigen presenting cells (APC) with concomitant upregulation of co-stimulatory receptors and secretion of cytokines, and pathogen-derived peptide presentation in MHC molecules is required. Activated APC initiate and direct the activation of antigen-specific T cells (cytotoxic and helper T cells), and subsequently helper T cells may promote antibody responses by specific B cells. The combination of the responses of these immune cells determines whether the immune response will be effective. As APC initiates the adaptive response, activation of APC can give an indication of the direction and effectiveness of the subsequent immune

response. Assays based on APC models could therefore bridge the gap between the physico- and immunochemical assays and the currently required *in vivo* potency tests.

In this study, we compared the activation of several APC platforms for their ability to discriminate vaccines of good or consistent quality from vaccines of poor or inconsistent quality. We used primary monocyte derived DC (moDC) and monocytes, two clinically relevant APC platforms. We also studied the human MUTZ-3 cell line displaying a DC phenotype, and a monocytic cell line of human origin, MonoMac6 (MM6). To determine whether these APC platforms could evaluate vaccine quality, vaccines of varying quality were required. Therefore, we manipulated the culture conditions of *B. pertussis*, resulting in wP vaccines containing differential levels of virulence proteins. The virulence proteins are important for inducing adaptive immunity. Based on the protein composition as well as *in vivo* potency experiments (Metz *et al.* in preparation), the ensuing vaccines were considered to be of a quality ranging from poor to good. Using these vaccines, we assessed the relevance of the APC platforms (i.e. their capacity to respond to TLR2 and TLR4 ligands), their sensitivity (i.e. their capacity to respond to the wP vaccine in a dose dependent manner) and their specificity (i.e. capacity to discriminate wP vaccines of a poor quality from a vaccine of good quality). This study provided proof of principle that two of the four tested APC platforms can be used for analysing wP vaccine quality *in vitro*.

Materials and methods

Production of whole cell *B. pertussis* vaccines of varying quality

B. pertussis strain 509 (Intravacc, Bilthoven, The Netherlands) was used to produce wP vaccine lots on laboratory scale. This clinical isolate was collected in 1963 and was used for the production of wP vaccine for the national vaccination program of the Netherlands until 2005. All cultures were grown in chemically defined medium[7,8] using a fully instrumented 3L bench-top bioreactor fitted with a six-bladed Rushton stirrer (Applikon, Schiedam, The Netherlands) with a 2L working volume. The reactor was inoculated from a pre-culture, seeded at 5% (v/v) and grown at 35°C. After 3-5 reactor volumes steady state was assumed and deliberate downregulation of virulence genes (t=0) was initiated by adding medium containing 50 mM $MgSO_4$, while at the same time $MgSO_4$ was added directly to the reactor to change the concentration to 50 mM $MgSO_4$ instantly. Just before the addition of $MgSO_4$ (0 hours) and at 2, 6, 12 and 24h after the start of the culture with $MgSO_4$, samples were taken, formaldehyde (16 mM) was added, and subsequently the samples were inactivated by heating (56°C) for 10 min. Three separate cultivation runs were performed. The vaccines are referred to as vaccine A_{ref} (t=0, the reference vaccine), vaccine C (t=6) and vaccine E (t=24). Unless mentioned otherwise, corresponding vaccine preparations were pooled and used for the stimulation of moDC, monocytes or the different cell lines at indicated OD_{590nm}. The *in vivo* Kendrick test revealed that the potency of vaccine A_{ref} was 7.0 IU/mL (95% interval 2 IU/mL and 27 IU/mL), the potency of vaccine C was 4.8 IU/mL (95% interval 2 IU and 10 IU) and the potency of vaccine E was 0.8 IU/mL (95% interval 0 IU/mL and 3 IU/mL) (details will be described in Metz *et al.*, in preparation). The test results of the vaccines all fulfilled the requirements with regard to parallelism, while vaccine A_{ref} was the only vaccine that deviated from the requirements with regard to linearity (p= 0.01). Nevertheless, we considered the performed potency testing of these experimental vaccines as valid. According to the criteria of the European Pharmacopoeia (potency at least 4 IU/mL and 95% interval with a lower limit of 2 IU/mL) the potencies of vaccine A and C were sufficient, whereas the potency of vaccine E was insufficient. Based on these results, the quality vaccine A_{ref} was considered good, the quality of vaccine E was considered poor and quality of vaccine C was considered intermediate.

Ethics statement

Donor blood was kindly provided by the internal blood donor system of the National Institute for Public Health and the Environment (RIVM) in the Netherlands. This study was conducted according to the principles expressed in the Decleration of Helsinki. All donors provided written informed consent for the collection of samples and subsequent analysis. The blood samples were processed anonymously.

APC platforms: moDC and monocytes

Peripheral blood mononuclear cells from healthy donors were isolated by density centrifugation on Lymphoprep (Nycomed) at 1000xg for 30 minutes. Cells were washed, harvested, and resuspended in PBS supplemented with 0.5% BSA and 2 mM EDTA. For isolation of monocytes, the cells were incubated with CD14 microbeads (Miltenyi Biotec) and sorted by MACS columns according to the protocol of the manufacturer. The primary monocytes were cultured in 24-wells plates at a concentration of 6×10^5 cells/ml in IMDM-1640 medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml L-glutamine (Gibco) and 10% FCS (Hyclone) and stimulated as described below. For generation of moDC the CD14 positive cells were cultured in 24-wells plates (Greiner) at a concentration of 4×10^5 cells/ml in IMDM-1640 medium (Gibco) supplemented with penicillin 100 U/ml, streptomycin 100 µg/ml, L-glutamine 0.3 mg/ml (Gibco), 1% FCS (Hyclone), GM-CSF (500 U/ml; PeproTech), and IL-4 (800 U/ml; Active Bioscience) for 6 days. The moDC were subsequently stimulated as described below.

APC platforms: MUTZ-3 and MM6

The MUTZ-3 cell line (kindly provided by prof. dr. R.J. Scheper, VUmc, Amsterdam, The Netherlands) was cultured in α-Minimum Essential Medium (Gibco), supplemented with 20% heat-inactivated FCS (Hyclone), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml L-glutamine (Gibco), 50 µM β-mercaptoethanol (Sigma) and 27.5 U/ml GM-CSF (PeproTech) in 12-well tissue culture plates (Greiner) at a concentration of $1–5 \times 10^5$ cells/ml. To induce differentiation into an immature dendritic cell phenotype, the MUTZ-3 cells were cultured in 12-well tissue-culture plates at a concentration of 1×10^5/ml in the presence of the growth factors GM-CSF (1000 U/ml), IL-4 (1000 U/ml) and TNF-α (2.5 ng/ ml; Miltenyi) for 6 days and stimulated as described below.

The MM6 cell line was grown in IMDM, supplemented with heat-inactivated 10% FCS (Hyclone), 20 µM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 mg/ml L-glutamine (Gibco). For stimulations, as described below, the MM6 cells were seeded in 24-well plates at 1×10^6 cells/ml.

Stimulation of APC platforms

Immature moDC, monocytes and MM6 cells were stimulated with *Escherichia coli* (*E. coli*) lipopolysaccharide (LPS) (100 ng/ml, Invivogen, ultrapure) and Pam3CSK4 (PAM) (1 µg/ml, Invivogen) as positive controls for activation, while immature MUTZ-3 cells were stimulated with TNF-α (75 ng/ml) and PAM (1 µg/ml) as a positive control. Immature moDC, monocytes, MUTZ-3 and MM6 cells were also stimulated with the wP vaccines A_{ref} at an indicated OD_{590nm} for 48h (moDC, monocytes and immature MUTZ-3) or 24h (MM6). In addition, immature moDC and MM6 cells were stimulated with vaccine A_{ref} (good quality), vaccine C (intermediate

quality), vaccine E (poor quality), vaccine A-run1 (good quality), vaccine A-run2 (good quality), and vaccine A-run3 (good quality) at indicated OD_{590nm}.

After stimulation, supernatants of moDC, monocytes and MM6 cells were collected and the cells were harvested by incubating with PBS at 4°C for 1h. Supernatants of MUTZ-3 cells were also collected and these cells were harvested by incubating with PBS supplemented with 0.5 µM EDTA at 37°C for 5 minutes.

Flow cytometric measurement of expression of surface receptors

MoDC and MUTZ-3 cells were washed and either left unstained or stained with anti-human CD14-PE (BD), anti-human CD80-FITC (BD), anti-human PD-L1-APC (eBioscience) and anti-human HLA-DR-Pacific Blue (Biolegend), or anti-human CD83-FITC (BD), anti-human CD40-PE (BD), anti-human DC-SIGN(CD209)-APC (BD) and anti-human CD86-Pacific Blue (Biolegend). Because of their monocytic phenotype, the monocytes and the MM6 cells were washed and stained with antibodies for the same receptors and markers, except PD-L1 and CD40. Instead these cell types were stained with anti-human CD38-APC (BD) and anti-human CD25-PE (eBioscience). Live dead staining Amcyan (Fischer) was added to all cells to exclude non-viable cells. Data was acquired on FACS Canto II (BD Biosciences) using FACS Diva software and analysed using FlowJo (TreeStar Inc.). Receptor expression on unstimulated cells was determined based on the mean fluorescent intensity (MFI) values of the receptors stained for compared to background MFI values of unstained cells.

Cytokine ELISAs

IL-6, IL-12p40, IL-1β and IL-10 secretion was measured in the culture supernatants using ELISA. The IL-6 ELISA kit was purchased from Sanquin, the IL-12p40 ELISA kit from Diaclone and the IL-1β and IL-10 ELISA kit from R&D systems.

Statistics

All statistical analyses were performed using GraphPad Prism 6.04. Statistical significance was determined by using unpaired t-tests (Figure 1, 4, 5). The Benjamini-Hochberg method was used for correction of multiple comparisons[9] (Figure 1). The Kruskal Wallis test was performed to determine dose dependency (Figure 2). The Wilcoxon signed rank test was used to determine whether responses deviated from 100% (Figure 3). P values <0.05 were considered statistically significant.

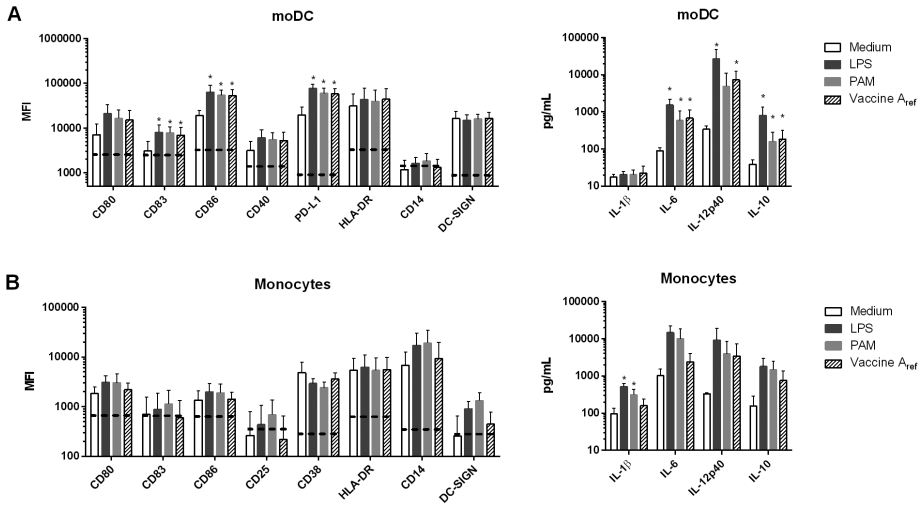
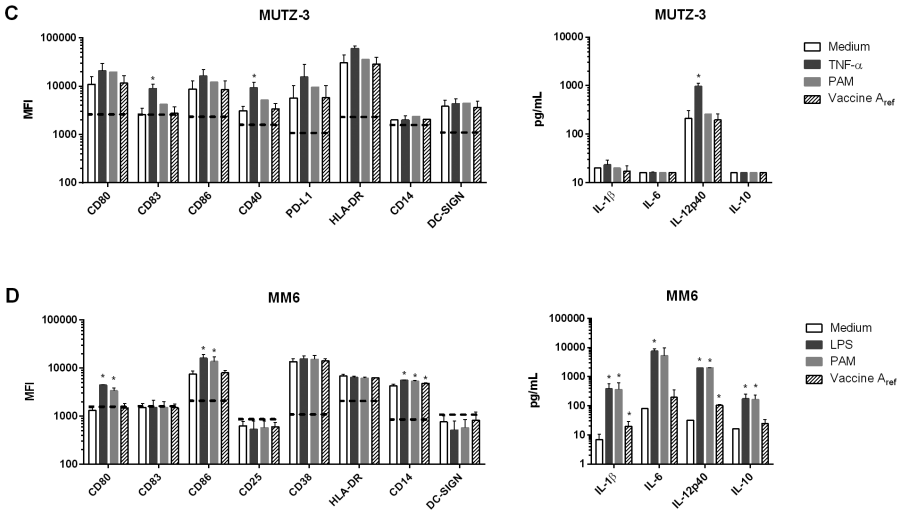


Figure 1 Response of the APC platforms to TLR4 and TLR2 agonists, TNF-α and the wP vaccine of good quality (vaccine A_{ref}). (A) moDC from healthy donors were stimulated with LPS, PAM, and vaccine A_{ref} (OD_{590nm} 0.00047) for 48h. moDC receptor expression data represent the average of five donors, the cytokine data represent the average of six donors. (B) Monocytes from 3 donors were stimulated with LPS, PAM, and vaccine A_{ref} (OD_{590nm} 0.00047) for 48h. (C) MUTZ-3 cells were stimulated with PAM, TNF-α and vaccine A_{ref} (OD_{590nm} 0.0015) for 48h (3 experiments). (D) MM6 cells were stimulated with LPS, PAM, and the vaccine A_{ref} (OD_{590nm} 0.00047) for 24h (3 experiments).

Results

TLR4 and TLR2 agonists and wP vaccine induced activation of moDC, monocytes and MM6 cells, but not MUTZ-3 cells

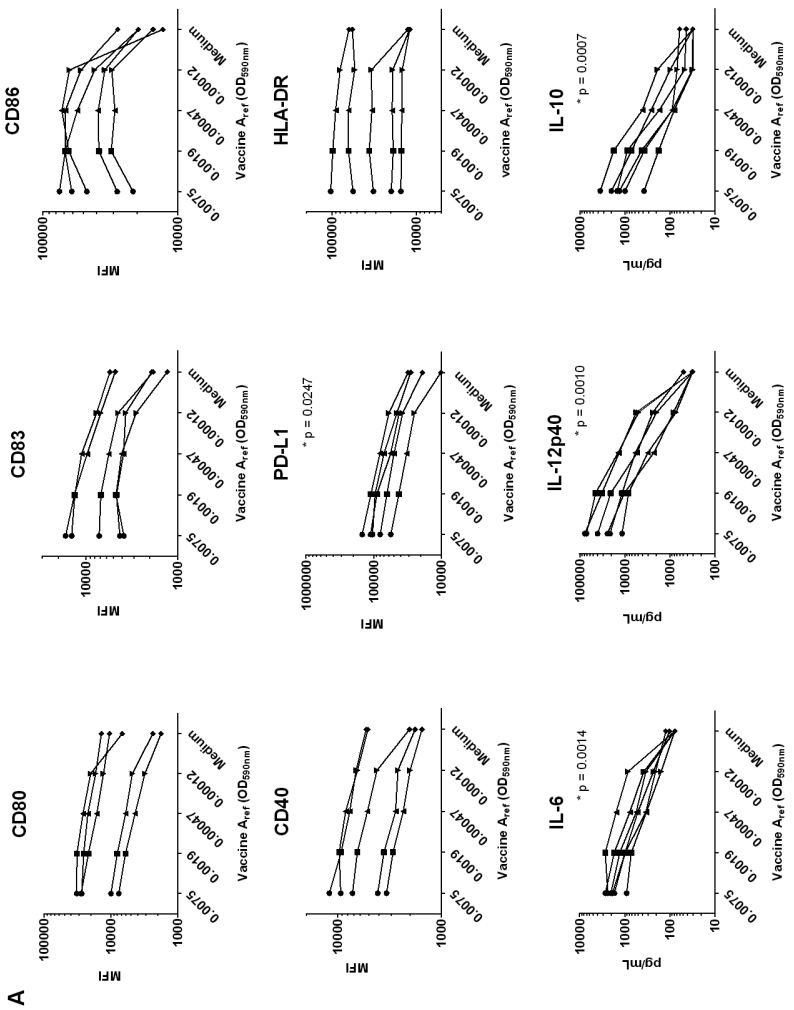
Since *B. pertussis* induced activation of DCs depends on TLR4 and TLR2 signalling[10], the responses of the APC platforms to the TLR4 ligand LPS, the TLR2 ligand PAM and reference vaccine A_{ref} were measured to determine the relevance of each cell model (Figure 1). The immature moDC expressed co-stimulatory receptors CD80, CD86, CD40, the co-inhibitory receptor PD-L1 and the DC markers HLA-DR and DC-SIGN. Stimulation of the moDC with LPS, PAM and vaccine A_{ref} induced up-regulation of CD83 and CD86 and PD-L1 (Figure 1A, left panel) and secretion of IL-6 and IL-10 (Figure 1A, right panel). In addition, LPS and vaccine A_{ref} induced significant secretion of IL-12p40 (Figure 1A, right panel). Furthermore, the PD-L1 expression and the secretion of IL-6, IL-12p40 and IL-10 was vaccine dose-dependent (Figure 2A), while



Receptor expression was determined for all cell types (left panel). The receptor expression is presented as mean fluorescence intensity (MFI). Cytokine secretion was measured in culture supernatants (right panels). The dashed lines indicate the background signal (MFI) of the unstained cells for the corresponding fluorochromes and the indicated cell type. * $p < 0.05$, significantly different from medium treated control cells.

the secretion of IL-1β was marginal and not vaccine dose-dependent (data not shown). The second primary cell model, the monocytes, expressed CD80, CD86, CD38, HLA-DR and CD14, although stimulation with LPS, PAM or vaccine A_{ref} did not induce significant changes in the expression of any of the analysed receptors (Figure 1B, left panel). LPS and PAM induced the secretion of IL-1β (Figure 1B right panel) and vaccine A induced a dose-dependent secretion of IL-1β (Figure 2B), though the responses varied considerably between the donors.

Immature MUTZ-3 expressed the co-stimulatory receptors CD80, CD86, CD40, the co-inhibitory receptor PD-L1 and the DC markers HLA-DR and DC-SIGN (Figure 1C, left panel). Activation of these cells by TNF-α induced upregulation of CD83 and CD40 (Figure 1C, left panel) and secretion of IL-12p40 (Figure 1C, right panel). In contrast, PAM did not induce changes in cytokine secretion by or receptor expression on MUTZ-3 cells (Figure 1C, both panels). Importantly, MUTZ-3 cells did neither respond to stimulation with vaccine A_{ref} at an OD_{590nm} of


A
CD80
CD83
CD86
CD40
PD-L1
HLA-DR
IL-6
IL-12p40
IL-10
MFI
pg/mL
* p = 0.0247
* p = 0.0014
* p = 0.0010
* p = 0.0007
Vaccine A$_{ref}$ (OD$_{590nm}$)
Medium

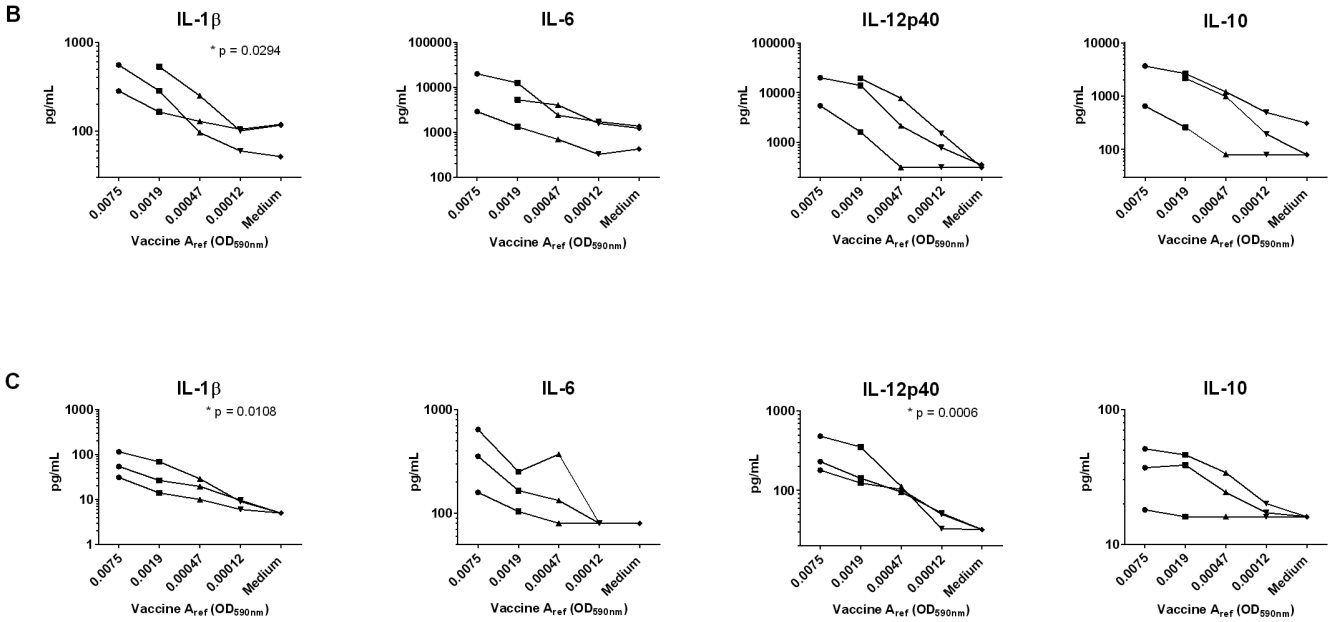


Figure 2 Dose-dependent up-regulation of surface receptors and cytokine secretion by moDC, monocytes and MM6 cells upon stimulation with the wP vaccine of good quality (vaccine A_{ref}). moDC (A), monocytes (B) and MM6 cells (C) were stimulated with medium or different concentrations of vaccine A_{ref} as indicated on the x-axis. Cytokine secretion by moDC, monocytes and MM6 cells and receptor expression on moDC was analysed. The receptor expression is presented as mean fluorescence intensity (MFI). Cytokine secretion was measured in culture supernatants. Lines represent independent donors (moDC, monocytes) or experiments (MM6 cells). * $p < 0.05$, significant difference between the concentrations of the vaccines.

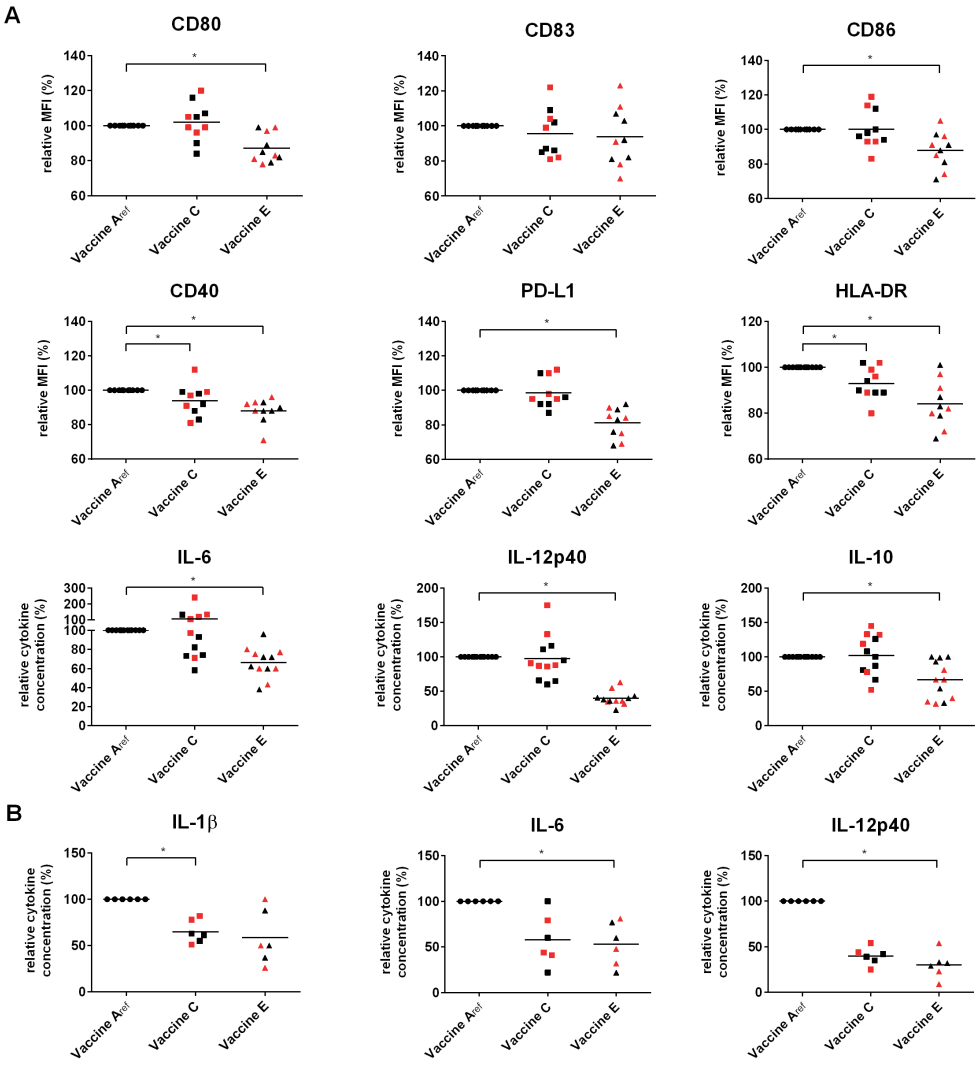

A
CD80
CD83
CD86
CD40
PD-L1
HLA-DR
IL-6
IL-12p40
IL-10
B
IL-1β
IL-6
IL-12p40
relative MFI (%)
relative cytokine concentration (%)
Vaccine Aref
Vaccine C
Vaccine E

Figure 3 Relative receptor expression and cytokine secretion by moDC and MM6 cells in response to the wP vaccines of intermediate and poor quality (vaccines C and E) compared to the consistently produced vaccine (vaccine A_{ref}). (A) moDC were stimulated with vaccine A_{ref}, C and E (OD_{590nm} 0.00047 and OD_{590nm} 000012 for all vaccines). moDC receptor expression data represent the average of five donors, the cytokine data represent the average of six donors (B) MM6 cells were stimulated with vaccine A_{ref}, C, and E (OD_{590nm} 0.00047 and OD_{590nm} 000012 for all vaccines) (3 experiments). Cytokine secretion by moDC and MM6 cells and receptor expression on moDC was analysed. The responses are expressed as the percentage of the response to vaccine A_{ref} (relative MFI or relative cytokine concentration = response to vaccine X/ response to the vaccine A_{ref}*100). Red symbols and black symbols represent the responses to the vaccines at an OD_{590nm} of 0.00047 and an OD_{590nm} of 0.00012, respectively. * $p < 0.05$, significantly different from responses to vaccine A_{ref}.

0.0015 (Figure 1C, both panels), nor to higher concentrations of the vaccine (data not shown). The second cell line, MM6, expressed CD86, CD38, HLA-DR and CD14 (Figure 1D, left panel). Stimulation with PAM and LPS resulted in activation of these cells, indicated by the increased expression of CD80, CD86 and CD14 (Figure 1D, left panel) and enhanced secretion of IL-1β, IL-12p40 and IL-10 (Figure 1D, right panel). LPS also induced secretion of IL-6 (Figure 1D, right panel). Vaccine A_{ref} induced upregulation of CD14 (Figure 1D, left panel) and induced enhanced secretion of IL-1β and IL-12p40 (Figure 1D, right panel). IL-1β and IL-12p40 were secreted in a vaccine A_{ref} dose dependent manner by MM6 cells (Figure 2C), while the CD14 expression was not dose-dependent (data not shown). Since the vaccine A_{ref} induced secretion of IL-10 (Figure 2C) and upregulation of CD14 was low and not dose-dependent, both markers are considered to be an inappropriate parameter for MM6 induced activation by vaccine A_{ref}.

Together these results indicate that MUTZ-3 cells and monocytes do not seem appropriate APC platforms to assess the quality of wP vaccines, due marginal responses to vaccine A_{ref} (both APC platforms) and considerable variation between experiments (monocytes), while moDC and MM6 cells do seem appropriate platforms to assess responses to wP vaccines. Therefore, moDC and MM6 cells were selected for further analysis.

The responses of moDC and MM6 cells to wP vaccines of varying qualities

To explore the capacity of the selected APC platforms to identify vaccines of poor and good quality, the responses of moDC and MM6 cells to vaccines of intermediate and poor quality (vaccine C and E respectively) were analysed relative to the response to the vaccine of a good quality (vaccine A_{ref}). Though vaccine E induced expression of CD80, CD86, CD40, PD-L1,

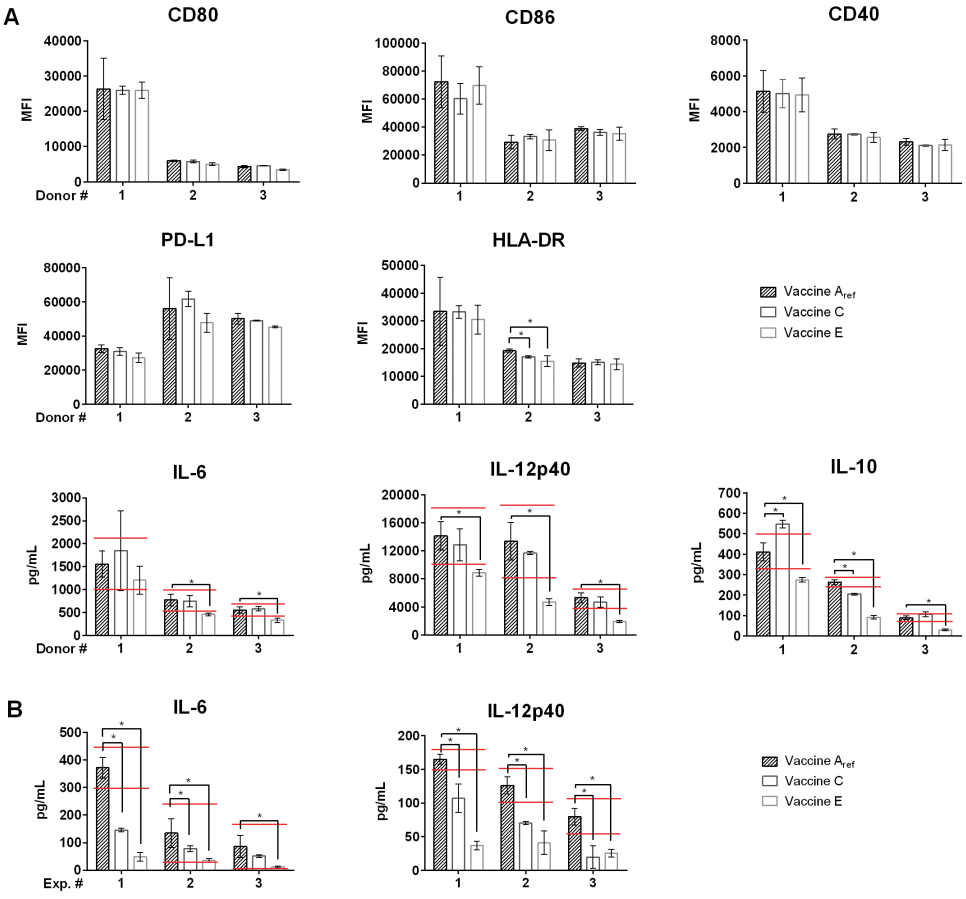


Figure 4 The responses of moDC and MM6 cells to the wP vaccines of good (vaccine A_{ref}), intermediate (vaccine C) and poor quality (vaccine E). MoDC (A) and MM6 cells (B) were stimulated with vaccine A_{ref}, C, and E (in triplicate, OD_{590nm} 0.00047). Cytokine secretion by moDC and MM6 cells and receptor expression on moDC was analysed. Receptor expression is presented as mean fluorescence intensity (MFI). Cytokine secretion was measured in culture supernatants. The lines mark the 2x SD of the response to vaccine A_{ref}. * $p < 0.05$, significantly different from responses to vaccine A_{ref}.

and HLA-DR on moDC, and the secretion of IL-6, IL-12p40 and IL-10, the expression of these receptors and secretion of these cytokines in response to vaccine E was lower than induced by vaccine A_{ref} (Figure 3A). Similarly, vaccine E induced less secretion of IL-6 and IL-12p40 by the MM6 cells than was secreted in response to vaccine A_{ref} (Figure 3B). The vaccine C induced

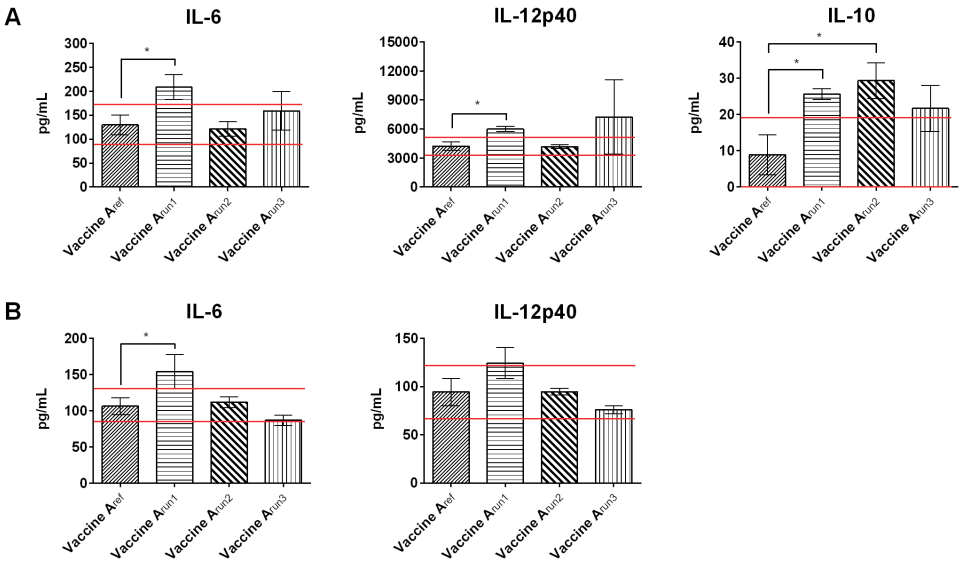


Figure 5 The responses of moDC and MM6 cells to the pooled wP reference vaccine (vaccine A_{ref}) and the separately produced wP vaccine runs (vaccine A_{run1}, vaccine A_{run2} and vaccine A_{run3}). moDC (A) and MM6 cells (B) were stimulated with vaccine A_{ref}, vaccine A_{run1}, vaccine A_{run2}, and vaccine A_{run3} (in triplicate, OD_{590nm} 0.00047). Cytokine secretion by moDC and MM6 cells was analysed in culture supernatant. The lines indicate the 2x SD of the response to vaccine A_{ref}. * $p < 0.05$, significantly different from vaccine A_{ref}.

expression of CD40 and HLA-DR on moDC and the vaccine C induced secretion of IL-1β by MM6 cells was significantly lower than induced by vaccine A.

Together, these results indicate that PD-L1, CD80, HLA-DR, CD86 and CD40 as well as the cytokines IL-6, IL-12p40 and IL-10 are appropriate markers for moDC and IL-12p40 and IL-6 are appropriate markers for MM6 cells to discriminate between a wP vaccine of good and poor quality. The responses of moDC and MM6 to a vaccine of intermediate quality (vaccine C) were marginally different from responses to a vaccine of good quality (vaccine A_{ref}).

Proof-of-principle for the use of *in vitro* APC platforms to assess wP vaccine quality

APC platform should consistently distinguish vaccines of a poor quality from vaccines of good quality in a reproducible manner. Therefore, we mimicked product testing by performing three

individual experiments with each stimulation in triplicate. The APC platforms were exposed to vaccines of good, intermediate and poor quality (vaccine A_{ref}, vaccine C and vaccine E) (Figure 4) and to separate vaccines of similar (good) qualities (vaccine A-run1, vaccine A-run2, vaccine A-run3) (Figure 5). The vaccine of poor quality consistently elicited a lower secretion of IL-12p40 and IL-10 by the moDC (Figure 4A) and a lower secretion of IL-6 and IL-12p40 by the MM6 cells (Figure 4B) than induced by the vaccine of a good quality. In contrast to the explorative experiments, the differences in vaccine quality were not reflected in the receptor expression by the moDC (Figure 4A). In general, the three vaccines of a good quality did not induce a significantly lower range of cytokine secretion by the moDC or MM6 cells than the reference vaccine, though one of these vaccines did induce a significantly higher cytokine secretion (Figure 5). Quality control tests often use a two or three fold standard deviation (SD) range (2SD and 3SD respectively) of a reference sample as a cut-off value to approve products under study. When applying the 2SD criteria (lines in Figure 4), vaccine E would consistently be rejected based on the IL-12p40 and IL-10 secretion by moDC and the IL-12p40 secretion by MM6 cells, since the mean ± the SD is lower than the 2SD range of the vaccine A_{ref} (Figure 4A and Figure 4B). Vaccine A-run1 is on the border of rejection since the mean ± the SD of cytokine concentrations is at or just above 2SD range of the vaccine A_{ref} (Figure 5A and Figure 5B). Together these findings indicate that the APC platforms moDC and MM6 cells are capable of discriminating wP vaccines of good and poor quality, though variation in response to vaccines of good quality has to be taken into account and standardisation will be necessary when these types of platforms are used.

Discussion

In this study, four human APC platforms (moDC, monocytes, MUTZ-3 cells and MM6 cells) were evaluated as an alternative *in vitro* approach for demonstration of consistency in vaccine production and vaccine quality testing, to eventually reduce and replace animal based lot release testing. This study demonstrated that two of the APC platforms, moDC and MM6 cells, mimicking innate immune activation in response to wP vaccines, suited criteria that are considered essential for other quality control tests, i.e. relevance, sensitivity and specificity.

The capacity of the APC platforms to respond to TLR4 and TLR2 agonists was investigated, since TLR4 and TLR2 signalling plays an important role in the activation of human DC by *B. pertussis*[10]. The relevance of TLR4 and TLR2 signalling for *B. pertussis* induced immune responses has also been determined in mice. These studies showed that wP vaccination of TLR4 deficient mice led to a significantly lower innate and adaptive immune response when compared to wild type mice[11] and an absence of protective immunity[12,13], while TLR2-deficiency had no significant effect on the immune response.[11] In another study, the effect of TLR4 deficiency on infection with *B. pertussis* was investigated.[14] TLR4 deficiency resulted in higher bacterial colonization, enhanced cellular infiltration and enhanced inflammatory cytokine secretion and antigen specific IFN-γ responses in the lungs.[14] The authors of this study suggested that, in addition to pro-inflammatory effects, TLR4 signalling by *B. pertussis* induces innate IL-10 production and thereby inhibits Th1 responses, induces regulatory T cell (Treg) responses and diminishes the pathology in the lungs. Since TLR4 mRNA as well as TLR4 receptor expression in MUTZ-3 cells is 5-18% of the expression observed in moDC[15], and the MUTZ-3 cells did not respond to LPS (data not shown) or the consistently produced vaccine (vaccine A_{ref}), we concluded that the MUTZ-3 cell line is not a relevant cell model for studying wP vaccine quality. However, since MUTZ-3 cells express a range of pathogen recognition receptors (PRR) and show functional responses to TLR2 agonists[15], it might be an interesting model for vaccines where other PRRs such as TLR2 plays an important role (e.g. Haemophilus influenzae type B[16]). Although the monocytes did respond to the TLR2 and TLR4 agonists and the wP vaccine, the responses were marginal, reflected in the absence of significant changes in receptor expression. The wP vaccine of good quality induced a dose dependent secretion of IL-1β, IL-6, IL-12p40 and IL-10, yet not all trends were significant. Due to the large variation in the responses of the monocytes derived from different donors and the marginal response to the TLR agonists and the wP vaccine, we concluded that this APC platform is unsuitable for studying wP quality.

Compared to the monocytes and MUTZ-3 cells, moDC displayed a stronger response to LPS and PAM and the wP vaccine of good quality (vaccine A_{ref}), reflected by the up-regulation of several co-stimulatory receptors, thereby confirming other studies.[17] In general, co-

stimulatory receptors on APC promote the activation of T cells while co-inhibitory receptors inhibit T cell activation.[18] Therefore, vaccine-induced changes in the expression of these co-stimulatory and co-inhibitory receptors on the APC platforms, may indicate the direction of the T cell response induced by vaccination. While the wP vaccine-induced expression of the co-stimulatory receptors such as CD86 by the moDC is indicative for optimal activation of T cells[18], the expression of the co-inhibitory receptor PD-L1 is indicative for the differentiation of T cells into Treg.[19] In addition to the receptor expression, the vaccine of good quality not only induced a higher secretion of pro-inflammatory cytokines by moDC, but also induced a higher secretion of the regulatory cytokine IL-10 than the vaccine of poor quality. Other studies suggest that the *B. pertussis* virulence factor FHA is one of the factors that positively influences IL-10 secretion by murine immune cells both *in vitro*[20] and *in vivo*.[21] Since the amount of virulence factors including FHA are reduced in the vaccine of poor quality (Hoonakker *et al.* in preparation), there might be a correlation between the FHA content in the vaccines and the secretion of IL-10 by the moDC. In general, pro-inflammatory as well as regulatory cytokines are essential for proper activation of T cells and thereby affect B cell activation and antibody production.[22] The expression of the co-inhibitory and co-stimulatory receptors on moDC and secretion of pro-inflammatory cytokines by moDC and MM6 and anti-inflammatory cytokines by moDC in response to the wP vaccine, suggests that the vaccine is capable of inducing pertussis-specific T and B cell responses, yet also induces regulatory feedback mechanisms to prevent overt immune-activation. Due to the relatively low variation in moDC and MM6 responses, the prominent response to LPS and the dose-dependent response to the wP vaccine, we considered the moDC and MM6 appropriate APC platforms for assessing wP quality.

Cytokine secretion proved to be a more robust platform read-out compared to receptor expression. The findings of this study illustrate that the MM6 cells and the moDC can distinguish between a wP vaccine of good and poor quality (vaccine A_{ref} and vaccine E), but not so much between wP vaccines of good and intermediate quality (vaccine A_{ref} and vaccine C) based on cytokine secretion. In case the moDC platform would be part of a quality test, we would therefore consider vaccine E to be of a different quality than vaccine A_{ref} and reject this vaccine. The same would be concluded based on the wP vaccine induced secretion of IL-12p40 by the MM6 cells. This means that the decision on the acceptance or rejection of vaccine A, C or E using the *in vitro* APC models corresponds well with the decision that would have been made based on their *in vivo* potencies as determined in the Kendrick test. In addition, when responses of the moDC and MM6 platforms to vaccines of similar qualities were studied, the three vaccines of good quality induced comparable responses in both APC platforms. Yet one vaccine run induced a response exceeding the reference vaccine (vaccine A_{ref}), in both moDC and MM6 cells. This highlights the importance of optimisation and internal standards when these types of APC platforms are used as quality tests. Overall, the findings illustrate that both

moDC as well as MM6 cells can be used for monitoring wP vaccine quality. The MM6 cell line is already utilized for quality testing of pyrogens[23], illustrating that this APC platform is suitable for standardized testing. On the other hand, a primary cell based APC platform, such as moDC, is of a higher clinical relevance[24].

The APC platforms used in this study might be of value for a new strategy within the vaccine field; the Consistency Approach. This approach is based on the principle that serial lots of vaccines have a quality consistent to that of a clinical or historical lot with proven safety and efficacy[25]. Consistency requires careful characterisation of the seed material, application of Good Manufacturing Practice (GMP) and Quality Assurance (QA) together with in-process and final lot testing. Functional immunological assays could complement this approach and ultimately reduce animal based lot release testing. Although previous studies have shown that APC platforms can be used *in vitro* to determine vaccine-induced responses in R&D[26,27], this is the first evaluation of these types of APC platforms for monitoring vaccine quality. Here, we provide proof-of-principle that moDC and MM6 cells can be used to monitor the quality of wP vaccine *in vitro*, although validation experiments will be necessary. In this study, the wP vaccine was used as a case study, but the APC platforms might form a valuable tool for quality testing of other vaccines that rely on animal testing for lot release, such as diphtheria, polio and tetanus.

Acknowledgements

This study was financially supported by the Dutch ministry of Health, Welfare and Sports and the Dutch ministry of Economic Affairs. We are grateful to Johan van der Gun (Bilthoven Biologicals), Arnoud Akkermans (National Institute for Public Health and the Environment (RIVM)), Bernard Metz (Intravacc), and Gideon Kersten (Intravacc) for helpful advice during the study. We thank Bas van de Waterbeemd (Intravacc) for preparing the *Bordetella pertussis* vaccines, prof. dr. R.J. Scheper (VUmc) for providing the MUTZ-3 cell lines and Jose Ferreira (RIVM) for advice on the statistical analysis.

References

1. Netherlands Food and Consumer Product Safety Authority. Zo doende. Utrecht: 2012.
2. van der Ark AA, Hozbor DF, Boog CJ, Metz B, van den Dobbelsteen GP, van Els CA. Resurgence of pertussis calls for re-evaluation of pertussis animal models. Expert review of vaccines. 2012;11(9):1121-37.
3. Seok J, Warren HS, Cuenca AG, Mindrinos MN, Baker HV, Xu W, *et al.* Genomic responses in mouse models poorly mimic human inflammatory diseases. Proceedings of the National Academy of Sciences of the United States of America. 2013. Epub 2013/02/13.
4. Libster R, Edwards KM. Re-emergence of pertussis: what are the solutions? Expert review of vaccines. 2012;11(11):1331-46.
5. Metz B, Jiskoot W, Mekkes D, Kingma R, Hennink WE, Crommelin DJ, *et al.* Quality control of routine, experimental and real-time aged diphtheria toxoids by *in vitro* analytical techniques. Vaccine. 2007;25(39-40):6863-71.
6. Metz B, Tilstra W, van der Put R, Spruit N, van den Ijssel J, Robert J, *et al.* Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid. Biologicals : journal of the International Association of Biological Standardization. 2013;41(4):231-7. Epub 2013/06/04.
7. Thalen M, van den IJ, Jiskoot W, Zomer B, Roholl P, de Gooijer C, *et al.* Rational medium design for *Bordetella pertussis*: basic metabolism. Journal of biotechnology. 1999;75(2-3):147-59.
8. Thalen M, Venema M, van den IJ, Berwald L, Beuvery C, Martens D, *et al.* Effect of relevant culture parameters on Pertussis Toxin expression by *Bordetella pertussis*. Biologicals : journal of the International Association of Biological Standardization. 2006;34(3):213-20.
9. Benjamini Y, Hochberg Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289-300.
10. Fedele G, Spensieri F, Palazzo R, Nasso M, Cheung GY, Coote JG, *et al. Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PloS one. 2010;5(1):e8734. Epub 2010/01/22.
11. Fransen F, Stenger RM, Poelen MC, van Dijken HH, Kuipers B, Boog CJ, *et al.* Differential effect of TLR2 and TLR4 on the immune response after immunization with a vaccine against Neisseria meningitidis or *Bordetella pertussis*. PloS one. 2010;5(12):e15692.
12. Higgins SC, Jarnicki AG, Lavelle EC, Mills KH. TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. J Immunol. 2006;177(11):7980-9.
13. Banus S, Stenger RM, Gremmer ER, Dormans JA, Mooi FR, Kimman TG, *et al.* The role of Toll-like receptor-4 in pertussis vaccine-induced immunity. BMC immunology. 2008;9:21.
14. Higgins SC, Lavelle EC, McCann C, Keogh B, McNeela E, Byrne P, *et al.* Toll-like receptor 4-mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to *Bordetella pertussis* by inhibiting inflammatory pathology. J Immunol. 2003;171(6):3119-27.
15. Rasaiyaah J, Noursadeghi M, Kellam P, Chain B. Transcriptional and functional defects of dendritic cells derived from the MUTZ-3 leukaemia line. Immunology. 2009;127(3):429-41.
16. Hoefnagel MH, Vermeulen JP, Scheper RJ, Vandebriel RJ. Response of MUTZ-3 dendritic cells to the different components of the Haemophilus influenzae type B conjugate vaccine: towards an *in vitro* assay for vaccine immunogenicity. Vaccine. 2011;29(32):5114-21. Epub 2011/06/01.
17. Re F, Strominger JL. Toll-like receptor 2 (TLR2) and TLR4 differentially activate human dendritic cells. The Journal of biological chemistry. 2001;276(40):37692-9.
18. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nature reviews Immunology. 2013;13(4):227-42.

19. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, *et al.* PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. The Journal of experimental medicine. 2009;206(13):3015-29.
20. McGuirk P, Mills KH. Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. European journal of immunology. 2000;30(2):415-22.
21. McGuirk P, McCann C, Mills KH. Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. The Journal of experimental medicine. 2002;195(2):221-31.
22. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. Nature reviews Immunology. 2013;13(6):412-26.
23. Hoffmann S, Peterbauer A, Schindler S, Fennrich S, Poole S, Mistry Y, *et al.* International validation of novel pyrogen tests based on human monocytoid cells. Journal of immunological methods. 2005;298(1-2):161-73.
24. Sallusto F, Lanzavecchia A. Monocytes join the dendritic cell family. Cell. 2010;143(3):339-40. Epub 2010/10/30.
25. Hendriksen C, Arciniega JL, Bruckner L, Chevalier M, Coppens E, Descamps J, *et al.* The consistency approach for the quality control of vaccines. Biologicals : journal of the International Association of Biological Standardization. 2008;36(1):73-7.
26. Giese C, Lubitz A, Demmler CD, Reuschel J, Bergner K, Marx U. Immunological substance testing on human lymphatic micro-organoids *in vitro*. Journal of biotechnology. 2010;148(1):38-45. Epub 2010/04/27.
27. Drake DR, Singh I, Nguyen MN, Kachurin A, Wittman V, Parkhill R, *et al. In vitro* Biomimetic Model of the Human Immune System for Predictive Vaccine Assessments. Disruptive science and technology. 2012;1(1):28-40.

5

Chapter 5

Adaptive immune response to whole cell pertussis vaccine reflects vaccine quality: a possible complementation to the Pertussis Serological Potency test

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Vaccine 2016 Aug; 34(37): 4429-4436

Abstract

Whole cell *Bordetella pertussis* (wP) vaccines are still used in many countries to protect against the respiratory disease pertussis. The potency of whole-cell pertussis vaccine lots is determined by an intracerebral challenge test (the Kendrick test). This test is criticized due to lack of immunological relevance of the read-out after an intracerebral challenge with *B. pertussis*. The alternative *in vivo* test, which assesses specific antibody levels in serum after wP vaccination, is the Pertussis Serological Potency test (PSPT). Although the PSPT focuses on a parameter that contributes to protection, the protective immune mechanisms after wP vaccination includes more elements than specific antibody responses only. In this study, additional parameters were investigated, i.e. circulating pro-inflammatory cytokines, antibody specificity and T helper cell responses and it was evaluated whether they can be used as complementary readout parameters in the PSPT to assess wP lot quality. By deliberate manipulation of the vaccine preparation procedure, a panel of high, intermediate and low quality wP vaccines were made. The results revealed that these vaccines induced similar IL-6 and IP-10 levels in serum four hours after vaccination (innate responses) and similar antibody levels directed against the entire bacterium. In contrast, the induced antibody specificity to distinct wP antigens differed after vaccination with high, intermediate and low quality wP vaccines. In addition, the magnitude of wP-induced Th cell responses (Th17, Th1 and Th2) was reduced after vaccination with a wP vaccine of low quality. T cell responses and antibody specificity are therefore correlates of qualitative differences in the investigated vaccines, while the current parameter of the PSPT alone was not sensitive enough to distinguish between vaccines of different qualities. This study demonstrates that assessment of the magnitude of Th cell responses and the antigen specificity of antibodies induced by wP vaccination could form valuable complementary parameters to the PSPT.

Introduction

Whole cell pertussis (wP) vaccines are used since the '50 and are still widely applied as an effective strategy in Latin America, Africa and Asia[1] to induce protection against whooping cough, caused by the Gram-negative bacterium *Bordetella pertussis*. Due to occasional side effects associated with wP vaccination, wP vaccines have been replaced by safer acellular pertussis (aP) vaccines in the 1990's in most industrialized countries. Though both aP vaccines and wP vaccines are effective in pertussis prevention campaigns, recent studies indicate that wP vaccination provides superior protection and durability of immunity.[2,3] It is therefore likely that wP vaccines will stay the vaccine of choice in many regions of the world in the near future.

Vaccine production is a biological process and is therefore associated with inherent variability in vaccine lot potency and safety, characteristics that are measured by regulatory required animal tests. The relevance of many of these *in vivo* models is disputable because they are not always susceptible to the pathogen of interest[4] and they do not always accurately mimic the human immune system.[5] Since mice are normally not susceptible for respiratory *B. pertussis* infection, the protective properties of wP vaccines are assessed by an intracerebral challenge of vaccinated mice, with lethality as end parameter.[4] Because it is unlikely that the pathology and immunology of a cerebral infection accurately reflects the responses induced by a natural respiratory infection in humans, and because of animal welfare reasons, there is a urgent need for alternative *in vivo* models and *in vitro* models that can provide a more accurate reflection of clinically relevant aspects of wP vaccine quality.

One of the alternatives under development is the Pertussis Serological Potency test (PSPT)[6-8], a test based on the detection of wP vaccine-induced *B. pertussis*-specific antibodies in mouse serum, a parameter that contributes to protection. However, the protective immune mechanisms after wP vaccination also depend on the vaccine's capacity to induce appropriate activation of innate immune responses and subsequent vaccine-specific T- and B cell responses. It has been shown that in humans *B. pertussis* infection as well as wP vaccination induce the formation of *B. pertussis*-specific antibodies[9,10], T helper (Th) 1 cells and/or Th17 directed cellular responses.[11-13] Similar adaptive immune mechanisms contribute to clearance of respiratory *B. pertussis* infection in mice.[14,15] In this study, we investigated whether other immune parameters can complement the current readout parameter of the PSPT, being *B. pertussis*-specific antibody responses, using wP vaccines of various protective properties (Metz *et al.* submitted). We studied the type of immune response these wP vaccines induce in two murine outbred strains (RIVM:NIH and CD1; strains used for wP potency testing) and evaluated whether the novel measured immune parameters reflect vaccine quality.

Materials and methods

Preparation of wP vaccines

In this study, experimental wP vaccine batches were prepared as described in detail elsewhere (Metz *et al.* submitted). Briefly, vaccines were generated from the *B. pertussis* strain 509 (Intravacc) cultured in a 3L bioreactor (2L working volume). The bioreactor was equipped with a six-bladed Rushton stirrer (Applikon, Schiedam, The Netherlands). After a pre-culture of the bacterium, the reactor was seeded at 5% (v/v), grown at 35°C and after 3-5 reactor volumes steady state was assumed. Subsequently, deliberate downregulation of virulence genes (t=0) was induced by addition of medium containing 50 mM $MgSO_4$. Meanwhile, $MgSO_4$ was added directly to the culture to instantly change the concentration to 50 mM $MgSO_4$. Samples were taken just before addition of $MgSO_4$ (0 hours (h)) and after 6 and 24h. To generate vaccines, the samples were inactivated by adding formaldehyde (16 mM) followed by heating at 56°C (for 10 minutes). Three individual runs were performed and corresponding vaccine preparations were pooled and referred to as vaccine A (t=0), vaccine C (t=6) and vaccine E (t=24). The vaccine were diluted to 8 IOU/mL and 0.5 IOU/mL based on the OD_{590nm} of the vaccines (1*OD_{590nm} = 20 IOU). The *in vivo* challenge test (Metz *et al.* submitted) revealed that the potency of vaccine A was the highest (7.0 IU/mL), the potency of vaccine C was intermediate (4.8 IU/mL) and the potency of vaccine E was the lowest (0.8 IU/mL) (Table 1). Vaccine A and C fulfilled the regulatory requirements with respect to potency, while vaccine E did not. In contrast to the behaviour of the vaccines in the intracerebral challenge test, differences in protective properties were not consistently found in respiratory infection experiments (data not shown). This discrepancy indicates that the intracerebral infection model has a higher sensitivity for subtle difference in wP vaccine quality, confirmed by earlier studies using similar vaccines.[16,17] If a respiratory challenge model would be used to detect qualitative differences between wP vaccines, adaptation and optimisation of this *in vivo* method will be required. In contrast, *in vitro* and immunochemical evaluation ([18], Hoonakker *et al.* submitted) showed that hTLR4 and moDC activating as well as the virulence protein content corresponds well with *in vivo* potencies (Table 1).

Table 1 Properties of vaccines used within this study

	Vaccine A	Vaccine C	Vaccine E	Reference
Virulence proteins content	High	Intermediate	Low	Hoonakker *et al.* 2015 submitted
Protective properties	7.0*	4.8*	0.8*	Metz *et al.* 2015 submitted
Activation of moDC	High	High/Intermediate	Low	Hoonakker *et al.* 2015 Biologicals
Activation of hTLR4	High	Intermediate	Low	Hoonakker *et al.* 2015 submitted
Activation of mTLR4	High	High	High	Hoonakker *et al.* 2015 submitted

* sufficient is a potency of at least 4 IU/mL and 95% interval with a lower limit of 2 IU/mL

Ethical statement and immunisation of mice

This study was approved by the local Committee on Animal Experimentation of PD-Alt under permit numbers 201300053 and 201400128. Groups of adult (20-24 gram) mice (n = 6 mice/group) of the outbred RIVM:NIH strain were vaccinated once i.p. with wP vaccine A, C or E at a dose of 0.25 IOU/mouse or 4 IOU/mouse, or were not vaccinated. Adult (20-24 gram) mice of the outbred CD1 strain (Harlan, the Netherlands) were injected once i.p. with wP vaccine A, C or E at a dose of 4 IOU/mouse wP vaccine (n = 12 mice/group) or PBS (n = 8 mice). Similar to the conventional PSPT, all groups consisted of an equal number of females and males. CD1 and NIH mice both originate from Swiss mice, but their lineages were separated in 1930.[19] These mouse strains are used for quality control testing of wP vaccines, i.e. for the Kendrick test[20] and the PSPT[7].

From the CD1 mice, blood samples were taken by orbital puncture 4h after vaccination. 28 days after vaccination, CD1 and RIVM:NIH mice were bled under anaesthesia (isoflurane in O_2) and sacrificed (cervical dislocation) and sera and spleens were isolated. Whole blood was collected in blood collection tubes (Z Serum Sep GOLD, Greiner Bio-One), centrifuged (15 min, 3500 r.p.m.) and serum was stored at −80 °C until further use.

Analysis of *B. pertussis*-specific antibodies

IgG1 and IgG2A antibodies were measured using ELISA plates coated with inactivated whole *B. pertussis* bacteria (Kh96/01 or BP18323 as indicated). Sera were diluted 1:100 followed by a dilution series of 1:3. *B. pertussis*-specific antibodies were detected by HRP-conjugated goat-anti-mouse IgG1 and IgG2A antibodies (Southern Biotech) and subsequent incubation with peroxidase substrate for 10 minutes, and 2M H_2SO_4. The presented values are the detected ODs at 450nm (ELISA reader, Bio-Tek) x dilution of the serum. The limit of detection for both antibody subclasses was at an OD of 0.1. The sera of vaccinated but non-responding mice (OD < 0.1) were excluded from the 2D electrophoresis analysis.

The antigen specificity of the IgG antibodies was analysed as described in detail by Raeven *et al.*[21]. Briefly, *B. pertussis* (strain 509) lysate was separated by 2D electrophoreses and transferred to a nitro cellulose membrane. For both mouse strains, blots were incubated with pooled sera of vaccinated or naïve mice (diluted 1:1000), followed by incubation with a goat-anti-mouse IgGtotal antibody labelled with IR800 (diluted 1:5000) and spots were analyzed using an Odyssey infrared imager and Delta2D software (Version 4.5). Spots were manually excised from the Coomassie stained gels and digested with trypsine in 50 mM triethylamomium bicarbonate buffer (pH 8.5). After vacuum drying and dissolving in formic acid/dimethyl sulfoxide/water (0.1/5/94.9%), peptides were analysed by LC-MS on a Orbitrap Fusion Lumos mass spectrometer (MS) according to the procedure described in detail by

Raeven *et al.*[21]. Before 2D electrophoresis, the identity and relative content of the proteins in the heat inactivated vaccines was analysed by MS by Metz *et al.* (submitted).

wP specific *in vitro* stimulation of splenocytes

Homogenized spleens were treated with erythrocyte lysis buffer. The splenocytes were cultured in 24-well plates (9×10^6 cells/well (Casy TTC Roche)) in complete medium (IMDM medium (Gibco), supplemented with 8% FCS, 1% L-Glutamine–Penicillin–Streptomycin solution (Sigma), and 20 µM β-mercaptoethanol (Sigma)). The cells were stimulated with wP vaccine (3 µg/ml) or medium and culture supernatant was collected after 3 or 6 days (as indicated).

Cytokine profiling using multiplex technology and ELISA

Cytokine levels in culture supernatant of the splenocytes and sera 4h after immunisation were measured using a customized Milliplex mouse cytokine 4-plex Luminex kit (IL-5, IL-10, IL-17 and TNFα) and 5-plex Luminex kit (IL-12p40, IL-12p70, IP-10, IL-6 and IL-1α) (Millipore) respectively, according to the manufacturer's protocol. Measurements and data analysis were performed with Bio-Plex 200, using Bio-Plex Manager software (version 5.0, Bio-Rad Laboratories). IFN-γ in culture supernatant was detected using a commercial ELISA kit (R&D systems), according to the manufacturer's protocol.

Intracellular cytokine staining of *in vitro* wP vaccine stimulated splenocytes

Intracellular cytokine staining (ICS) was performed on *in vitro* cultured splenocytes, isolated and stimulated as described above. Before intracellular cytokine staining, the splenocytes of the CD1 mice were re-cultured in U-bottom 96-well plates and restimulated overnight (ON) with wP vaccine (3 µg/ml) or medium for intracellular cytokine analysis using flowcytometry. A pilot experiment showed that a 3 days culture (9×10^6 cells/well) followed by ON restimulation resulted in the highest sensitivity and percentage of viable cells (data not shown). After culture and stimulation, splenocytes were treated with 10 µg/ml Golgi-plug (BD Biosciences), 1 µg/ml αCD28 (BD Pharmingen), and 1 µg/ml αCD49d (BD Pharmingen) for 5h. Splenocytes were then stained with Pacific blue-conjugated anti-CD4 (Biolegend), FITC-conjugated anti-CD44 (BD Biosciences), and with LIVE/DEAD Aqua (Invitrogen). Subsequently, splenocytes were fixed, permeabilised using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (BD Biosciences), according to the manufacturer's protocol, followed by staining with PE-conjugated anti-IFNγ (BD Biosciences), APC-conjugated anti-IL-5 (Biolegend), and PerCP-Cy5.5-conjugated anti-IL-17a (eBioscience). Data were acquired using a FACS Canto II (BD Biosciences), gated (Supplementary Figure S1) and analysed using FlowJo software (Tree Star).

Statistical analysis

After log-transformation (Figure 1, 3 and 4 and Table 2), significant differences were analysed using a Student's t-test. The Benjamini-Hochberg method was used for correction of multiple comparisons.[22]

Results

wP vaccine quality does not affect early innate cytokine levels measured in serum

Because of the pivotal role of innate immune responses for protective immunity, cytokine/chemokine levels in sera of CD1 mice were studied shortly (4h) after vaccination. Immunisation with all wP vaccines induced elevated levels of IP-10 and IL-6 (Figure 1), while none of the vaccines induced detectable levels of IL-12p40 or IL-12p70 in serum (data not shown). Vaccine A (high quality) and E (low quality) slightly reduced the level of IL-1α in serum compared to naïve mice, while there was no effect of vaccine C (intermediate quality) on the level of this cytokine. The levels of the assessed cytokines were not significantly different between the vaccine groups, indicating that wP vaccine quality did not affect the detected innate immune cell response in these mice.

B. pertussis-specific IgG levels are not related to wP vaccine quality

Evaluation of the conventional readout parameter of the PSPT, revealed that immunisation with all of the wP vaccines resulted in IgG1 and IgG2 antibodies directed against the *B. pertussis* bacterium in both outbred mouse strains (Table 2). In general, there were no significant differences between the vaccine groups, except that vaccine C induced a higher IgG1 antibody response in RIVM:NIH mice compared to those induced by vaccine A (Table 2). No significant differences in total IgG levels were observed (data not shown). These data indicate that the experimental wP vaccines used within this study induced potent *B. pertussis*-specific IgG responses, but that in general these responses were not affected by vaccine quality.

Table 2 *B. pertussis* specific IgG levels[1] in sera 28 days after vaccination

Vaccination	RIVM/NIH[2]		CD1[3]	
	IgG1	IgG2A	IgG1	IgG2A
Unvaccinated	n.d.	n.d.	n.d.	n.d.
Vaccine A - 0.25 IOU	55 ± 23*	17 ± 15		
Vaccine C - 0.25 IOU	47 ± 20*	9 ± 2		
Vaccine E - 0.25 IOU	37 ± 37*	18 ± 12		
Vaccine A - 4 IOU	772 ± 519*	921 ± 590*	2283 ± 934*	1100 ± 953*
Vaccine C - 4 IOU	1904 ± 457*†	1436 ± 382*	2862 ± 1250*	1769 ± 1206*
Vaccine E - 4 IOU	658 ± 485*	760 ± 340*	1558 ± 759*	1319 ± 892*

[1] OD measured at 450nm x dilution of sera
[2] antibodies against *B. pertussis* strain 18323
[3] antibodies against *B. pertussis* strain Kh96/01
n.d. not detectable
* $p < 0.05$ vaccination vs naïve mice
† $p < 0.05$ vaccination vs vaccine A

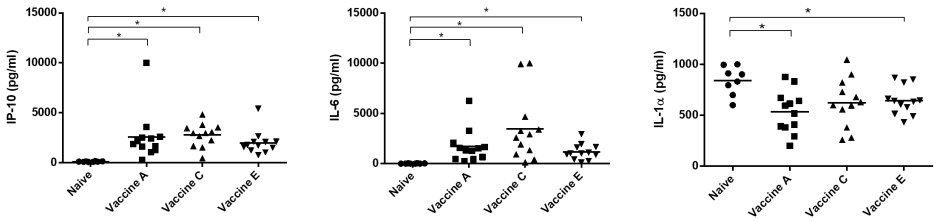


Figure 1 Innate cytokine levels in sera shortly after wP vaccination. CD1 mice were immunised i.p. with wP vaccine A, C, E (4 IOU/mouse, 12 mice per group) or were injected i.p. with PBS (8 mice). After 4h, blood samples were collected and cytokines levels in sera were determined using a multiplex assay. The measured cytokine levels (mean per group) are shown. Each dot represents one mouse. * significant difference between indicated groups for $p < 0.05$.

Antigen specificities of antibodies after wP vaccination are affected by vaccine quality

A combination of 2D electrophoresis and Western blotting was used to identify the specificity of wP vaccine-induced antibodies directed against particular *B. pertussis* proteins.[21] The analysis revealed much overlap between the specificities of antibodies produced by both mouse strains (RIVM:NIH and CD1). In sera of mice immunised with vaccine A, C and E, we identified significant antibodies levels directed against 18, 17 and 17 (RIVM:NIH), and 17, 19 and 14 antigens (CD1 mice), respectively (Figure 2B and 2C, Supplementary Figure S2 and S3). In agreement with the study by Raeven *et al.* using wP vaccine established from BP1917 in combination with 2D electrophoresis[21], we could not detect antibodies directed against PRN, FHA and PTx. In contrast, an earlier study by Stenger *et al.*[23] demonstrated that antibodies against FHA and PRN were detected upon immunization with a multivalent vaccine containing BP509 and BP134, using mouse multiplex immunoassay. The discrepancy might be the result of the used vaccine strain, other vaccine components, the sensitivity of the detection methods or a combination of these factors, though antibodies against aP proteins were detected in sera of aP vaccinated mice using 2D electrophoresis.[21] The identified proteins are involved in various processes including pathogenesis, cell adhesion, glycolysis, fatty acid synthesis and (ion) transport (Uniprot taxonomic identifier 257313A).

For many of the identified proteins, antibodies were detected in the sera after immunization with all of the wP vaccines, though levels were higher after immunisation with vaccine A compared to vaccine C and vaccine E. Vaccine A immunized mice of both strains produced higher antibody levels directed against 7 proteins (Figure 2B and 2C, numbers 1, 3, 6-10) than immunized with vaccine C or E. In addition, vaccine A immunized RIVM:NIH mice produced

A

Protein content vaccins

Vaccine A	Vaccine C	Vaccine E
2.87	1.76	0.40
0.44	0.75	0.06
0.15	0.13	0.09
0.05	0.07	0.08
0.15	0.20	0.30
0.08	0.05	0.00
0.31	0.84	1.48
0.14	0.24	0.13
9.73	12.02	11.86
0.45	0.81	0.77
0.26	0.12	0.00
-	-	-
0.02	0.01	0.01
0.00	0.00	0.00
0.38	0.52	0.41
0.01	0.00	0.01
0.00	0.00	0.00

Relative protein concentration in vaccines

0	0.25	0.5	2.25	5

Protein number	Protein[1]	Excession number[2]	ID	Number of spots	BKG[3]	RIVM:NIH mice Vaccine A	RIVM:NIH mice Vaccine C	RIVM:NIH mice Vaccine E	Number of spots	BKG[3]	CD1 mice Vaccine A	CD1 mice Vaccine C	CD1 mice Vaccine E
1	Putative parvulin-type peptidyl-prolyl cis-trans isomerase	P40415	BP3561	2	0.005	55.6	6.3	1.9	2	0.005	42.0	10.6	3.1
2	Autotransporter	Q79GN7	vag8	6	0.016	7.4	6.5	1.7	1	0.003	3.3	4.2	1.6
3	N-acetyl-gamma-glutamyl-phosphate reductase	Q7VUW0	argC	1	0.003	27.9	4.5	4.1	1	0.003	9.4	7.8	5.5
4	Electron transfer flavoprotein alpha-subunit	Q7VZE9	etfA	1	0.003	17.4	4.4	6.3	1	0.003	0.7	0.7	5.1
5	Isocitrate dehydrogenase [NADP]	Q7VVZ2	icd	2	0.005	4.9	1.1	1.0	-		0.0	0.0	0.0
6	Putative exported protein	Q7VUX9	BP2936	1	0.003	11.9	1.0	0.8	1	0.003	5.4	1.1	0.4
7	Fructose-bisphosphate aldolase	Q7VY44	fba	1	0.003	12.4	0.9	2.4	1	0.003	4.0	2.3	1.5
8	Putative exported solute binding protein	Q7VUV7	BP2963	1	0.003	40.9	1.6	2.2	1	0.003	12.4	3.3	2.9
9	60 kDa chaperonin	P48210	GroEL	2	0.005	30.7	5.9	20.9	3	0.008	32.6	4.7	17.8
10	Ketol-acid reductoisomerase	Q7VZU4	ilvC	3	0.008	23.4	1.5	11.8	2	0.005	13.4	3.3	5.6
11	BrkA autotransporter	Q45340	BrkA	1	0.003	14.7	6.0	10.4	1	0.003	6.6	9.3	3.8
12	Putative LPS	-	-	4	0.010	1.8	6.6	2.3	11	0.029	3.1	6.5	30.0
13	3-oxoacyl-[acyl-carrier protein] reductase	Q7VW31	fabG	1	0.003	1.5	7.5	1.0	1	0.003	3.2	14.1	0.4
14	Outer membrane porin protein BP0840	Q04064	BP0840	1	0.003	27.5	27.8	68.7	3	0.008	44.1	43.3	112.0
15	ATP synthase subunit beta	Q7VU44	atpD	2	0.005	6.4	7.1	22.0	1	0.003	4.1	7.3	6.0
16	Putative membrane protein	Q7VZ75	BP1057	1	0.003	3.4	2.7	14.5	1	0.003	11.5	9.3	2.9
17	BrkA autotransporter and tracheal colonization factor	Q45340/ Q79GX8	BrkA/ tcfa	1	0.003	1.9	2.5	1.9	1	0.003	1.0	16.9	0.3
18	Unidentified protein 1	-	-	6	0.016	13.6	6.1	9.7	8	0.021	13.7	13.7	54.8
19	Unidentified protein 2	-	-	9	0.023	20.9	12.2	27.8	10	0.026	5.4	24.0	106.4
20	Unidentified protein 3	-	-	1	0.003	1.0	6.4	1.1	-		0.0	0.0	0.0
21	Unidentified protein 4	-	-	3	0.008	3.1	6.3	9.1	1	0.003	1.7	7.9	1.6
22	Unidentified protein 5	-	-	3	0.008	6.4	19.9	27.1	4	0.011	9.5	35.6	23.5
23	Unidentified protein 6	-	-	1	0.003	1.1	1.4	6.8	-		0.0	0.0	0.0
24	Unidentified protein 7	-	-	1	0.003	1.7	1.6	3.2	1	0.003	2.1	11.5	1.8

B

C

Fluorescence intensities

0.0	2.0	4.0	23.0	50.0

[1] Proteins identified by LC-MS in spots excised from 2D-gel
[2] Accession number of proteins by Uniprot based on Tohama sequence (http://www.uniprot.org/)
[3] BKG: Average background of the immunoblot of naïve mice (= 0.0026) x number of spots

Figure 2 Antigen-specificity of IgG antibodies in sera of wP vaccinated mice. Lysate of *B. pertussis* strain 509 was separated by 2D electrophoresis and incubated with pooled sera of wP vaccinated (4 IOU/mouse) or naïve RIVM:NIH or CD1 mice. Non-responders were not included (OD < 0,1), being one mouse in the vaccine A group (RIVM:NIH), one mouse in the vaccine C group (CD1) and one mouse in the vaccine E group (CD1). Spots were manually excised from the Coomassie stained blots and analysed by LC-MS (supplementary Figure S2). Antibody specificity to 23 *B. pertussis* proteins were identified in sera of wP vaccinated mice. Based on experiment by Raeven *et al.*[21] one spot (number 12) was considered to correspond to antibody bound LPS. Proteins were ranked based on the responses of the RIVM:NIH mice according to the following order: vaccine A > C ≥ E, vaccine A > C < E, vaccine A < C > E, vaccine A ≤ C < E. The protein numbers in this figure and the spot numbers in Figure S2 correspond. (A) The relative content of the proteins identified after immunoblotting was analysed by Mass Spectrometry (adapted from Metz *et al.* submitted) and is shown for vaccine A, C and E as a heat map. (B) and (C) show the intensity of the spots after immunoblotting analysed for RIVM:NIH and CD1 mice separately. The intensities of the antibody bound spots were analysed in three individual blots in gray values using an Odyssey infrared imager. The intensities of multiple spots for one protein were enumerated and corrected for the background of this protein. A two fold increase above background was considered a significant induction of specific antibodies.

A
B
C
Medium
wP
IL-5 (pg/ml)
IL-17 (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-10 (pg/ml)
Naïve
Vaccine A - 0.25 IOU
Vaccine A - 4 IOU
Vaccine A
Vaccine C
Vaccine E

Figure 3 Th cell cytokine responses after immunisation with wP vaccines. Mice were injected i.p. with wP vaccine A, C, E (4 IOU/mouse), vaccine A (0.25 IOU/mouse) or PBS. The splenocytes were collected 28 days after vaccination and cultured in medium (medium) or medium supplemented with 3 µg/mL wP vaccine (wP). (A) Cytokine levels in culture supernatant after 6 days of cultivation of splenocytes obtained from untreated and immunised RIVM:NIH mice, receiving an indicated dose of vaccine A (n = 4-6 mice/group). (B) Cytokine levels in the supernatant of splenocytes of RIVM:NIH mice, immunised with 4 IOU/mouse of the indicated wP vaccines or left untreated (n= 4-6 mice/group), after 6 days of *in vitro* culture with wP vaccine. (C) Cytokine levels in the supernatant of splenocytes of CD1 mice, immunised with a 4 IOU/mouse of the indicated wP vaccines or left untreated (n = 8-12 mice/per group), after 3 days (optimal for additional ICS analysis) of *in vitro* culture with wP vaccine. The measured cytokines levels are presented as mean ± SD. * significantly different from naïve mice with $p < 0.05$. † significant difference between indicated groups for $p < 0.05$.

higher levels against 5 additional proteins (numbers 2, 4, 5, 11 and 18) and CD1 mice against 1 additional protein (number 16) compared mice that were administered with vaccine C or E.

Interestingly, there were a couple of proteins for which - after immunisation with vaccine C - antibody levels were lower or higher compared to vaccine A and E immunized mice. Lower amounts of antibodies induced by vaccine C were directed against protein number 9, 10, 11 and 19 (RIVM:NIH) and protein number 9 and 10 (CD1), while levels induced by vaccine C and directed against protein number 12, 13, 17 and 20 (RIVM:NIH) and protein number 11, 13, 21, 22 and 24 (CD1) were higher than induced by vaccine A en E. Moreover, antibody levels against specific proteins were dominant in sera of vaccine E immunized mice, being number 14, 15, 16, 22-24 (RIVM:NIH) and 4, 12, 14 and 18 and 19 (CD1).

Since the protein composition of the wP vaccines differs, availability of antigens could cause the distinct pattern of antibody responses. However the relative protein concentrations (Figure 2A) and spot intensities did not always follow the same order (e.g. protein number 7). Remarkably, antibody levels against protein number 14 were relatively high after immunization with all wP vaccines, while the level of this protein in the vaccines was below the level of detection suggesting that it has strong immunogenic properties. Nevertheless, for a number of proteins (e.g. number 1 and 6) antibody levels corresponded with the relative protein concentrations in the respective vaccines (Figure 2A). Collectively, these data show that the antigen-specificity of IgG antibodies varied between the groups immunised with the three wP vaccines under study. Based on this evaluation, proteins could be selected for an assay that enables detection of vaccine quality dependent antibody levels or specificities.

Magnitude of wP vaccine-specific Th cell cytokine responses is associated with vaccine quality

Analysis of the produced cytokines in culture supernatant of splenocytes indicative for the presence of several Th cell subsets (IL-5 for Th2 cells, IL-17 for Th17 cells, TNF-α and IFN-γ for Th1 cells and IL-10 for Treg cells), revealed that immunisation of RIVM:NIH mice with vaccine A resulted in wP specific and dose-dependent secretion of IL-5, IL-17 and TNF-α by splenocytes, but had no significant effect on IFN-γ and IL-10 production (Figure 3A and 3B). Similarly, immunisation of CD1 mice with wP vaccine A resulted in vaccine-specific secretion of IL-5 and IL-17 (Figure 3C). For both mouse strains, the magnitude of the IL-17 response induced by vaccine C and E was significantly lower than induced by vaccine A (Figure 3B and 3C). Compared to vaccine A, the level of TNF-α produced by splenocytes was significantly lower when RIVM:NIH mice were immunised with vaccine E and IL-5 levels were significantly lower when CD1 mice were immunised with vaccine C or E. In addition, immunisation of RIVM:NIH mice with vaccine C resulted in significantly reduced *B. pertussis*-specific IL-10 and TNF-α

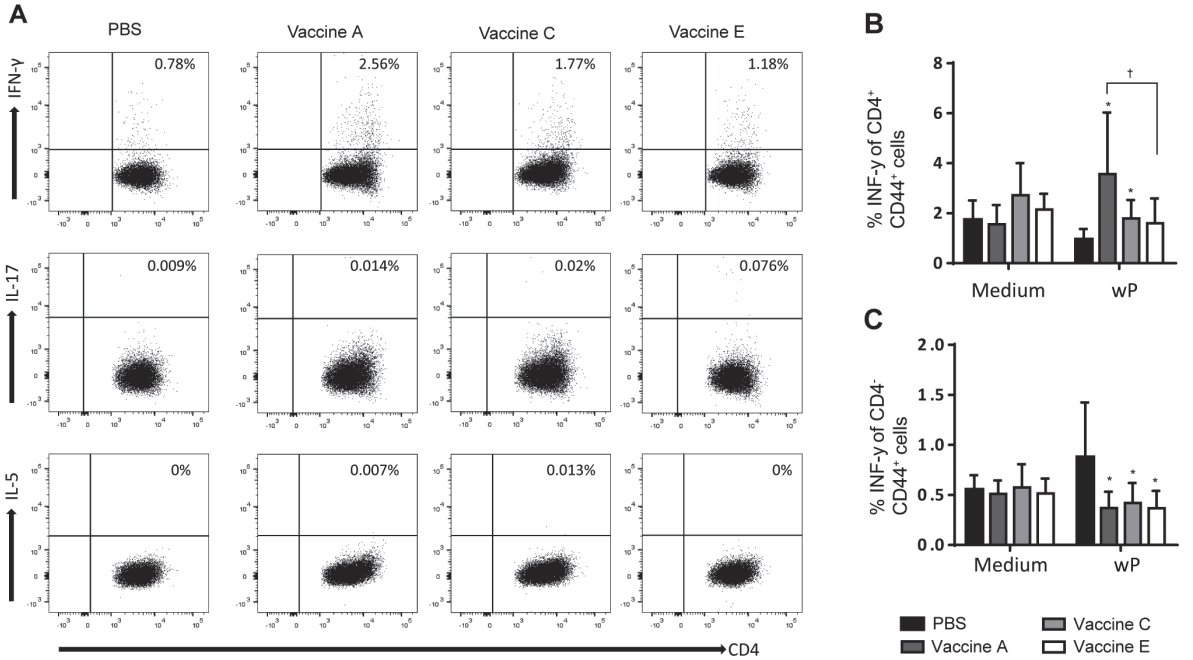


Figure 4 Intracellular cytokine staining of splenocytes of wP vaccinated mice. Groups of 12 CD1 mice were immunised i.p. with wP vaccine A, C, E (4 IOU/mouse). A group of 8 mice were injected i.p. with PBS (naïve mice). Splenocytes of mice were collected 28 days after vaccination and cultured for 3 days in medium or medium supplemented with 3 µg/mL wP vaccine. Then, cells were analysed by flowcytometry after an additional ON restimulation. (A) Dotplots of $CD4^+CD44^+$ T cells from one representative mice vaccinated as indicated and stimulated *in vitro* with wP vaccine. (B and C) Summary of the percentage IFN-γ positive cells within the $CD4^+CD44^+$ T cells or $CD4^-CD44^+$ cells, respectively, of mice vaccinated as indicated and stimulated *in vitro* with medium or wP vaccine.* significantly different from corresponding naïve mice with $p < 0.05$. † significant difference between indicated groups for $p < 0.05$.

responses compared to vaccines A and E. However, IL-10 levels did not exceed the IL-10 levels produced by naïve mice, indicating that the IL-10 production was not vaccine specific. In both mouse strains, no significant induction of IFN-γ secretion was detected in culture supernatants of vaccinated mice. Stimulation of the splenocytes with a cocktail of the aP vaccine antigens FHA, PTx and PRN resulted in a similar pattern, but generally lower levels of these cytokines (Figure S4).

Analysis of intracellular cytokine secretion at a single cell level revealed that the percentage of IFNγ-secreting Th cells ($CD4^+CD44^+$) was significantly lower when CD1 mice were immunised with vaccine E compared to vaccine A (Figure 4A and B), while there was no vaccine specific effect on the percentage of IL-17 and IL-5 positive $CD4^+CD44^+$ T cells (Figure 4A). This suggests that the kinetics of the analysed cytokines may differ. In addition, IFN-γ producing and activated non-CD4 cells ($CD4^-CD44^+$ cells) were identified, possibly $CD8^+$ T cells or NK cells, irrespective of the presence of wP stimulation in both naïve and vaccinated CD1 mice (Figure 4C). This constitutive production of IFN-γ may have masked the contribution of wP vaccine specific IFN-γ-producing $CD4^+$ T cells in supernatant analyses. Taken together, these data indicate that wP vaccine quality is reflected in the magnitude of pertussis cytokine responses indicative for specific Th cell subsets, i.e. Th1 and Th17 responses (both mouse strains) and Th2 responses (CD1).

Discussion

We studied whether serological properties in combination with additional biomarkers of the immune response to wP vaccination may reflect vaccine quality and can be used as a complementation to the current readout parameters in the Pertussis Serological Potency test (PSPT). Three major observations were made. Firstly, wP vaccination increased the levels of circulating pro-inflammatory cytokines and chemokines shortly after vaccination, and it induced IgG responses directed against the entire bacterium. However, these parameters did not discriminate between vaccines of varying qualities. Secondly, the wP vaccine of high quality (vaccine A) was a potent inducer of especially Th17 and Th1 cytokines, but also of Th2 responses, and induced a larger number of antibodies with distinct antigen specificities compared to the vaccines of lower quality. Thirdly, the effects were found in two outbred mouse strains. These outbred mouse strains (NIH and CD1) are commonly used for quality control testing (e.g. for the Kendrick test[20] and the PSPT[7]), since they provide a more relevant reflection of the variation within a human population than inbred mouse strains.[19,24,25] The data obtained provide evidence that determining the antigen specificity of wP vaccine-induced antibodies and the magnitude of wP vaccine-induced Th cell responses can provide useful and immunologically relevant additional parameters for assessing vaccine quality in the PSPT. Nevertheless, validation studies are needed before these parameters can be implemented.

Clearance of a respiratory B pertussis infection has been shown to depend on induction of appropriate Th cell responses, particularly Th1[15,26] and Th17-type responses[27,28], in combination with B cell and antibody responses. Here, we showed that a low wP vaccine quality (vaccine E) is associated with a reduced magnitude of such pertussis Th17 responses, and also Th1 and Th2 responses, and with a distinct antibody specificities compared to the responses induced by a vaccine of optimal quality (vaccine A). The observed differences in Th cell responses and antibody specificity are most likely not the result of variations in vaccine-induced innate immune responses, since we could not detect differences in the levels of cytokines in sera shortly after vaccination (Figure 1). However, since there are indications that lymphocytes contribute to protection against intracerebral infection[29,30], the Th1 and Th17 responses induced by the wP vaccines might have played a role in protective properties of the vaccines against intracerebral infection observed by Metz *et al.* (submitted). A study by Raeven *et al.* has recently demonstrated that antibodies induced by an experimental wP vaccine derived from *B. pertussis* strain BP1917 are primarily directed against BrkA, GroEL and Vag8[21]. In our study, both GroEL and Vag8 (protein number 9 and 2 respectively) as well as several other proteins were dominant in the humoral response of vaccine A compared to vaccine C and E immunized RIVM:NIH and CD1 mice. Remarkably, distinct antibody profiles were induced with different antigen-specificities after immunization with vaccine C or E. These characteristic antibody

patterns could partly be explained by the distinct amounts of specific antigens, such as lower levels of Vag8, BP3561 and argC (protein number 2, 1 and 3 respectively), in these vaccines and might have influenced vaccines potency. Of these proteins, Vag8 is described as a virulence protein[31] that contributes to resistance to complement mediated killing.[32] Furthermore, this protein has a role in vaccine-induced immunity.[33] To elucidate the exact contribution of Vag8 and other proteins to vaccine potency, further studied will be needed. Although these results show that vaccine quality can be recognised based on the pattern of antibody-specificity, 2D electrophoresis might be too complicated for routine testing. The analysis, however, might enable the selection of one of several candidate proteins, which can be used to establish a routine quality control test.

There are certain virulence pertussis antigens, e.g. filamentous hemagglutinin, pertussis toxin and pertactin described that exhibit Th cell immune-modulatory properties.[34] Antibodies directed against these proteins were not detected, but the different quantities of these proteins in the vaccines might have narrowed or changed the antigen-specificity of Th or Treg cell response and thereby affected the magnitude of the induced *B. pertussis*-specific T cell response. Further identification of the antigen specificity of wP-induced T cells will be needed to reveal the contribution of antigen-specific immunomodulation of the wP vaccine-induced T cell responses.

The PSPT was developed as an alternative to the current regulatory required intracerebral challenge test for wP vaccines.[6-8] The official medicines control laboratories network estimated that in Europe approximately 100.000 mice are required annually for wP potency testing (personal communication). The PSPT not only reduces distress levels inflicted on the animals, but also reduces animal number with approximately 25%.[7] Since B and T cell responses both contribute to whole cell vaccine induced protection, this study demonstrates the relevance of measuring T cell responses as well as antibody specificity and thereby contributes to the improvement of the current PSPT, though thorough and manufacturer specific evaluation, optimisation and validation studies will be required. Based on the results of this study, we hypothesise that Th17 cells and antibodies directed against Vag8, BP3561 and argC might contribute to wP vaccine induced protection, though further studies into the role of these immune components are required. In the scope of this study, application of parameters such as the magnitude of wP vaccine specific IL-17 responses detected in the supernatant and levels of antibodies directed against specific proteins (e.g. Vag8), might increase the probability of the implementation of the PSPT for wP vaccine lot release testing.

Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

Acknowledgements

This study was part of the programme Dutch ministry of Health, Welfare and Sports and the Dutch ministry of Economic Affairs that aims to develop alternatives to animal experiments. We are grateful to Johan van der Gun (Bilthoven Biologicals), Rob Vandebriel, Arnoud Akkermans, Jolanda Brummelman (RIVM), René Raeven and Gideon Kersten (Intravacc) for helpful advice during the study. We thank Michel Weyts (Intravacc) for preparing the experimental wP vaccines, and Jose Ferreira (RIVM) for advice on the statistical analyses.

Supplementary information

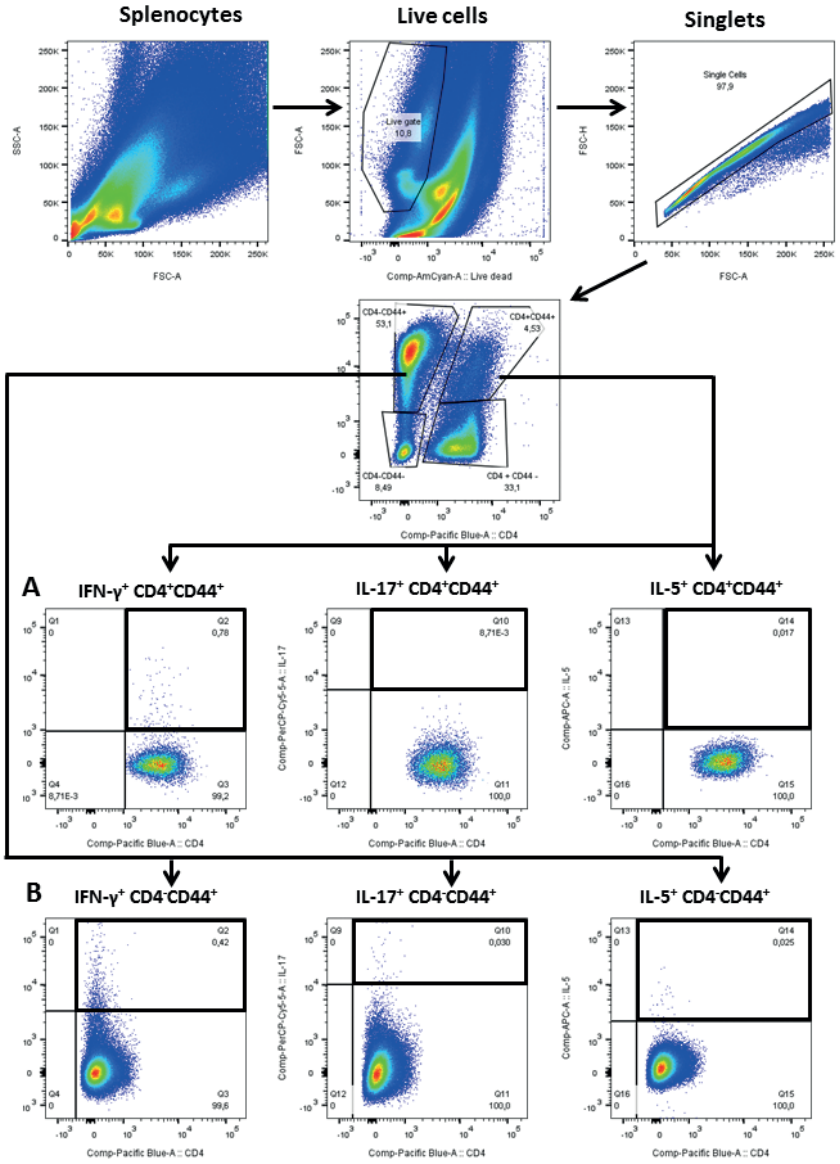


Figure S1 Gating strategy ICS data. To identify the CD4$^+$CD44$^+$ T cells for Figure 3. The live cells were first gated based on low Amcyan expression followed by exclusion of doublets based on FSC-A/FSC-H. Subsequently, activated (CD44$^+$) CD4$^+$ and CD4$^-$ expressing cells were selected. Within this population, the wP specific cytokine producing CD4$^+$ T (A) and CD4$^-$ (B) cells were analysed.

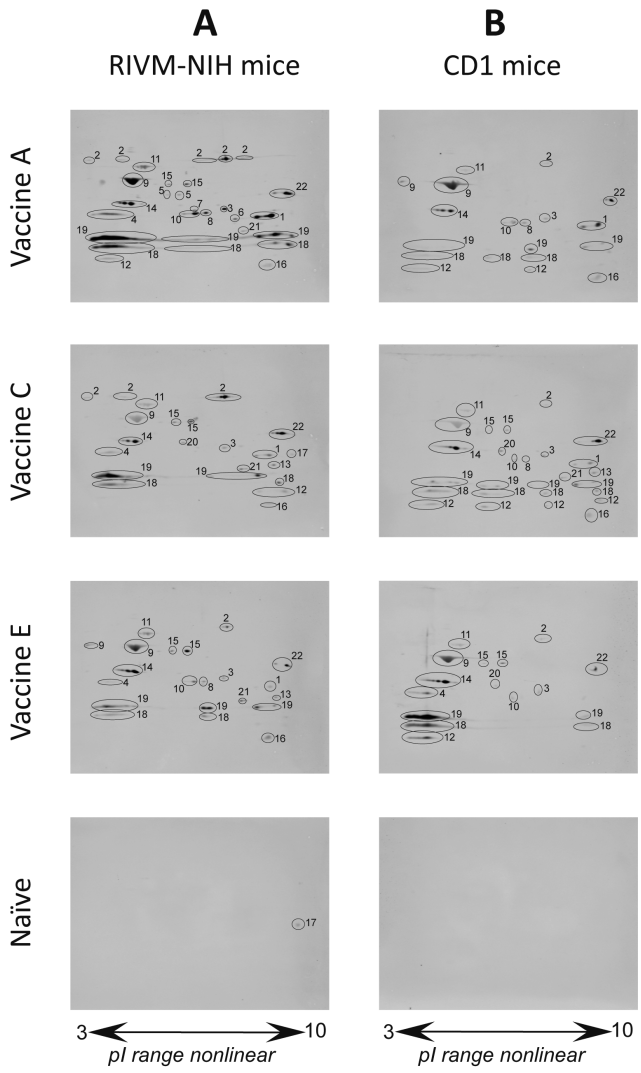


Figure S2 Immunostaining of 2D electrophorese gels of sera of wP vaccinated RIVM:NIH and CD1 mice. (A and B) Lysate of *B. pertussis* strain 509 was separated by 2D gel electrophoresis and the protein were blotted. Blots were incubated with the pooled sera of naïve or vaccine A, vaccine C or vaccine E immunised mice 28 days after vaccination (RIVM:NIH mice 4-6 mice per group, CD1 mice 11-12 mice/group). Pools of immunised mice only included sera for which IgG levels were detected in the IgG ELISA (Table 2). Subsequently, the bound antibodies were detected with IR800-labeled anti-IgG. The proteins in the spots encircled were analysed with LC-MS. The spot numbers in this figure and the protein numbers in Figure 2 correspond. The experiments were performed three times with similar results. One representative blot is shown per vaccine group.

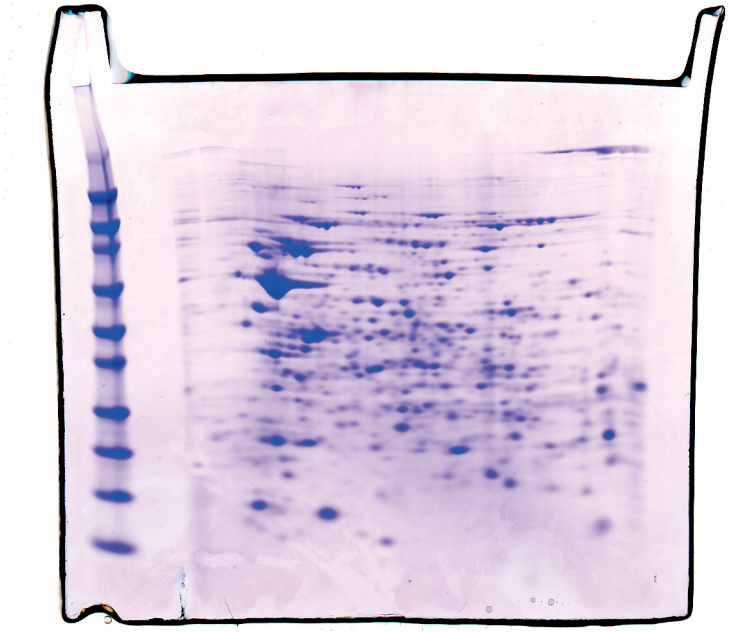
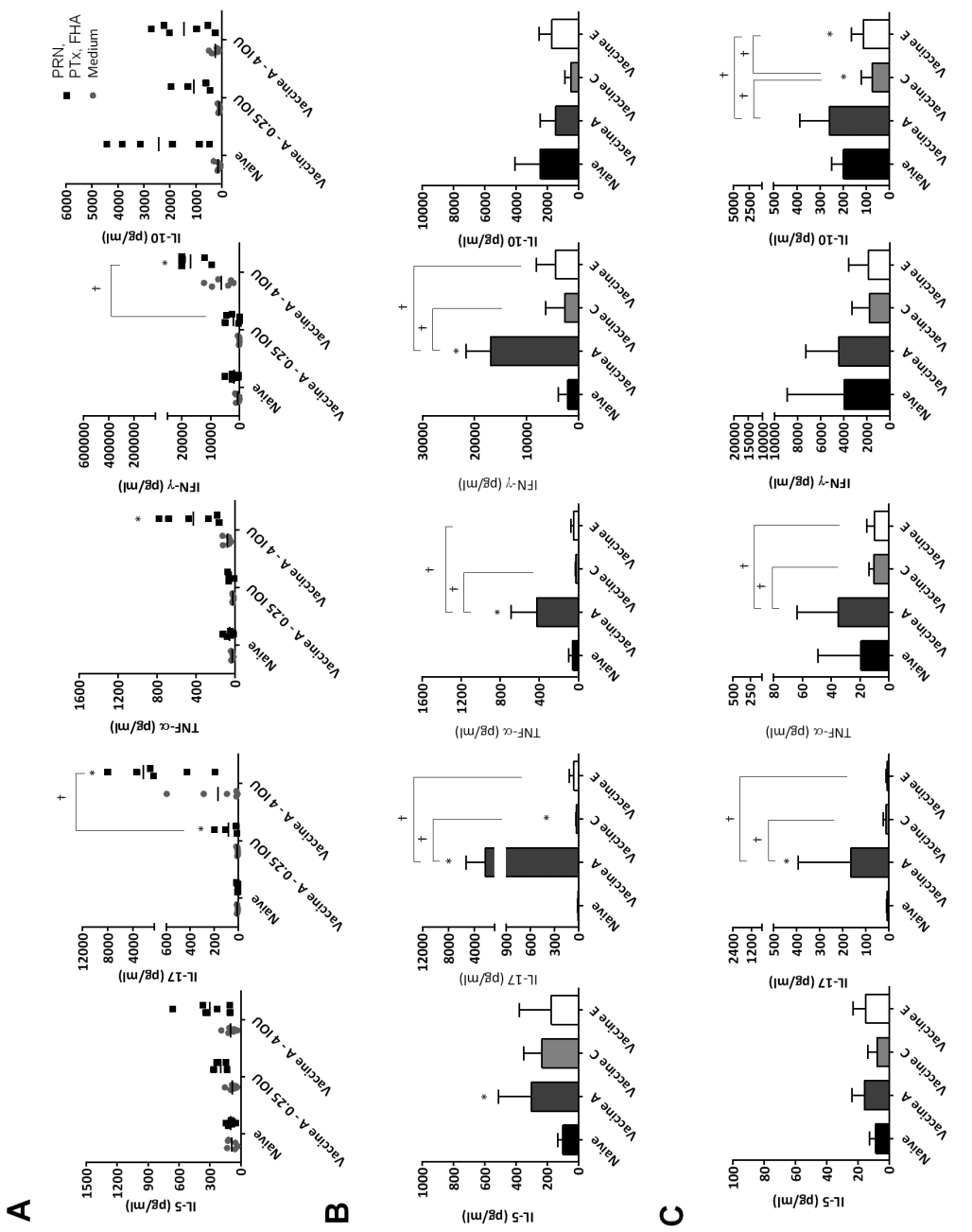


Figure S3 Coomassie staining of 2D electrophorese separated *B. pertussis* proteins. Lysate of *B. pertussis* strain 509 was separated by 2-dimensional gel electrophoresis and the protein were blotted. One 2D gel of strain BP509 was stained with Coomassie to identify proteins in Figure 2.

Figure S4 (right) Th cell cytokine responses after immunisation with wP vaccines. Mice were injected i.p. with wP vaccine A, C, E (4 IOU/mouse), vaccine A (0.25 IOU/mouse) or PBS. The splenocytes were collected 28 days after vaccination and cultured in medium or medium supplemented with a cocktail of 1 µg/mL PRN, 1 µg/mL PTx, 1 µg/mL FHA. (A) Cytokine levels in culture supernatant after 6 days of cultivation of splenocytes obtained from untreated and immunised RIVM:NIH mice, receiving an indicated dose of vaccine A (n = 4-6 mice/group). (B) Cytokine levels in the supernatant of splenocytes of RIVM:NIH mice, immunised with 4 IOU/mouse of the indicated wP vaccines or left untreated (n= 4-6 mice/group), after 6 days of *in vitro* culture with PRN, PTx and FHA. (C) Cytokine levels in the supernatant of splenocytes of CD1 mice, immunised with 4 IOU/mouse of the indicated wP vaccines or left untreated (n = 8-12 mice/per group), after 3 days (optimal for additional ICS analysis) of *in vitro* culture with PRN, PTx and FHA. The measured cytokines levels are presented as mean ± SD. * significantly different from naïve mice with $p < 0.05$. † significant difference between indicated groups for $p < 0.05$.


A
B
C
PRN, PTx, FHA
Medium
IL-5 (pg/ml)
IL-17 (pg/ml)
TNF-α (pg/ml)
IFN-γ (pg/ml)
IL-10 (pg/ml)
Naive
Vaccine A - 0.25 IOU
Vaccine A - 4 IOU
Vaccine A
Vaccine C
Vaccine E

References

1. Libster, R. and K.M. Edwards, Re-emergence of pertussis: what are the solutions? Expert Rev Vaccines, 2012. 11(11): p. 1331-46.
2. Witt, M.A., P.H. Katz, and D.J. Witt, Unexpectedly limited durability of immunity following acellular pertussis vaccination in preadolescents in a North American outbreak. Clin Infect Dis, 2012. 54(12): p. 1730-5.
3. Gambhir, M., *et al.*, A change in vaccine efficacy and duration of protection explains recent rises in pertussis incidence in the United States. PLoS Comput Biol, 2015. 11(4): p. e1004138.
4. van der Ark, A.A., *et al.*, Resurgence of pertussis calls for re-evaluation of pertussis animal models. Expert Rev Vaccines, 2012. 11(9): p. 1121-37.
5. Seok, J., *et al.*, Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc Natl Acad Sci U S A, 2013.
6. van der Ark, A., *et al.*, Development of pertussis serological potency test. Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. Biologicals, 1994. 22(3): p. 233-42.
7. van der Ark, A., *et al.*, The Pertussis Serological Potency Test. Collaborative study to evaluatereplacement of the Mouse Protection Test. Biologicals, 2000. 28(2): p. 105-18.
8. von Hunolstein, C., *et al.*, Evaluation of two serological methods for potency testing of whole cell pertussis vaccines. Pharmeuropa Bio, 2008. 2008(1): p. 7-18.
9. Cherry, J.D., *et al.*, A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. Vaccine, 1998. 16(20): p. 1901-6.
10. Mahon, B.P., *et al.*, Atypical disease after *Bordetella pertussis* respiratory infection of mice with targeted disruptions of interferon-gamma receptor or immunoglobulin mu chain genes. J Exp Med, 1997. 186(11): p. 1843-51.
11. Ausiello, C.M., *et al.*, Vaccine- and antigen-dependent type 1 and type 2 cytokine induction after primary vaccination of infants with whole-cell or acellular pertussis vaccines. Infect Immun, 1997. 65(6): p. 2168-74.
12. Higgs, R., *et al.*, Immunity to the respiratory pathogen *Bordetella pertussis*. Mucosal Immunol, 2012. 5(5): p. 485-500.
13. Ryan, M., *et al.*, Distinct T-cell subtypes induced with whole cell and acellular pertussis vaccines in children. Immunology, 1998. 93(1): p. 1-10.
14. Mills, K.H., *et al.*, A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun, 1998. 66(2): p. 594-602.
15. Ross, P.J., *et al.*, Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog, 2013. 9(4): p. e1003264.
16. Bellalou, J. and E.H. Relyveld, Studies on culture conditions of *Bordetella pertussis* and relationship to immunogenicity of vaccines. Ann Microbiol (Paris), 1984. 135B(1): p. 101-10.
17. Dolby, J.M., J.P. Ackers, and D.E. Dolby, The effect of the antigen which elicits the bactericidal antibody and of the mouse-protective antigen on the growth of *Bordetella pertussis* in the mouse brain. J Hyg (Lond), 1975. 74(1): p. 71-83.
18. Hoonakker, M.E., *et al.*, *In vitro* innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle. Biologicals, 2015. 43(2): p. 100-9.

19. Chia, R., *et al.*, The origins and uses of mouse outbred stocks. Nat Genet, 2005. 37(11): p. 1181-6.
20. van Straaten-van de Kappelle, I., *et al.*, Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biologicals, 1997. 25(1): p. 41-57.
21. Raeven, R.H., *et al.*, Immunoproteomic Profiling of *Bordetella pertussis* Outer Membrane Vesicle Vaccine Reveals Broad and Balanced Humoral Immunogenicity. J Proteome Res, 2015.
22. Benjamini, Y. and Y. Hochberg, Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society. Series B (Methodological), 1995. 57(1): p. 289-300.
23. Stenger, R.M., *et al.*, Impaired long-term maintenance and function of *Bordetella pertussis* specific B cell memory. Vaccine, 2010. 28(40): p. 6637-46.
24. WHO, Manual for Quality Control of Diphtheria, Tetanus and Pertussis Vaccines. http://apps.who.int/iris/bitstream/10665/80681/1/WHO_IVB_11.11_eng.pdf, 2013: p. 12.
25. Aldinger, K.A., *et al.*, Genetic variation and population substructure in outbred CD-1 mice: implications for genome-wide association studies. PLoS One, 2009. 4(3): p. e4729.
26. Mills, K.H., *et al.*, Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun, 1993. 61(2): p. 399-410.
27. Dunne, A., *et al.*, Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against *Bordetella pertussis*. J Immunol, 2010. 185(3): p. 1711-9.
28. Andreasen, C., D.A. Powell, and N.H. Carbonetti, Pertussis toxin stimulates IL-17 production in response to *Bordetella pertussis* infection in mice. PLoS One, 2009. 4(9): p. e7079.
29. Adams, G.J. and J.W. Hopewell, Enhancement of intracerebral infection of mice with *Bordetella pertussis*. J Med Microbiol, 1970. 3(1): p. 15-27.
30. Dolby, J.M., D.E. Dolby, and C.J. Bronne-Shanbury, The effects of humoral, cellular and non-specific immunity on intracerebral *Bordetella pertussis* infections in mice. J Hyg (Lond), 1975. 74(1): p. 85-102.
31. Finn, T.M. and D.F. Amsbaugh, Vag8, a *Bordetella pertussis* bvg-regulated protein. Infect Immun, 1998. 66(8): p. 3985-9.
32. Marr, N., *et al.*, *Bordetella pertussis* autotransporter Vag8 binds human C1 esterase inhibitor and confers serum resistance. PLoS One, 2011. 6(6): p. e20585.
33. de Gouw, D., *et al.*, Proteomics-identified Bvg-activated autotransporters protect against *Bordetella pertussis* in a mouse model. PLoS One, 2014. 9(8): p. e105011.
34. McGuirk, P., C. McCann, and K.H. Mills, Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J Exp Med, 2002. 195(2): p. 221-31.

6

Chapter 6

The cAMP assay: a functional *in vitro* alternative to the *in vivo* Histamine Sensitization test

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Vaccine 2010 Feb; 28(5): 1347-1352

Abstract

Safety requirements stipulate the performance of the *in vivo* Histamine Sensitization (HIST) test for quality control of acellular pertussis (aP) vaccines. For reasons of reproducibility and animal welfare concern, an *in vitro* assay was developed. The assay reflects the mechanism of histamine sensitization and is based on cAMP production in A10 cells to residual pertussis toxin (PTx). We showed that PTx induces cAMP levels in a dose dependent manner while the sensitivity of the assay equals the sensitivity of the HIST. Neither the individual components nor the combination vaccine DTaP-IP did affect the assay. The cAMP assay meets the criteria for specificity and sensitivity and therefore might be a promising candidate to replace the HIST.

Introduction

Whooping cough is a disease caused by the gram negative bacterium *Bordetella pertussis*. The disease mainly affects children and can ultimately be lethal. For that reason vaccination against pertussis is part of most national immunisation programmes worldwide. The first vaccines were based on heat or chemically inactivated whole bacteria. Due to the frequent side effects of these vaccines, which are partly attributed to residual active pertussis toxin (PTx), "safer" acellular pertussis (aP) vaccines were developed. During pathogenesis, PTx binds to receptors on respiratory cells, macrophags and lymphocytes.[1] It acts as a cooperative adhesin and as an exotoxin. *In vitro*, exotoxin function is established by translocation of one of PTx' subunit into the cytosol where it becomes enzymatically active generally leading to the uncoupling of G proteins from their receptors.[2] Although animal studies demonstrate that PTx causes systematic symptoms such as lymphocytosis, insulinemia/hypoglycaemia, histamine sensitivity and delay of neutrophil recruitment, the exact contribution of PTx to whooping cough in humans remains unclear.[2,3]

As it has been described that inactivated PTx (PTd) is essential for an effective immune response[4,5], all aP vaccines on the market contain this antigen. Due to incomplete inactivation or reversion of PTd to active PTx a low amount of active PTx can remain in the vaccine. Therefore, most pharmacopoeias, such as the European Pharmacopoeia (Ph. Eur.), specify the Histamine Sensitization (HIST) test for the detection of residual active PTx.[6] This *in vivo* test is based on the empirical finding that vaccination of mice with vaccines containing active PTx decreases the lethal histamine dose.[7] The HIST has three disadvantages: 1. poor reproducibility of test results, 2. high variability with regard to sensitivity of different mouse strains[8] and 3. animal welfare concerns as the test may be accompanied by severe animal suffering. Although several alternatives such as the mouse weight gain test, the leukocytosis promoting test[9,10], the chinese hamster ovary test[11,12] and HPLC assays[13,14] have been developed, all of them have one or more disadvantageous that makes them less suitable as a release test.

Recently, an *in vitro* alternative to the HIST test was described by Van Meijeren.[15] Based on the use of a rat vascular smooth muscle cell line (A10 cells), the assay reflects the mechanism of histamine sensitization *in vivo*. This *in vitro* assay is founded upon the PTx catalysed transfer of an ADP-ribose moiety (of NAD^+) to uncoupled G proteins.[4] The resulting inability of these proteins to inhibit adenylate cyclase causes a stimulation of adenylate cyclase to result in an unrestrained conversion of ATP into cAMP. Because isoprenaline, as a β_2-adenoceptor agonist, better corresponds with cellular mechanisms than histamine, isoprenaline was used to mimic histamine stimulation *in vivo*. After stimulation, cAMP levels produced by A10 cells can be measured with the aid of a (commercially available) cAMP ELISA. Although the principle of the

assay was demonstrated by Van Meijeren, the assay was not ready for use yet. The present study aimed to improve the sensitivity and determine the effect of the adjuvant $AlPO_4$ and other constitutes of the aP vaccine, e.g. filamentous hemaglutinin and pertactin, on cAMP production. Furthermore, we aimed to identify sources of variation with the intention of increasing standardization.

Materials and Methods

Cells

Rat vascular smooth muscle cells (A10 cells) obtained from the American Type Culture Collection (ATCC) were cultured in Dulbecco Modified Eagle's Medium (Gibco) containing 4,5 g L^{-1} glucose, 1.0 mM sodium pyruvate, 1.5 g L^{-1} sodium bicarbonate, supplemented with 10% fetal calf serum (v v^{-1}), 100 units ml^{-1} of penicillin and 100µg ml^{-1} streptomycin at 37°C and 5% CO_2. The cells were harvested every 2-4 days with trypsine-EDTA (trypsine 0.05%) (ICN Biomedicals). A10 cells were controlled for mycoplasma contamination by ATCC and certified to be free. Cells were banked at the NVI and used in experiments under GMP conditions.

PTx treatment and cAMP assessment

The day prior to the experiment A10 cells were harvested and sub cultured in 6-well plates (Greiner Bio-one, The Netherlands) at a density of $2.5x10^5$-$3.0x10^5$ cells $well^{-1}$. The next day, the medium was exchanged for 2 mL fresh medium, which contained the samples of interest.

Samples were incubated for 2 hours, after which the cells were treated with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) for 5 minutes, followed by stimulation with isoprenaline for 15 minutes (both chemicals were obtained from Sigma, The Netherlands). Macroscopic examination revealed no significant morphological changes after PTx or isoprenaline addition.

After the optional addition of isoprenaline, cellular processes were stopped by aspiration of the medium, washing with Phosphate Buffered Saline (PBS) and the addition of 1 mL ice cold 65% ethanol. Afterwards, cells were harvested with cell scrapers (Greiner Bio-one, The Netherlands) and the suspension was transferred to microcentrifuge tubes. This procedure was repeated with 0.5 mL ice cold 65% ethanol.

For destruction of cell membranes and total release of cAMP, the cell suspension was sonicated for 5 seconds at 47% capacity of a Soniprep 150 (Beun de Ronde, The Netherlands). To control for cell number, cAMP samples were separated from protein samples by centrifugation at 2000 g and 4°C for 15 minutes. After aspiration of the supernatant, protein pellets were dissolved in 400 µL distilled water.

The supernatants, containing the cAMP, were evaporated at 60°C under a flow of nitrogen. The dried cAMP samples were re-suspended in the assay buffer obtained from a commercial cAMP ELISA kit (Amersham Bioscience, United Kingdom). cAMP and protein samples were stored overnight at - 80°C and -30°C respectively. Afterwards, the cAMP values were determined using the cAMP ELISA kit, whereas protein levels were assessed using a commercially available

Pierce BCA™ protein assay kit (Thermo Scientific, U.S.A.). Standard protocol for the ELISA was followed, except that the enzyme substrate for the cAMP ELISA was incubated for 15 minutes instead of 60 minutes. A standard protocol was also used for the protein assessment, including the removal of surplus fluid with filter paper.

Samples, positive and negative controls

Unless otherwise mentioned cells with passage number 8 were used for the experiments. Cells treated with only medium and isoprenaline are indicated as positive controls. Samples without stimulation of isoprenaline served as negative controls to test for non-specific cAMP production. For assessment of test specificity, sensitivity and standardization, cells were treated with different quantities (0, 25, 50, 100 and 200 ng PTx) of active PTx (Sanofi, Canada), heat inactivated PT (PTd) or specific components of the acellular pertussis vaccine: filamentous hemaglutinin (FHA), pertactin (PRN), fimbriae (FIM) (Sanofi, Canada), the adjuvant $AlPO_4$ (Netherlands Vaccine Institute, The Netherlands) or the complete DTaP-IP vaccine (Netherlands Vaccine Institute, The Netherlands). Furthermore, DTaP-IP vaccine was spiked with 0, 25, 50, 100 and 200 ng PTx.

Statistical analysis

Data are presented as mean values ± the standard deviation. Since some of the obtained data were not normally distributed and the sample sizes were relatively small, the data were analyzed by non-parametrical statistics. All tests were calculated using the software package SPSS 16.0. Two independent samples were compared using the Mann–Whitney U test. Multiple dependent samples were analyzed with the Willcoxon signed ranks test. Variances were compared with Levene's test for variance. Differences were considered significant if their probability of occurring by chance was less than 5%.

Results

Sensitivity of PTx induced cAMP production

The sensitivity of PTx induced cAMP production by A10 cells was determined after isoprenaline stimulation. Cells were cultured in the presence of 0-200 ng PTx. A10 cells not stimulated with isoprenaline, but treated with similar amounts of PTx, served as negative controls. An isoprenaline specific PTx induction of cAMP was noted in a dose-dependent manner (Figure 1A; Spearman correlation, n = 55, ρ = 0.872, $p < 0.001$). The experiments revealed that cAMP produced in the presence of 25, 50, 100 and 200 ng PTx significantly differed from cAMP levels produced in the presence of 0 ng PTx (Figure 1A; Mann–Whitney U test, $n_{0ng} = 22$, $n_{25ng} = 4$, $n_{50ng} = 11$, $n_{100ng} = 7$, $n_{200ng} = 11$, $U_{0ng\text{-}25ng} = 1$, $p < 0.001$, $U_{0ng\text{-}50ng} = 2$, $p < 0.001$, $U_{0ng\text{-}100ng} = 0$, $p < 0.001$, $U_{0ng\text{-}200ng} = 0$, $p < 0.001$). Without isoprenaline stimulation, the addition of medium or pertussis toxin resulted in almost undetectable cAMP levels (Figure 1B).

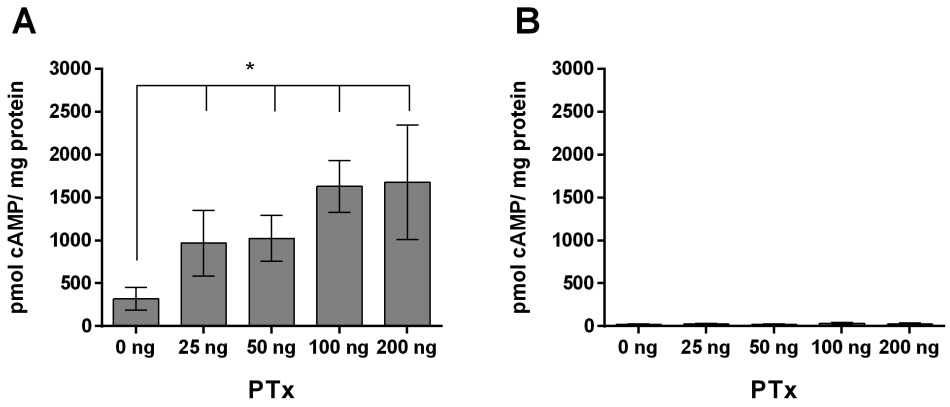

Figure 1 Sensitivity of the cAMP assay for varying quantities of PTx. cAMP production of A10 cells per mg protein after 120 minutes incubation with varying concentrations of PTx (x-axis) followed by the addition of: (A) the stimulating agonist isoprenaline and (B) no stimulation. * $p < 0.05$, significantly different from cAMP levels produced by medium treated control cells.

Specificity of PTx induced cAMP production

The specificity of cAMP production by A10 cells was determined by the exposure of A10 cells to the aP vaccine components FHA, PRN, FIM or $AlPO_4$ and subsequent stimulation of the cells with isoprenaline (Figure 2). The added quantities of each component equalled the quantities normally present in one human dose of aP vaccine (10 µg FHA, 2.5 µg FIM, 1.5 µg PRN, 10 µg PTd, 1500 µg $AlPO_4$). Medium treated cells served as positive controls. In addition, PTx specific cAMP induction was examined by treating cells with 50 ng PTx. The influence of additional

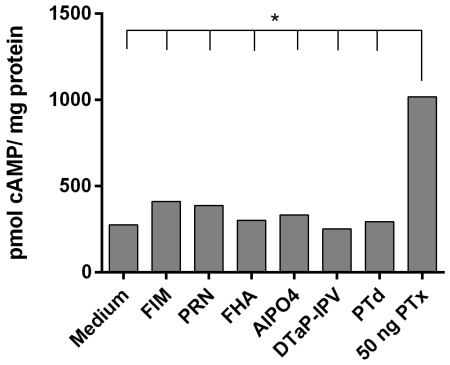

Figure 2 Specificity of the assay for PTx. cAMP production of A10 cells per mg protein after 120 minutes incubation with varying aP vaccine components (FIM, PRN, FHA, $AlPO_4$), the DTaP-IP vaccine, PTd, 50 ng PTx and medium (control) (x-axis) followed by the addition of the stimulating agonist isoprenaline. * $p < 0.05$, significantly different from cAMP levels produced by medium treated control cells.

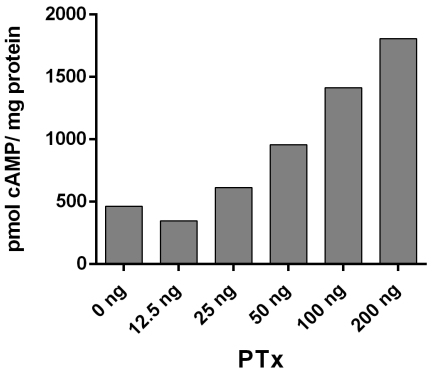

Figure 3 PTx spiking of DTaP-IP vaccine. cAMP production of A10 cells per mg protein after 120 minutes incubation with varying concentrations of PTx (x-axis) in the presence of one human dose (0.5 mL) DTaP-IP vaccine followed by the addition of the stimulating agonist isoprenaline. Standard deviations are not given because the data rely on results from one experiment.

components of the complete vaccine was determined by the addition of a reference DTaP-IP vaccine. This vaccine contains FHA, PRN, FIM or $AlPO_4$ and heat inactivated PTx in similar quantities as mentioned for the separated components.

Similarly treated cells, which were not stimulated with isoprenaline, were used to determine non-specific cAMP production. Production of cAMP in A10 cells after isoprenaline stimulation in the presence of 50 ng PTx differed significantly from cAMP levels produced in the presence of FHA, FIM, PRN, $AlPO_4$, DTaP-IP vaccine or PTd (Figure 2; Mann–Whitney U test, $n_{medium} = 6$, $n_{FHA} = 2$, $n_{FIM} = 2$, $n_{PRN} = 2$, $n_{ALPO4} = 2$, $n_{DTaP-IPV} = 2$, $n_{PTd} = 2$, $n_{50ng\ PTx} = 11$, $U_{FHA\text{-}50ng\ PTx} = 0$, $p = 0.026$, $U_{FIM\text{-}50ng\ PTx} = 0$, $p = 0.026$, $U_{PRN\text{-}50ng\ PTx} = 0$, $p = 0.026$, $U_{AlPO4\text{-}50ng\ PTx} = 0$, $p = 0.026$ $U_{DTaP\text{-}IPV\text{-}50ng\ PTx} = 0$, $p = 0.026$, $U_{PTd\text{-}50ng\ PTx} = 0$, $p = 0.026$). Furthermore, cAMP levels produced by cells treated with the different aP components, apart from PTx itself, did not significantly differ from cAMP levels produced by the medium treated positive control cells (Figure 2; Mann–Whitney U test, $U_{FHA\text{-}medium} = 4.5$, $p = 0.714$, $U_{FIM\text{-}medium} = 1$, $p = 0.143$, $U_{PRN\text{-}medium}$ 3, $p = 0.429$, $U_{AlPO4\text{-}medium} = 3$, $p = 0.429$, $U_{DTaP\text{-}IPV\text{-}medium} = 5$, $p = 0.857$, $U_{PTd\text{-}medium} = 5$, $p = 0.857$, $U_{PTx\text{-}medium} = 0$, $p < 0.001$). In agreement with the previous experiments, non specific cAMP production was nearly absent (data not shown).

cAMP production after PTx spiking of DTaP-IP vaccine

cAMP production in A10 cells treated with increasing quantities of PTx in the presence of a reference DTaP-IP vaccine (which passed the HIST), followed by isoprenaline stimulation were determined. Cells treated in a similar manner, but not stimulated with isoprenaline served as negative controls. Spiking of DTaP-IP vaccine with increasing quantities of PTx resulted in a PTx dose- dependent increase in cAMP level (Figure 3; Spearman correlation, n = 6, ρ = 0.943, p = 0.005). The negative control cells confirmed the earlier observation that non-specific cAMP production is very low (data not shown).

Standardization of the cAMP protocol

Following demonstration of sensitivity and specificity, we studied approaches for further standardization of the assay. For this purpose, A10 cells were treated with 50 ng PTx followed by isoprenaline stimulation. Cells treated with medium and stimulated with isoprenaline served as positive controls.

Experiments demonstrated that sonication is essential, because the absence of sonication resulted in variable protein levels (data not shown). The second potential source of variation is the use of filter paper to remove surplus liquid during the protein determination. An alternative evaporation method, using a flow of nitrogen, lead to slightly increased cAMP levels (Figure 4), although cAMP levels not significantly differed from the standard cAMP levels (Mann–Whitney U test, $n_{\text{standard 50ng PTx}} = 6$, $n_{\text{nitrogen evaporation 50ng PTx}} = 4$, U = 7, p= 0.325). The introduction of nitrogen evaporation also resulted in decreased variation in the protein levels measured and decreased variation in corrected cAMP levels, although not significantly different from the variation observed during the standard situation (Levene's test for variances, $F_{\text{standard 50ng PTx -nitrogen evaporation 50ng PTx}} = 2.489$, p = 0.153). The incubation time of the enzyme substrate used during the cAMP ELISA was thought to be a third potential source of variation. However, extending the incubation of enzyme substrate from 15 to 60 minutes resulted in slight but not significant

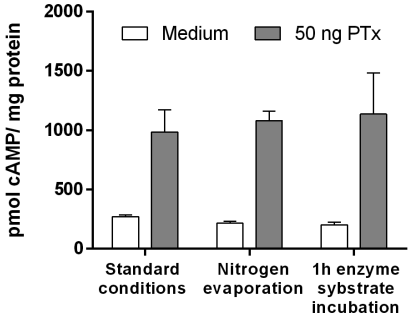

Figure 4 Standardisation of the protocol. cAMP production of A10 cells per mg protein after 120 minutes incubation with 50 ng PTx (x-axis) followed by the addition of the stimulating agonist isoprenaline under standard conditions, in the presence of nitrogen evaporation and after 1 hour of enzyme substrate incubation.

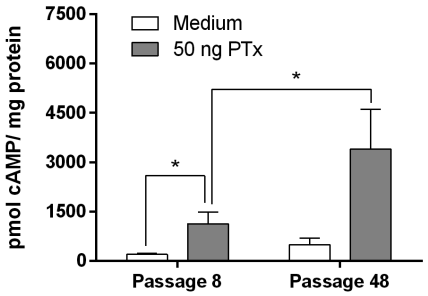

Figure 5 The effect of cell passage number. cAMP production of 8th and 48th passage cells (x-axis) per mg protein after 120 minutes incubation with medium (control) or 50 ng PTx followed by the addition of the stimulating agonist isoprenaline. * $p < 0.05$, significantly different from cAMP levels produced by medium treated control cells. ** $p < 0.05$, significant difference between cAMP levels produced by 8th passage cells and 48th PTx treated cells.

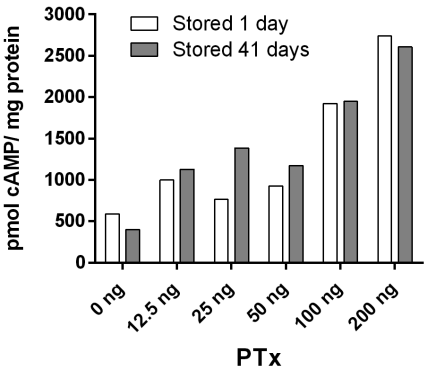

Figure 6 The effect of cAMP storage. cAMP production of A10 cells per mg protein after 120 minutes incubation with varying concentrations of PTx (x-axis) followed by the addition of the stimulating agonist isoprenaline. cAMP level were determined after 1 day and 41 days of storage at - 80°C.

elevated cAMP levels (Mann–Whitney U test, $n_{\text{standard 50ng PT}} = n_{\text{1h incubation 50ng PTx}} = 6$, $U_{\text{standard 50ng PTx -1h incubation 50ng PTx}} = 12$, $p = 0.337$), while the variance in cAMP levels was not significantly different (Levene's test for variances, $F_{\text{standard 50ng PT -1h incubation 50ng PTx}} = 0.100$, $p = 0.759$).

A10 cell passage effect on PTx induced cAMP production

For practical reasons the applicability of cells with a high passage number was determined. In this respect, a comparison was made between cAMP levels formed by cells with a passage number of 8, as used during the other experiments, and cAMP levels produced by cells with the relative high passage number of 48. cAMP levels produced by cells of both passage numbers were determined after treatment with 50 ng PTx and subsequent isoprenaline stimulation. Cells of similar passage numbers treated with medium only and stimulated with isoprenaline were named positive controls.

Light microscopic examination of untreated cells revealed no obvious morphological differences between cells of passage number 8 and 48. cAMP levels produced by 48 passage cells treated with PTx were significantly higher than cAMP levels produced by 8 passage cells after PTx treatment (Mann–Whitney U test, $n_{\text{passage 8, 50ng PTx}} = 6$, $n_{\text{passage 48, 50ng PTx}} = 4$, $U_{\text{passage 8, 50ng PTx- passage 48, 50ng PTx}} = 0$, $p = 0.010$) (Figure 5). Significant differences between cAMP levels produced by positive control cells and 50 ng PTx treated cells were detected for cells of passages 8 (Mann–Whitney U test, $n_{\text{passage 8, 50ng PTx}} = 6$, $n_{\text{passage 8, control}} = 3$, $n_{\text{passage 48, 50ng PTx}} = 4$, $n_{\text{passage 48, control}} = 2$, $U_{\text{passage 8, 50ng PTx-passage 8, control}} = 0$, $p = 0.024$), whereas no significant differences were observed between cAMP levels produced by positive control cells and PTx treated cells with passage number 48 ($U_{\text{passage 48, control-passage 48, 50ng PTx}} = 0$, $p = 0.133$). In this respect, it is important to note the variation in cAMP levels produced by cells of passage 48 is higher compared to passage 8 (although this difference in variation was not significant; Levene's test for variances $F_{\text{passage 8, 50ng PTx-passage 48, 50ng PTx}} = 4.845$ $p = 0.059$).

Possible re-determination of stored cAMP samples

For practical reasons, the possibility of re-determining cAMP samples after storage was examined. For this purpose, A10 cells were treated with 0-200 ng PTx and stimulated with isoprenaline after which cAMP levels were determined following the standard protocol. The surplus of each cAMP sample was stored at - 80°C and cAMP levels were re-determined after 41 days. No significant differences were detected between paired cAMP determinations (Figure 6, Wilcoxon signed rank test, $n = 2$, $Z = - 0.734$, $p = 0.563$), although it is important to notice that this is based on a small sample size ($n0ng = n_{12,5ng} = n_{25ng} = n_{50ng} = n_{100ng} = n_{200ng} = 2$).

Discussion

This paper describes an *in vitro* model as an alternative to the Histamine Sensitization test (HIST) in the lot release quality control of acellular pertussis vaccines. The cell based cAMP assay is principally founded upon recent studies concerning the mechanism of the HIST.[15] In these studies, Vleeming *et al.* and van Meijeren *et al.* reported that decreased blood pressure and reduced contractile properties of resistance arteries are major mechanisms involved in PTx induced HIST.[15-19] The reduced contractile properties occurred independent from the nitric oxide synthase system. It was hypothesized that the decrease contractile properties could be caused by a decreased sensitivity for Ca^{2+} [17], as previous studies have shown that the reduced contraction occurs independent of Ca^{2+} concentration.[20,21]

The majority of the biological effects of PTx are thought to be mediated through ADP-ribosylation of the α subunits of G proteins.[4,22] This leads to an ineffective protein unable to perform its normal functions such as the inhibition of adenylate cyclase. The inability to inhibit adenylate cyclase results in an unrestrained conversion of ATP into cAMP. In turn high levels of the nucleotides cAMP and cGMP are able to regulate enzyme activities involved in muscle contraction, which can ultimately result in decreased sensitivity to Ca^{2+} and decrease contraction abilities.[23,24] The *in vitro* model using A10 cells (A10 cell-line) mimics part of the pharmacological pathway of PTx as the biological endpoint of the assay is PTx induction of cAMP.[15]

In the present study we evaluate the applicability of the *in vitro* cAMP assay for aP vaccine lot release testing, paying particular attention to aspects such as specificity, sensitivity and standardization. We confirmed the earlier observation that addition of PTx can significantly enhance cAMP levels produced by A10 cells. However, such an induction was only observed after stimulation with isoprenaline and not with histamine.[15] Isoprenaline has a high affinity for β_2-adrenoceptors, receptors that are known to stimulate adenylate cyclase resulting in the generation of cAMP.[25] The absence of cAMP increase after stimulation with histamine and the cAMP increase after isoprenaline stimulation indicates that H_2 receptors are absent and β_2-adrenoceptors are present in A10 cell membranes. Although affecting different receptors, isoprenaline and histamine can both stimulate adenylate cyclase through G proteins. In this respect, it is also important to mention that (a) mainly H_1 receptors instead of H_2 receptors are involved in PTx induced HIST in rats and mice[15,16] and (b) Van Meijeren *et al.* also showed that histamine is only secondarily involved in this process.[17] Consequently it seems to be more relevant to concentrate on the known ADP-ribosylation effect of PTx.

According to most pharmacopoeias such as the Ph. Eur.[6] mouse strains are suitable for the HIST if at least 50% of the mice challenged with 50 ng of PTx are sensitized for histamine[**]. Consequently, an alternative for the HIST test should at least be able to detect 50 ng of PTx. We showed that the cAMP levels produced by control cells were significant lower that cAMP levels produced by cells treated with 25, 50, 100 and 200 ng PTx, demonstrating that the sensitivity of the assay equals the sensitivity of the HIST test.

In addition, we showed that the assay is able to discriminate between PTx and PTd. We demonstrated that PTx induced cAMP levels after isoprenaline challenge were significantly higher than PTd induced cAMP levels after isoprenaline challenge. Levels of cAMP produced in the presence of PTd (in quantities normally present in one human dose) and subsequent stimulation with isoprenaline did not significantly differ from cAMP levels produced by control cells. In fact, the addition of 25 ng PTx to the reference vaccine (which passed the HIST), demonstrates that even small amounts of PTx can be detected in the presence of large quantities of PTd, which reflects the situation in aP vaccines.

Specificity of the assay for PTx was assessed by comparing PTx induced cAMP levels after isoprenaline challenge with cAMP levels generated after the addition of several aP vaccine antigen components. These components included FHA, PRN and FIM. The experiments showed that cAMP levels produced in the presence of these antigens (at concentrations normally present in one human dose of aP vaccine) did not significantly differ from cAMP levels produced by control cells, but were significantly lower than cAMP levels produced by cells treated with 50 ng PTx. This indicates that these components do not affect assay results.

We also investigated the effect of the adjuvant $AlPO_4$ on the assay. From other studies it is known that mineral salts like $AlPO_4$ can affect cell functioning and consequently influence cell based assays such as the Chinese Hamster Ovary test[26,27]. Our experiments showed that cAMP levels produced in the presence of high $AlPO_4$ concentrations were not significantly different from cAMP levels generated by the control cells, whereas cAMP levels produced after the addition of a high dose of $AlPO_4$ alone were significantly lower than cAMP levels produced in the presence of 50 ng PTx. From these results it can be concluded that high concentrations of $AlPO_4$ do not increase basal cAMP levels and do not affect the cAMP assay.

[**] The Ph. Eur. guideline for the HIST was adapted in 2013. According to the this updated guideline of the European Pharmacopoeia, a mouse strain is generally considered suitable for the HIST if the LD_{50} for the BRP PTx is between 6 IU and 50 IU. A vaccine is accepted if not more than 5% of the immunized mice die when challenged with histamine. A detailed description of the requirements is provided in Table 3, Chapter 8.

The additional data obtained from a study focused on the establishment of a standard curve for PTx in the presence of DTaP-IP vaccine confirmed that neither $AlPO_4$ nor other antigens have an effect on cAMP production. DTaP-IP vaccine is an example of a vaccine in which different antigens are combined into one product. cAMP levels produced in the presence of one human dose of DTaP-IP vaccine were not significantly higher than cAMP levels produced by control cells. These experiments further revealed that in the presence of the reference vaccine, cAMP levels were correlated with the amount of PTx added. This shows that the assay is specifically affected by PTx in a dose dependent manner, even in the presence of DTaP-IP vaccine. In following studies we will confirm these data by comparing spiked cAMP results with data coming from the *in vivo* HIST.

Because large amounts of PTd are present in this reference vaccine, the experiment also reinforces the conclusion that the assay is not biased by PTd or any of the other components. Furthermore, such a spiked (standard) curve would be highly desirable for monitoring of the test system and maintenance of quality of a release assay.

One of the problems with the assay performance was intra- and inter-assay variations. Thorough examination of the protocol revealed that sonication, filter paper use in protein determination and the incubation time of enzyme substrate in the cAMP ELISA could be potential causes of variation. The experiments revealed that sonication, in particular, seemed to be essential for consistent results.

Because occasionally variation in determined protein replicates was present, the procedure for protein determination was examined. During the protein determination residual fluid was removed using filter paper. As the use of filter paper may have caused imprecise results, the possibility of evaporation with nitrogen was investigated. Compared to filter paper, evaporation with nitrogen resulted in similar cAMP levels with less variation. However, this decrease in variation was not statistically significant, probably due to the low number of samples.

For logistical reasons it might be advantageous to perform cAMP assays at a later time or to repeat the determinations. Therefore we studied the possibility of re-determining cAMP samples stored at - 80°C for several days. Methods for storage and freezing of biological samples for cyclic nucleotide assays are described by Mayer *et al.*[28]. Nevertheless, to our knowledge nothing is known about the effect of storage time on cAMP values. Generally we demonstrated that cAMP levels assessed after 41 days of storage at - 80˚C were not significantly different from cAMP levels determined after one day of storage at - 80˚C. These findings indicate that the storage method probably not affects cyclic nucleotide cAMP levels.

For both logistical reasons and to examine the robustness of the test system, we investigated the effect of A10 cell passage number on the test results. Passage number and culture time are known to influence cell functions such as cell senescence, apoptosis and expression of proteins[29-32]. Light microscopic examination of cells of passage 8 and 48 revealed no obvious morphological differences. cAMP levels produced by PTx stimulated cells passed for 48 times were significant higher than cAMP levels generated by cells passed for 8 times (passage number normally used). This indicates that the enzyme activity and cellular mechanisms might have changed during subsequent passages. Further study is required to examine what range of cell passage numbers can be used without any effect on cAMP levels.

Overall, this *in vitro* assay is a promising alternative to the HIST test. We showed that the method is specific and sensitive for PTx, while reproducibility is acceptable but can be improved. Nevertheless, further research is required to validate and finally implement the assay.

Acknowledgement

We are grateful to Johan van der Gun (NVI) for helpful advice throughout the study. We thank Sanofi for the provision of pertussis toxin, thank Han de Vries (Department Behavioural Biology, Utecht University) and Lonneke Levels (NVI) for comments on the statistical analysis and thank Keith Redhead (Schering Plough - Intervet) for helpful comment on drafts of the manuscript. Finally, we are especially grateful for the financial support of an anonymous donator and the ministry of Health, Welfare and Sport (VWS), which enabled the performance of this study.

References

1. Kerr JR, Matthews RC. *Bordetella pertussis* infection: pathogenesis, diagnosis, management, and the role of protective immunity. Eur J Clin Microbiol Infect Dis. 2000;19(2):77-88.
2. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other Bordetella subspecies. Clin Microbiol Rev. 2005;18(2):326-82.
3. Carbonetti NH. Immunomodulation in the pathogenesis of *Bordetella pertussis* infection and disease. Curr Opin Pharmacol. 2007;7(3):272-8.
4. Black WJ, Munoz JJ, Peacock MG, Schad PA, Cowell JL, Burchall JJ, Lim M, Kent A, Steinman L, Falkow S. ADP-Ribosyltransferase activity of pertussis toxin and immunomodulation by *Bordetella pertussis*. Science 1988;240(4852):656-59.
5. Sato H, Ito A, Chiba J, Sato Y. Monoclonal antibody against pertussis toxin: effect on toxin activity and pertussis infections. Infect Immun. 1984;46(2):422-8.
6. Eur.Ph. Diphtheria, tetanus, pertussis acellular component and poliomyelitis inactivated vaccine adsorbed. 2009;6.4(1934).
7. Parfentjev IA, Goodline MA. Histamine shock in mice sensitized with Hemophilus pertussis vaccine. J Pharmacol Exp Ther 1948;92(4):411-3.
8. Van Straaten-van de Kappelle I, Van der Gun JW, Marsman FR, Hendriksen CFM and Van de Donk HJM. Collaborative Study on Test Systems to Assess Toxicity of Whole Cell Pertussis Vaccine. Biologicals 1997;25(1):41-58.
9. Morse SI, Morse JH. Isolation and properties of the leukocytosis- and lymphocytosis-promoting factor of *Bordetella pertussis*. J Exp Med 1976;143(6):1483-502.
10. Morse SI. Lymphocytosis-promoting factor of *Bordetella pertussis*: isolation, characterization, and biological activity. J Infect Dis 1977;136:S234-8.
11. Gillenius P, Jäätmaa E, Askelöf P, Granström M, Tiru M. The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J Biol Stand 1985;13(1):61-6.
12. Fujiwara H, Iwasa S. The quantitative assay of the clustering activity of the lymphocytosis-promoting factor (pertussis toxin) of *Bordetella pertussis* on Chinese hamster ovary (CHO) cells. J Biol Stand 1989;17(1):53-64.
13. Cyr T, Menzies AJ, Calver J, Whitehouse LW. A quantitative analysis for the ADP-ribosylation activity of pertussis toxin: an enzymatic-HPLC coupled assay applicable to formulated whole cell and acellular pertussis vaccine products. Biologicals 2001;29(2):81-95.
14. Yuen CT, Canthaboo C, Menzies JA, Cyr T, Whitehouse LW, Jones C, Corbel MJ, Xing D. Detection of residual pertussis toxin in vaccines using a modified ribosylation assay. Vaccine 2002;21(1-2):44-52.
15. Van Meijeren CE. Development of an *in vitro* assay to assess pertussis toxin in acellular pertussis vaccines. PhD Thesis 2005 Utrecht University, Utrecht.
16. Vleeming W, Hendriksen CF, van de Kuil A, van den Hout JW, de Wildt DJ. Mepyramine but not cimetidine or clobenpropit blocks pertussis toxin-induced histamine sensitization in rats. Br J Pharmacol 2000;129(8):1801-7.
17. van Meijeren CE, Vleeming W, van de Kuil T, Gerards AL, Hendriksen CF, de Wildt DJ. Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system? Eur J Pharmacol 2004;493(1-3):139-50.

18. Van Meijeren CE, Vleeming W, van de Kuil T, Manni J, Kegler D, Hendriksen CF, de Wildt DJ. *In vivo* pertussis toxin treatment reduces contraction of rat resistance arteries but not that of mouse trachea. Eur J Pharmacol 2004;488(1-3):127-35.
19 van Meijeren CE, Vleeming W, Dormans JA, van de Kuil T, Opperhuizen A, Hendriksen CF, de Wildt DJ. Pertussis toxin relaxes small arteries with no vascular lesions or vascular smooth muscle cell injury. Exp Toxicol Pathol 2004;56(3):139-43.
20. Spitzbarth-Régrigny E, Petitcolin MA, Bueb JL, Tschirhart EJ, Atkinson J, Capdeville-Atkinson C. Pertussis toxin-sensitive G(i)-proteins and intracellular calcium sensitivity of vasoconstriction in the intact rat tail artery. Br J Pharmacol 2000;131(7):1337-44.
21. Petitcolin MA, Spitzbarth-Régrigny E, Bueb JL, Capdeville-Atkinson C, Tschirhart E. Role of G(i)-proteins in norepinephrine-mediated vasoconstriction in rat tail artery smooth muscle. Biochem Pharmacol 2001;61(9):1169-75.
22. Gierschik P. ADP-ribosylation of signal-transducing guanine nucleotide-binding proteins by pertussis toxin. Curr Top Microbiol Immunol 1992;175:69-96.
23. Abdel-Latif AA. Cross talk between cyclic nucleotides and polyphosphoinositide hydrolysis, protein kinases, and contraction in smooth muscle. Exp Biol Med (Maywood) 2001;226(3):153-63.
24. Pfitzer G. Invited review: regulation of myosin phosphorylation in smooth muscle. J Appl Physiol 2001;91(1):497-503.
25. Raymond JR, Hnatowich M, Lefkowitz RJ, Caron MG. Adrenergic receptors. Models for regulation of signal transduction processes. Hypertension 1990;15(2):119-31.
26. Iwasa S, Fujiwara H. Comparison of the detoxification courses of the histamine-sensitizing, lymphocytosis-promoting and CHO cell-clustering activities in the process of the production of acellular pertussis vaccine. Biologicals 1990;18(2):127-30.
27. Van Straaten-van de Kappelle I, Wiertz EJ, Marsman FR, Borsboom DJ, van de Donk HJ, Kreeftenberg JG. The modified leukocytosis promoting factor (LPF)-test: a valuable supplement to the mouse weight gain (MWG)-test in toxicity control of whole cell pertussis vaccine. Biologicals 1992;20(4):277-82.
28. Mayer SE, Stull JT, Wastila WB. Rapid tissue fixation and extraction techniques. Methods Enzymol. 1974;38:1-9.
29. Sorrentino JA, Millis AJ. Structural comparisons of fibronectins isolated from early and late passage cells. Mech Ageing Dev 1984;28(1):83-97.
30. Zhang J, Patel JM, Block ER. Enhanced apoptosis in prolonged cultures of senescent porcine pulmonary artery endothelial cells. Mech Ageing Dev 2002;123(6):613-25.
31. Briske-Anderson MJ, Finley JW, Newman SM. The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. Proc Soc Exp Biol Med. 1997;214(3):248-57.
32. Wagner M, Hampel B, Bernhard D, Hala M, Zwerschke W, Januarisen-Dürr P. Replicative senescence of human endothelial cells *in vitro* involves G1 arrest, polyploidization and senescence-associated apoptosis. Exp Gerontol 2001;36(8):1327-47.

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Chapter 7

Reporter cell lines for detection of pertussis toxin in acellular pertussis vaccines as a functional animal-free alternative to the *in vivo* Histamine Sensitization test

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Submitted to Vaccine

Abstract

Detoxified pertussis toxin (pertussis toxoid) is a major antigen in acellular pertussis vaccines. Testing these vaccines on the presence of residual pertussis toxin (PTx) and reversion to toxicity is performed by the regulatory required *in vivo* Histamine Sensitization test (HIST). Lack of mechanistic understanding of the HIST, technical handicaps and animal welfare concerns, have promoted the development of alternative methods. As the majority of the cellular effects of PTx depend on its ability to activate intracellular pathways involving cAMP, the *in vitro* cAMP-PTx assay was developed. Although this assay could be used to detect PTx activity, it lacked sensitivity and robustness for use in a quality control setting. In the present study, novel reporter cell lines (CHO-CRE and A10-CRE) were generated that stably express a reporter construct responsive to changes in intracellular cAMP levels. These reporter cell lines were able to detect PTx in a dose-dependent manner when combined with fixed amounts of forskolin. The CHO-CRE cell line enabled detection of PTx in the context of a multivalent vaccine containing aP, with a sensitivity equal to the HIST. However, the sensitivity of the A10-CRE cells was insufficient for this purpose. The experiments also suggest that CHO-CRE reporter cell line might be suitable for assessment of cellular effects of PTd reverted to PTx. The CHO-CRE reporter cell line provides a platform that meets the criteria for specificity and sensitivity and is a promising *in vitro* model with potential to replace the HIST.

Introduction

Acellular pertussis (aP) vaccines form the second generation of pertussis vaccines to protect children and adults against whooping cough. aP vaccines minimally contain inactivated pertussis toxin (referred to as pertussis toxoid (PTd)) and one to four other proteins (filamentous hemagglutinin, pertactin, fimbriae type 2 and/or type 3). PTd is thought to be key for vaccine-induced protective immunity[1,2], while pertussis toxin (PTx) is considered responsible for the occasional side effects after wP and aP vaccination. Consequently, regulatory authorities require monitoring for inactivation and reversion to toxicity through the murine histamine sensitization test (HIST). The HIST is based on the principle that PTx reduces the lethal dose of histamine 30-300 fold.[3,4] However, since the test lacks mechanistic understanding, suffers from standardization problems and causes severe animal suffering, there is an urgent need for an alternative test method.

Alternative methods for the HIST should take into consideration the four main steps involved in PTx-mediated toxicity, being cell binding, internalisation, translocation and enzymatic activity. PTx is an AB_5 toxin of which the B-oligomer is responsible for binding of PTx to glycoproteins on cell membranes, resulting in transport of the toxin to Golgi and the endoplasmatic reticulum, upon which the A subunit is released into the cytosol. In the cytosol, the A-subunit ADP-ribosylates the α-subunit of inhibitory G (G_i)-coupled receptors.[5,6] Consequently, the α-subunit can no longer inhibits adenylate cyclase (AC), an enzyme that converts ATP into cAMP.

A combination of a HPLC assay measuring the enzymatic activity of PTx[7] and a fetuin-binding assay is under development as an alternative to the HIST.[8, 9] However, these assays do not assess the internalisation and translocation of the toxin. A second alternative method is based on PTx-induced clustered growth of CHO cells[10], which requires an enzymatically active A-subunit[11], but also cell binding, internalisation and translocation of the A-subunit to the cytosol. Current application of this test is limited to bulk products, since adjuvant-salts in final products have cytotoxic effects. Furthermore, quantification of PTx levels is restricted to manual reading due to lack of a quantitative read-out parameter, which might increase variability in test outcomes.

Previously, we have developed an A10 cell-based assay that determines the effect of PTx on G_i-coupled receptors and AC function by measuring intracellular cAMP levels, requiring cell binding, internalisation, translocation and intracellular enzymatic activity.[12] To improve sensitivity and reduce variability of this assays, we generated reporter cell lines carrying a cAMP-reporter construct, using the A10 cell lines and the PTx sensitive CHO cell line. For these reporter cell lines, optimal assay conditions and sensitivity for PTx alone and in the context of an aP containing multivalent vaccine were studied. The findings show that particularly CHO-CRE reporter cells can be used to assess residual PTx activity in aP vaccines in a quantitative manner.

Materials and Methods

Cell lines and culture conditions

A10 rat vascular smooth muscle cells (ATCC, CRL-1476) and Chinese Hamster Ovary (CHO-K1) cells (ECACC, 85051005) were cultured (37°C, 5% CO_2) in DMEM with pyruvate (Gibco) and F-12 Ham's (Sigma Aldrich), respectively. Media were supplemented with 10% fetal calf serum (v/v), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.3 µg/ml L-glutamine (Gibco). Cells were passaged every 2-4 days with trypsine-EDTA.

Stable transfection of A10 and CHO cells with CRE-reporter

A10 and CHO cells were transfected with pNL(NIucP/CRE/Hygro) (Promega) using Viafect (Promega) as described by the manufacturer. The vector contains a minimal promotor and a cAMP responsive element (CRE) controlling the transcription of NanoLucP luciferase.[13] Stable transfectants were selected with 500 µg/ml hygromycin and single clones were obtained by limiting dilution. Two clones of each of the resulting CHO-CRE and A10-CRE reporter cell lines, clone 10 and 17 and clone 20 and 28 respectively, were selected for further analysis.

Reporter assay

For optimisation experiments (Figure 1-3), A10-CRE and CHO-CRE cells were cultured overnight (ON) in 96-well F-bottom plates at a concentration of 1 x 10^4 and 2.5 x 10^4 cells/well, respectively. Cells were exposed to medium, PTx (BRP1; the reference preparation of the European Directorate for the Quality of Medicines & Health Care (EDQM)) or DMSO (Merck) and stimulated with norepinephrine, isoprenaline or forskolin (Sigma-Aldrich).

For experiments with vaccines (Figure 4, 6 and Supplementary Figure S3, S4, S5, S6), A10-CRE and CHO-CRE cells were cultured in 24-well plates ON at 0.625 x 10^4 and 2 x 10^4 cells/well, respectively. After the example of a previous collaborative study organised by EDQM (BSP114, phase 2)[14], wells in which cells were exposed to vaccine carried inserts (0.4 µm, Pore Polycarbonate Membrane, Corning), to prevent direct contact between aluminium-salts of the vaccine and cells, thereby circumventing the cytotoxic effect of commercially available multivalent vaccine containing aP.

The DTaP-IP vaccine (diphtheria and tetanus toxoid, aP, inactivated polio, Netherlands Vaccine Institute, The Netherlands) used in this study, contains glutaraldehyde-formaldehyde inactivated PTx and complied with the HIST for absence of residual toxicity and reversion to toxicity (data not shown). Vaccine and medium were 'spiked' with the indicated concentrations of PTx for 1 hour at 4°C and were either used directly or stored at 4˚C or 37˚C for 4 weeks to evaluate reversion to toxicity. Before exposure to the cells, the vaccines were centrifuged at

2000 x g and the supernatant was replaced by the same volume of medium (after the example of BSP114 phase 2).[14] For all experiments, plates were left at room temperature (RT) for 10 minutes, followed by addition of Nano-Glo luciferase substrate (Promega). Luciferase activity was measured for 0.1 s. using a Berthold Centro lb960 reader.

CHO cell clustering assay

Clustering capacities of the CHO-CRE cell clone and parental CHO cells were studied by culturing the cells (2 x 10^4 cells/well) in a 24-well plate for 3h, followed by exposure to indicated concentrations of PTx for 48h. 24-well inserts were used, when cells were exposed to DTaP-IP vaccine. CHO cell clustering was visualised using an Olympos CKX41 microscope and Olympos UC30 camera.

Immunoblotting of multivalent DTaP-IP vaccine

For immunoblotting, PTx-spiked or non-spiked DTaP-IP vaccines were desorbed for 2 days using 3.4 mM Trisodium citrate dihydrate (Sigma-Aldrich). Subsequently, the samples were concentrated using Amicon Ultra 0.5 mL centrifugal filters with a 3kDa cut-off (Millipore), boiled in loading buffer and separated using denaturing 10% NuPage polyacrylamide gels with MES running buffer. Gels were either stained with Coomassie or transferred to nitrocellulose membranes. Blots were incubated with a monoclonal α-S1 IgG antibody (ab37686, Abcam), goat-anti-mouse IgG antibody labelled with IR800 and scanned using the Odyssey infrared imager.

Statistical analysis

All data were log-transformed and statistical significance was determined by using unpaired t-tests (Figure 1A, 2-4, 6) or column statistics (t-test) (Figure 1B). The Benjamini-Hochberg method was used for correction of multiple comparisons.[15] P values <0.05 were considered statistically significant.

Results

Reporter cell responses to isoprenaline, norepinephrine, forskolin and PTx

In accordance with previous results[12], PTx alone had no effect on cAMP levels in A10-CRE and CHO-CRE cells (data not shown, fold increase was at or below 1.05). Therefore, the α/β-adrenoceptor agonists isoprenaline, norepinephrine and the AC agonist forskolin were analysed for their capacity to enhance the cellular effect of PTx (Figure 1). Without pre-exposure to PTx, only forskolin enhanced the cAMP levels in CHO-CRE [10^{-4} -10^{-7} M], whereas all agonists increased the cAMP levels in A10-CRE cells (Figure 1A).

Pre-exposure of both cell types to PTx primarily enhanced the response to low concentrations of forskolin (i.e. 10^{-6}-10^{-7} M) (Figure 1B). For A10 cells, PTx effects were also observed after isoprenaline and norepinephrine stimulation, while for CHO-CRE cells these agonists had a negligible effect on cAMP levels (fold increase was not significantly above stimulation with PTx alone, being 1.05) (Figure 1B). Based on these findings, stimulation with 0.1 μM forskolin was considered optimal for detection of PTx and used in the next experiment.

Optimal time of forskolin stimulation

Next, the effect of duration of forskolin stimulation (0.25-24h) was analysed (Figure 2). For CHO-CRE cells, enhanced responses to 2 and 20 IU/mL PTx were observed between two and seven hours of forskolin stimulation. Detection of 0.2 IU/mL PTx and a dose-dependent response required at least four hours of forskolin stimulation (Figure 2A and B). For A10-CRE, a minimum of two hours of stimulation was sufficient for detection of 0.2-20 IU/mL PTx, though marked effects of 0.2 IU/mL PTx required four hours of stimulation (Figure 2C and 2D). After 24 hours of stimulation, PTx activity could still be detected by CHO-CRE cells, although dose-dependent activation was largely lost. In contrast, A10-CRE cells were no longer capable of detecting the effects of PTx at this time point. Optimal forskolin stimulation was reached after four and six hours for A10 and CHO cells, respectively (Figure 2A-D). Using these incubation times, forskolin concentrations were optimized even further (Supplementary Figure S1), resulting in concentrations of 0.5 μM (CHO-CRE) and 0.25 μM (A10-CRE) that were used accordingly in all subsequent experiments.

Optimal time of reporter cell exposure to PTx

As a next step, the duration of PTx exposure was evaluated in combination with forskolin stimulation. Both reporter cell types detected 0.2-20 IU/mL PTx after one to four hours of PTx exposure (Figure 3). The CHO-CRE cell clones discriminated between 0.2 IU/mL and higher concentrations of PTx for all of the studied times of exposure, while discrimination between 2 and 20 IU/mL was only possible after one hour (clone 17) and two hours (clone 10) of PTx exposure (Figure 3A, B).

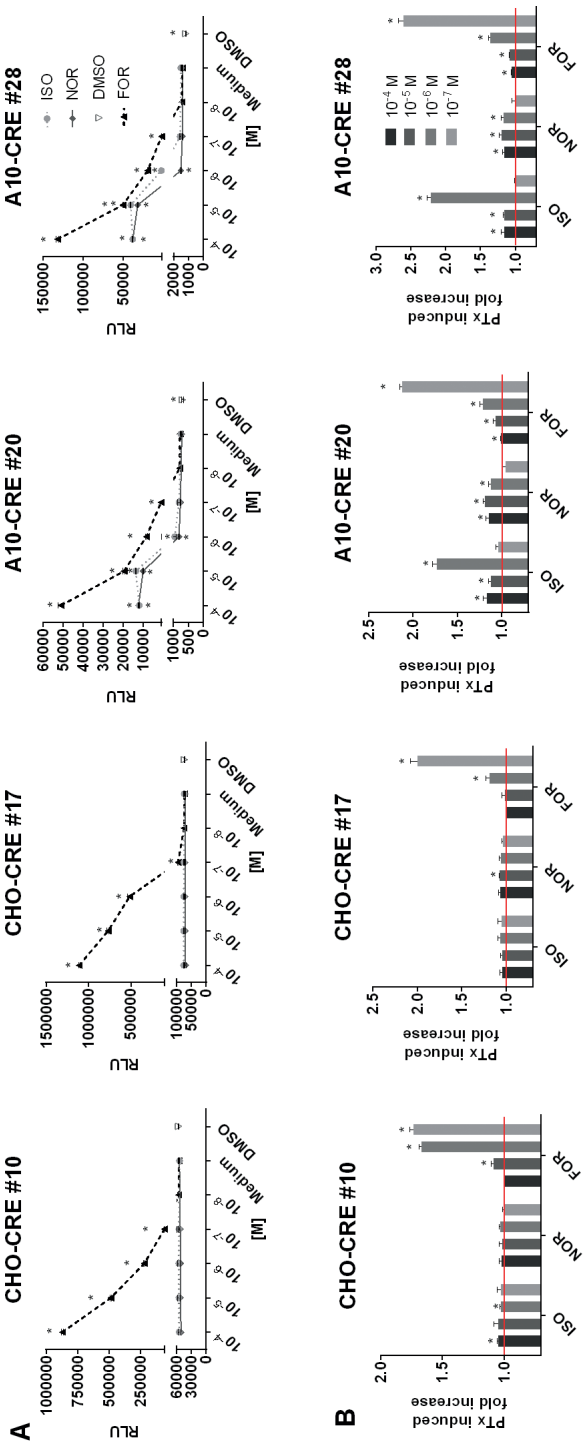


Figure 1 CHO-CRE and A10-CRE cell responses to isoprenaline, norepinephrine, forskolin and PTx. CHO-CRE clones 10 and 17 and A10-CRE clones 20 and 28 were exposed to medium or 2 IU/ml PTx for 2 hours, followed by stimulation with the agonists isoprenaline (ISO), norepinephrine (NOR) or forskolin (FOR) for 4 hours at indicated concentrations. (A) Responses of medium exposed CHO-CRE and A10-CRE clones to the indicated agonists. Relative Light Units (RLU) represent the average of three wells ± SD. One representative experiment out of two is shown. * $p < 0.05$, significantly different from responses to medium. (B) Responses of CHO-CRE and A10-CRE clones to PTx compared to cells exposed to medium for indicated concentrations of the agonists. The responses are expressed as the fold increase (luciferase activity of cells exposed to PTx and agonist/average of luciferase activity of cells exposed to medium and agonist). The average fold increase of three wells ± SD is shown for one out of two representative experiments. * $p < 0.05$, significantly different from 1.

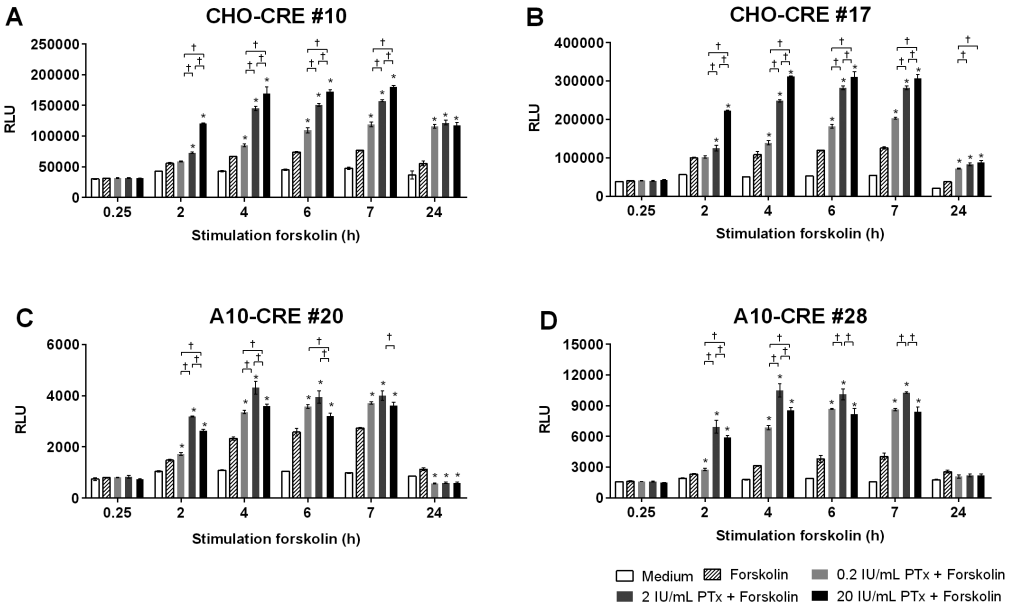


Figure 2 Effect of forskolin incubation time on detection of PTx by CHO-CRE and A10-CRE clones. The cells were exposed to medium or PTx (0.2, 2 or 20 IU/mL PTx) for 2 hours and subsequently stimulated with forskolin [0.1 μM] for 0.25-24h. RLU of CHO-CRE clones 10 (A) and 17 (B) and A10-CRE cell clones 20 (C) and 28 (D) are shown, representing the average of three wells ±SD. One representative experiment out of two is shown. * $p < 0.05$, significantly different from responses to cells exposed to medium and stimulated with forskolin. † significant difference between indicated samples for $p < 0.05$.

Using A10-CRE cells (clone 20 and 28), a distinction between 0.2 and 2 IU/mL PTx could only be made after one and two hour of PTx exposure, whereas a dose dependent discrimination between 2 and 20 IU/mL PTx proved difficult for any of the times of PTx exposure. In fact, an inverted response was observed after four hours of PTx pre-treatment, where 0.2 IU/mL PTx elicited the highest level, 2 IU/mL PTx an intermediate level and 20 IU/mL PTx the lowest level of cAMP. Based on these results, 2 hour (CHO-CRE) and 1 hours (A10-CRE) of PTx exposure were selected as optimal and shown to allow detection of even higher concentrations of PTx (200 IU/mL; Supplementary Figure S2). However, only A10-CRE cells enabled discrimination between 20 and 200 IU/mL of PTx.

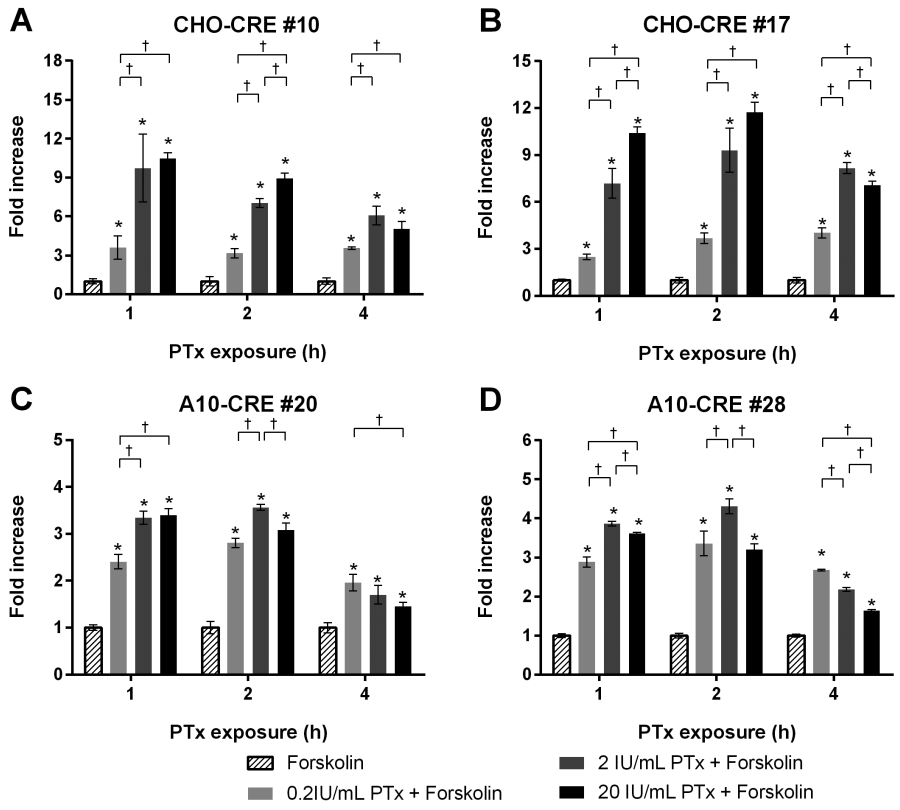


Figure 3 Effect of the time span of PTx exposure. CHO-CRE clones 10 and 17 and A10-CRE cell clones 20 and 28 were exposed to medium or PTx (0.2-20 IU/mL) for 1, 2 or 4 hours. Subsequently, CHO-CRE cells were stimulated with forskolin [0.5 µM] for 6 hours (A, B), while A10-CRE cells were stimulated with forskolin [0.25 µM] for 4 hours (C, D). Measured responses were corrected for background by subtracting the response of cells exposed to only medium. Responses are expressed as fold increase (RLU of cells exposed as indicated/average RLU of cells stimulated with only forskolin). The average fold increase of three wells ± SD is shown for one out of two representative experiments. * $p < 0.05$; significantly different from responses of cells exposed to medium and stimulated with forskolin. † significant difference between indicated samples for $p < 0.05$.

Detection of PTx in PTx-spiked aP vaccines

The LD_5 of the HIST was calculated to be 1.11 IU of the BRP1 PTx (corresponding to 2.22 IU/mL and 14.8 ng/mL of PTx)[16] and is therefore considered to be the maximum amount of PTx accepted for aP vaccines. Since testing of aP containing vaccines with our reporter cells required 24 well plates (due to the use of inserts), pilot experiments were performed that

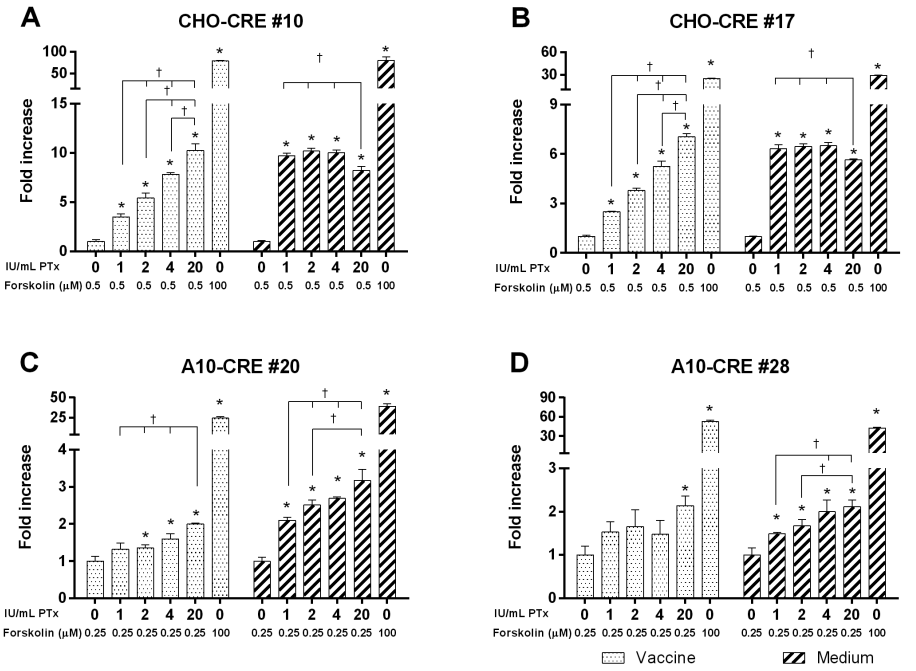


Figure 4 Detection of PTx in a DTaP-IP vaccine. CHO-CRE clones 10 and 17 were exposed to PTx-spiked vaccines (0, 1, 2, 4 or 20 IU/mL) or medium containing the same amounts of PTx for 4 hours and stimulated with indicated concentrations of forskolin for 6 hours (A, B). A10-CRE cell clones 20 and 28 were exposed to vaccines or medium were spiked with PTx (0, 1, 2, 4 or 20 IU/mL) for 2 hours and stimulated with indicated concentrations of forskolin for 4 hours (C, D). The measured responses were corrected for background by subtracting the response of cells exposed to only medium. The responses are expressed as fold increase (RLU of cells exposed as indicated/average RLU of cells stimulated with only forskolin). The average fold increase of three wells ± SD is shown for one out of two representative experiments. * $p < 0.05$, significantly different from responses of cells exposed to medium and stimulated with forskolin. † significant difference between indicated samples for $p < 0.05$.

revealed different optimal cell densities than used for 96 well plates (0.625×10^4 cells/well for A10 cells) and 2×10^4 cells/well for CHO-CRE cells) (Supplementary Figure S3) and PTx-spiked vaccine exposure times (4h for CHO-CRE cells, 2h for A10-CRE cells) (Supplementary Figure S4).

Under these conditions, vaccine samples containing 1, 2, 4 and 20 IU/mL PTx elicited a significantly increased and dose-dependent production of cAMP by both CHO-CRE clones (Figure 4A and B), while similar concentrations of PTx alone caused enhanced, but not dose-

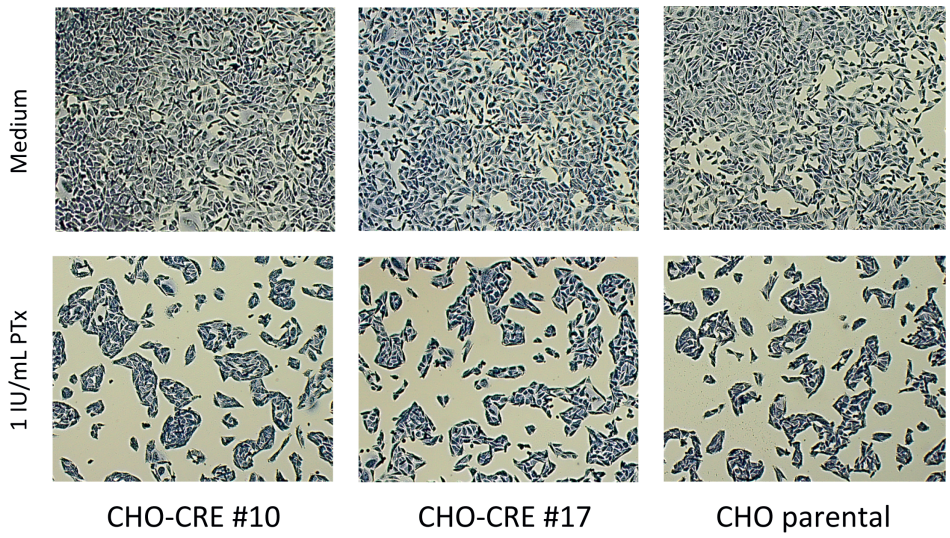


Figure 5 PTx-induced clustering of CHO-CRE cell clones 10 and 17 compared to parental CHO cells. Cells were exposed to medium or PTx for 48 hours, then fixed with methanol and stained with Giemsa stain. One out of two representative experiments is shown (magnification 10x).

dependent, cAMP levels. A10-CRE clones detected PTx in the presence of DTaP-IP vaccine, but significantly increased responses were observed only for 2-20 IU/mL of PTx (clone 20) and 20 IU/mL of PTx (clone 28). In contrast, PTx in medium (1-20 IU/mL) did trigger a dose-dependent rise in cAMP levels (Figure 4C an D). The vaccine itself had no effect on basal cAMP levels or cAMP levels after forskolin stimulation (Supplementary Figure S5), confirming the results of previous experiments.[12] These findings indicate that both CHO-CRE clones and A10-CRE clone 20 were capable of detecting PTx in the presence of an DTaP-IP vaccine, of which only the CHO-CRE cells could do so in a dose-dependent manner. Furthermore, CHO-CRE cells were more sensitive to low concentrations of PTx.

CHO cell clustering capacity of CHO-CRE clones

To examine whether the introduction of the vector into the CHO cell genome had affected the clustering capacities of the cells, a CHO cell clustering assay was performed. This revealed that PTx exposure elicited a clustered growth pattern of both CHO-CRE cell clones which was very similar to the pattern observed for the parental CHO cells (Figure 5). This indicates that the stable genomic integration of the vector did not change the cellular response of these cells to PTx. Furthermore, it enables a direct comparison between the CHO-reporter and the CHO clustering assays.

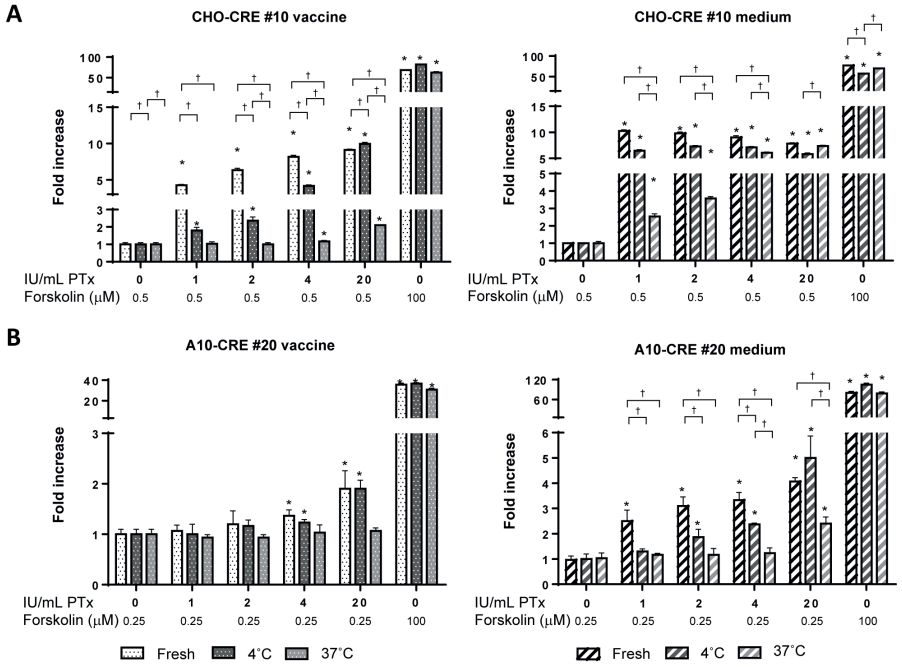
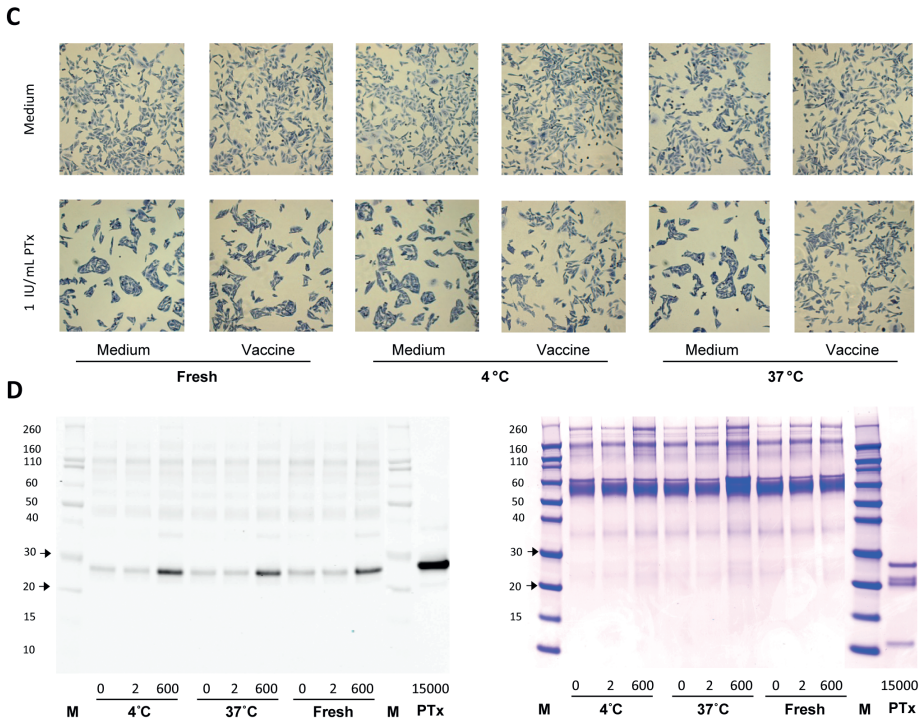


Figure 6 Effect of storage on residual toxicity in a DTaP-IP vaccine. Vaccines or medium were spiked with PTx (0, 1, 2 4 or 20 IU/mL) and stored at 4˚C and 37˚C for 4 weeks. The same samples were prepared freshly on the day of use. CHO-CRE clone 10 was exposed to the indicated samples for 4 hours and stimulated with forskolin for 6 hours (A). A10-CRE clone 20 was exposed to the indicated samples for 2 hours and stimulated with forskolin for 4 hours (B). The measured responses were corrected for background by subtracting the response of cells exposed to corresponding medium or vaccine exposed cells. The responses are expressed as fold increase (RLU of cells exposed as indicated/average RLU of cells stimulated with only forskolin). The average fold increase of three wells ±SD is shown for one experiment. * $p < 0.05$, significantly different from corresponding

Detection of reversion of PTd to PTx

To explore whether the CRE-reporter cell lines are able to detect reversion to toxicity within DTaP-IP vaccines, vaccines were spiked as described before and stored at 4˚C and 37˚C. Although the response to non-spiked vaccine stored at 37°C was slightly higher than induced by the non-spiked vaccine stored at 4°C (Figure 6 and Supplementary Figure 6A, B), the levels of cAMP elicited by non-spiked vaccines remained substantially lower than induced by the



non-spiked medium or vaccine exposed and forskolin stimulated cells. † significant difference between indicated samples for $p < 0.05$. Clustering of parental CHO cells was analysed by exposure for 48 hours to medium or vaccine spiked with PTx (0, 1, 2, 4 or 20 IU/mL), fixed with methanol and stained with Giemsa stain (magnification 10x) (C; complete data set is shown in Figure S6C). Vaccines or medium were spiked with PTx (0, 2 and 600 IU/mL) and stored at 4˚C and 37˚C for 4 weeks. The same samples were prepared freshly on the day of use. Pure PTx (15000 IU/mL) served as a positive control. The proteins in the vaccines were separated on a denaturing polyacrylamide gel and either analysed by immunoblotting using an S1-specific antibody (left) or stained with Coomassie (right) (D).

vaccine containing 1 IU/mL PTx (fresh), suggesting that there is limited or no reversion to toxicity after storage, thereby confirming the results of the HIST.

In contrast, storage caused a reduction in the cAMP levels produced by CHO-CRE cells in response to 1-4 IU/mL PTx (4˚C) and 1-20 IU/mL PTx (37˚C), compared to vaccines freshly spiked with the same amounts of PTx (Figure 6A). Responses to 1-2 IU/mL PTx spiked vaccines

stored at 37˚C were no longer above background. A similar reduction in the cAMP signal was observed for 1-4 IU/mL PTx spiked into medium and stored at 37˚C. A10-CRE cells detected 4 and 20 IU/mL PTx in the presence of DTaP-IP vaccines (fresh and stored at 4˚C), while cAMP levels in response to vaccine spiked with 1-2 IU/mL of PTx (fresh and stored at 4˚C) or 1-20 IU/mL (stored at 37˚C) were not above background (Figure 6B). For A10-CRE cells, storage also reduced the response to PTx spiked into medium, i.e. for 1-4 IU/mL PTx stored at 4˚C and 1-20 IU/mL stored at 37˚C.

Storage at 37˚C also affected the clustering response of parental CHO cells. The clustered pattern was absent or diminished when the vaccine contained 1-2 IU/mL or 4 IU/mL PTx, respectively. Vaccine samples spiked with 1-20 IU/mL PTx and being fresh or stored at 4˚C, all resulted in a clustered growth pattern, while non-spiked vaccine (fresh and stored at 4˚C and 37˚C) did not. Storage at 4˚C or 37˚C had no effect on the clustering for any of the PTx concentrations spiked into medium (Figure 6C).

The PTx/PTd content of the vaccines after storage was analysed further by Western blot, revealing that the used monoclonal α-S1 particularly binds to the single S1 subunit (26 kDa) in PTx, but also in PTd, with a comparable intensity in freshly spiked vaccines and vaccines stored at 4˚C or 37˚C (Figure 6D). Remarkably, there is slight recognition of proteins with a higher molecular weight in all of the vaccine samples, suggesting either the presence of multimers of PTd or non-specific binding of the secondary antibody.

These results indicate that it is unlikely that storage of this vaccine caused reversion to toxicity and in fact indicates that storage at 37˚C and to a lesser extent at 4˚C might reduce residual PTx activity. These results therefore suggest that both types of reporter cells have the potential to detect the cellular effects of reversion of PTd to PTx, though CHO-CRE cells provide a more sensitive platform.

Discussion

In the present study, cAMP-reporter cell lines based on CHO and A10 cells were generated and evaluated for their capability to replace the regulatory required *in vivo* HIST for demonstration of the toxic effects of PTx in aP vaccines. The study shows that these reporter cells can detect residual PTx in the context of a multivalent DTaP-IP vaccine in a dose-dependent and sensitive manner.

Since cellular effects of PTx cannot be detected by exposure to PTx alone, we investigated the role of $\beta_{1\text{-}3}$ adrenoceptor agonist isoprenaline[17], the $\alpha_{1,2}$ and $\beta_{1\text{-}3}$ adrenoceptor agonist norepinephrine[18, 19] and forskolin, a direct stimulator of ACs, for discrimination between PTx exposed and non-exposed cells. We showed that CHO-CRE cells do not respond to isoprenaline and norepinephrine, thereby confirming earlier observations[17] and suggesting an absence or insensitivity of $\beta_{1\text{-}3}$ receptors on these cells. In contrast, A10 cells did respond to both hormones, which is also consistent with previous studies.[20,21] More importantly, the findings revealed that for both cell lines the effects of PTx on cellular functioning were most pronounced after stimulation with forskolin.

After demonstrating the duration of PTx exposure and duration of stimulation of forskolin optimal for the detection of PTx, the sensitivity of the reporter cells for this toxin in the absence and presence of a multivalent vaccine was studied. The experiments in this study showed that the CHO-CRE and A10-CRE cells detect at least 0.2 IU/mL PTx in medium, while the sensitivity of the CHO-CRE cells to PTx in the presence of the multivalent DTaP-IP vaccine was superior (1-20 IU/mL) to the sensitivity of A10-CRE cells (4-20 IU/mL PTx). For CHO-CRE cells, a dose-response relationship between cAMP levels and the amount of PTx was observed. Subsequent stability experiments revealed that CHO-CRE reporter cells detected no or a marginal level of reversion of PTd into PTx in the vaccine after storage at 37°C for four weeks (far below 1 IU/mL), while storage at this temperature did not result in clustering of parental CHO cells. Remarkably, storage at 37°C and to a lesser extent at 4°C reduced the capacity of residual PTx both in the vaccine as well as in medium to induce a cAMP increase (Figure 6). In contrast, Oh *et al.*[22] showed that storage of glutaraldehyde-formaldehyde derived PTd at 37°C for 2 to 8 weeks resulted in enhanced ADP-ribosylation and binding analysed by an HPLC method and fetuin binding assay, respectively. The discrepancy between these and our results may be due to differences in the PTx inactivation process. Although we cannot rule out that storage causes a change in the toxoid which is not detected by our assay, our results indicate that such changes most likely will not affect intracellular cAMP levels and therefore most likely not influence cellular functioning.

This work also showed that conditions optimal for detection of PTx in the presence of a DTaP-IP vaccine differ from those for detection of PTx in medium, most likely because the PTx in our vaccines is bound to $AlPO_4$ and gradually becomes available by competition between vaccine components and FCS in the medium. This is confirmed by the observation that PTx spiked into vaccines takes longer to be detected by the reporter cells than PTx spiked into medium. We cannot rule out that some vaccine antigens including PTx remain bound to the adjuvant and will not become available for the cells. However, the experiments in this study showed that the sensitivity of the reporter cells is sufficient to detect levels of PTx at or below the LD_5 spiked into vaccine, even though some of the PTx may have remained bound to the adjuvant. For application as a quality control test, we suggest to spike a safe in-house lot of aP and relate newly produced lots of aP vaccine to this standard, rather than to PTx spiked into medium. This is line with the specifications of the WHO[23], describing that an alternative method should have a similar sensitivity as the *in vivo* test and stating that the amount of residual PTx should not exceed the level found in a lot shown to be safe in the clinic.

In conclusion, a CHO-CRE reporter cell line was generated that has a sensitivity and specificity for PTx similar to the HIST. Although the CHO-CRE reporter assay and the CHO cell clustering assay both reflect binding, internalisation, translocation and enzymatic activity of the toxin and have a similar sensitivity, the CHO-CRE reporter cells allow simple, quantitative and dose-dependent detection of PTx, features that are not offered by the CHO cell clustering assay. The results indicate that it is questionable whether reversion is a relevant risk to the investigated aP vaccine, however the CHO-CRE cells presumably can detect cellular effects if reversion would occur. Because of its sensitivity and its mechanistic relevance, the CHO-CRE reporter assay forms a suitable *in vitro* alternative to replace the current *in vivo* HIST.

Conflict of interest statement

The authors declare no financial or commercial conflicts of interest.

Acknowledgements

This study was part of a project funded by Dutch Ministry of Health, Welfare and Sports and the Dutch Ministry of Economic Affairs that aims to develop alternatives to animal use. We are grateful to Nicole Ruiterkamp, Johan van der Gun (Bilthoven Biologicals, The Netherlands), Christina Bache, Björn Becker (Paul Ehrlich Institute, Germany) and Elly van Riet (Intravacc) for helpful advice during the study. We thank Bilthoven Biologicals for providing the DTaP-IP vaccine and Jose Ferreira (RIVM) for advice on the statistical analyses.

Supplementary information

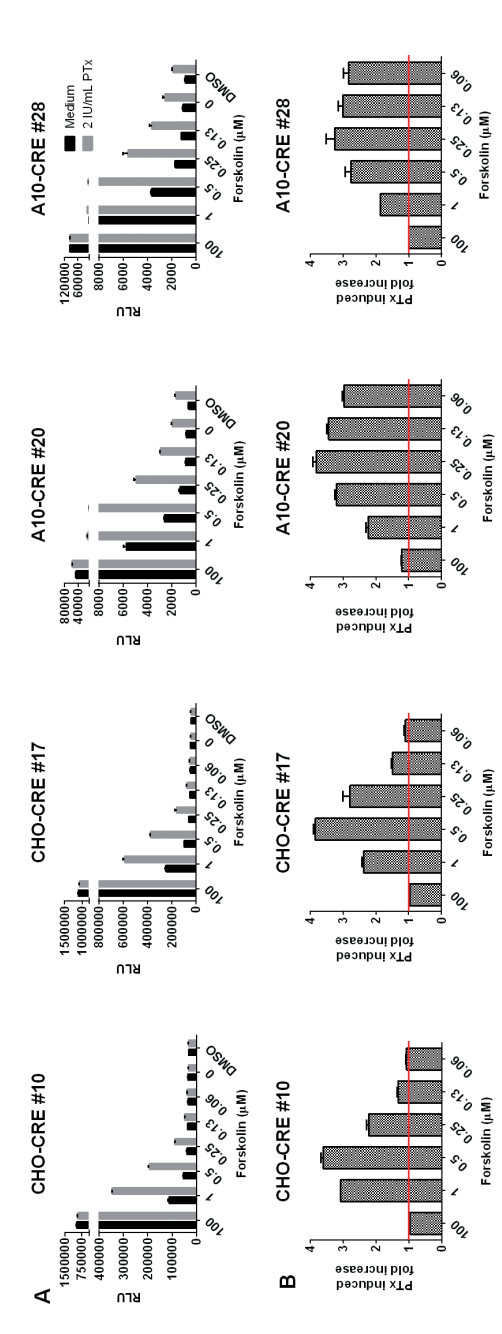


Figure S1 Evaluation of forskolin concentration to optimize the window for PTx-induced cAMP levels. CHO-CRE clones were exposed to medium or PTx (2 IU/mL) for 2 hours and subsequently stimulated with indicated concentrations of forskolin for 6 hours. A10-CRE cell clones were exposed to medium or PTx (2 IU/mL) for 1 hour and stimulated with indicated concentrations of forskolin for 4 hours. (A) Shown are the measured RLUs. (B) Responses are expressed as fold increase (luciferase activity of cells exposed to PTx and agonist/average of luciferase activity of cells exposed to medium and agonist). The average fold increase of three wells ± SD is shown.

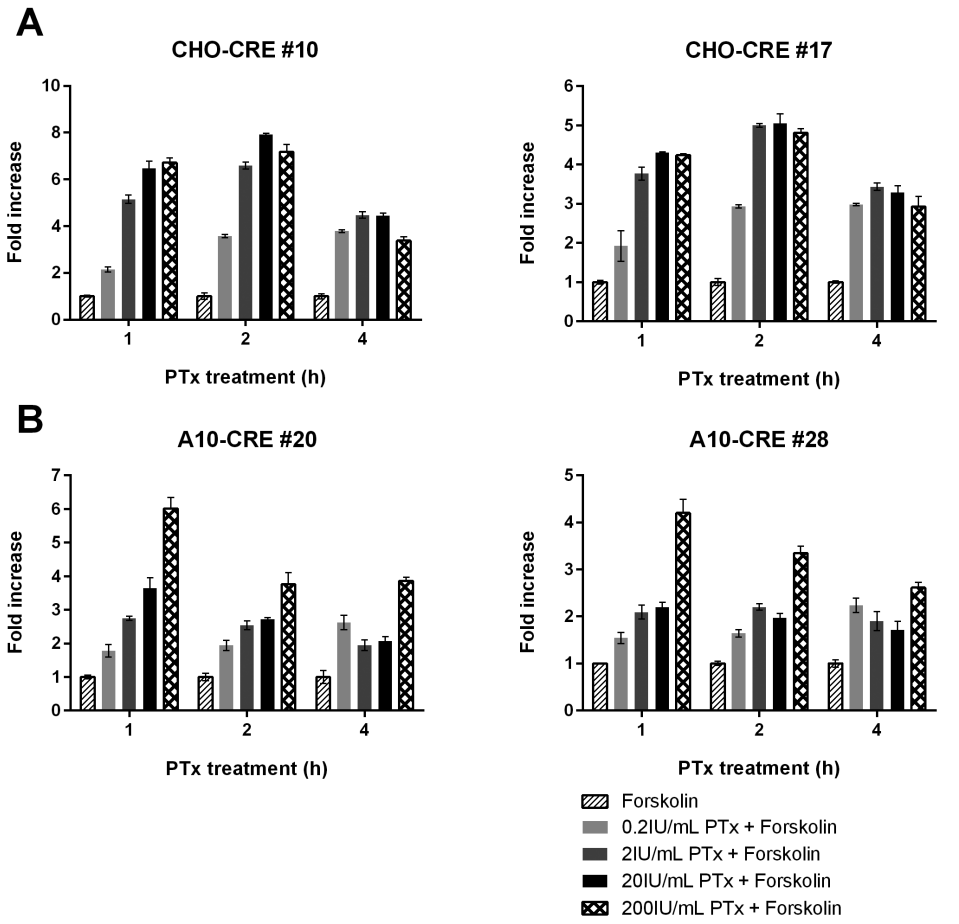


Figure S2 Effect of high concentrations of PTx. CHO-CRE clone 10 and 17 and A10-CRE cell clone 20 and 28 were exposed to medium or PTx (0.2-200 IU/mL) for 1, 2 or 4 hours. Subsequently, CHO-CRE cells were stimulated with forskolin [0.5 µM] for 6 hours, while A10-CRE cells were stimulated with forskolin [0.25 µM] for 4 hours. The measured responses were corrected for background by subtracting the response of cell exposed to only medium. The responses are expressed as fold increase (RLU of cells exposed as indicated/average RLU of cells stimulated with only forskolin). The average fold increase of three wells ±SD is shown for one out of two representative experiments.

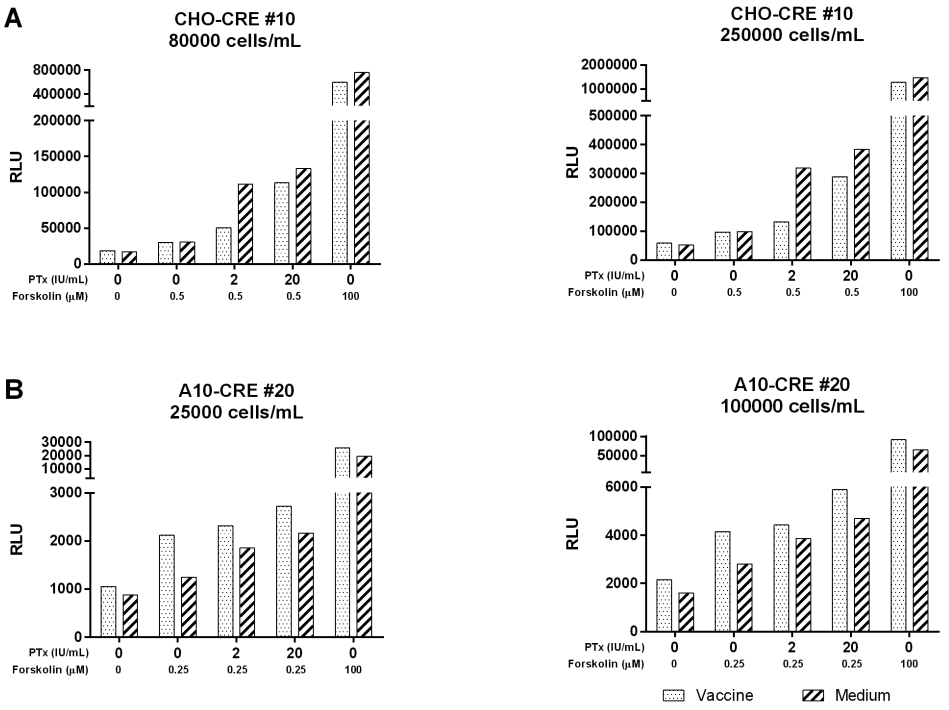


Figure S3 Optimization of cell densities in 24-well plates. CHO-CRE clone 10 was exposed to medium or vaccine spiked with PTx (0, 2 or 20 IU/mL) for 2 hours and subsequently stimulated with indicated concentrations of forskolin for 6 hours. A10-CRE clone 20 was exposed to medium or vaccine spiked with PTx (0, 2 or 20 IU/mL) for 1 hours and subsequently stimulated with indicated concentrations of forskolin for 4 hours. Shown are RLU of CHO-CRE cell clone 10 (A) and A10-CRE cell clone 20 (B). Single samples were analysed due to limited availability of the DTaP-IP vaccine.

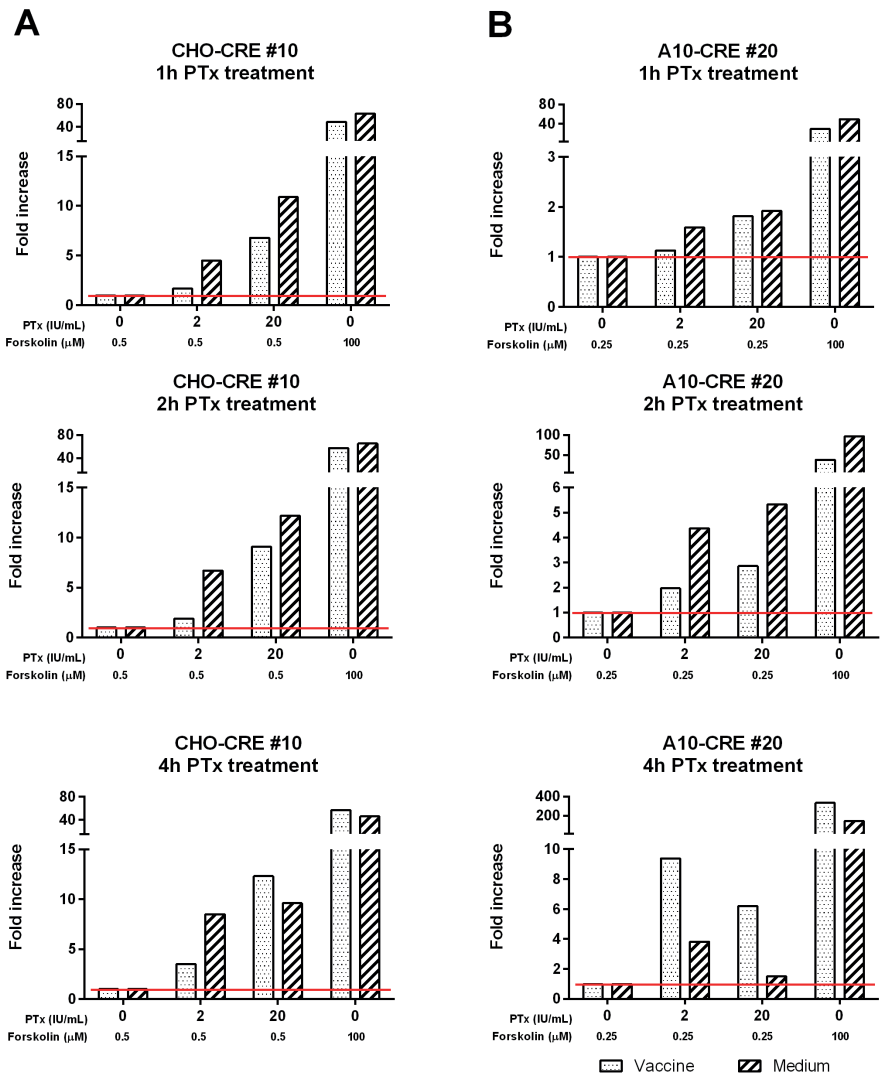


Figure S4 Optimization of PTx incubation times for detection of PTx in spiked DTaP-IP vaccines in a 24-well format. CHO-CRE clone 10 (2 x 10^4 cells/well) was exposed to both vaccine or medium spiked with PTx (0, 2, 20 IU/mL) for the indicated periods of time and subsequently stimulated with forskolin for 6 hours (A). A10-CRE clone 20 (0.625 x 10^4 cells/well) was exposed to both vaccine or medium spiked with PTx (0, 2, 20 IU/mL) for the indicated periods of time and subsequently stimulated with forskolin for 4 hours (B). The measured responses were corrected for background by subtracting the response of cells exposed to only medium. The responses are expressed as fold increase (RLU of cells exposed as indicated/average RLU of cells stimulated with only forskolin). The average fold increase of three wells ±SD is shown.

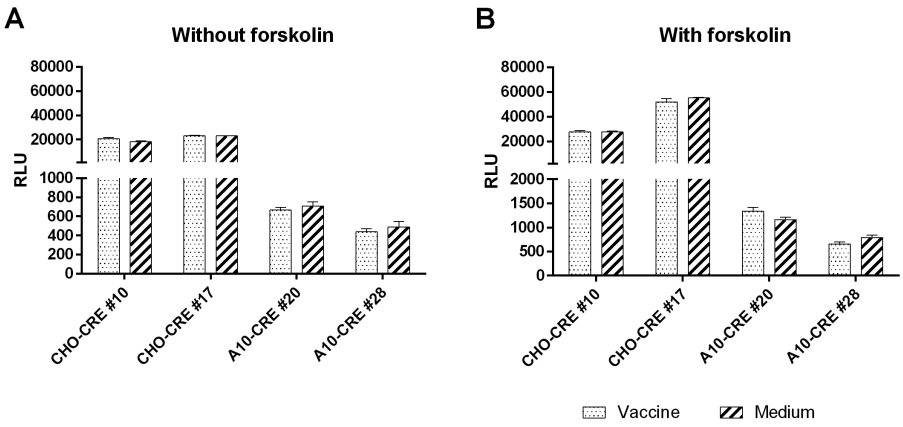
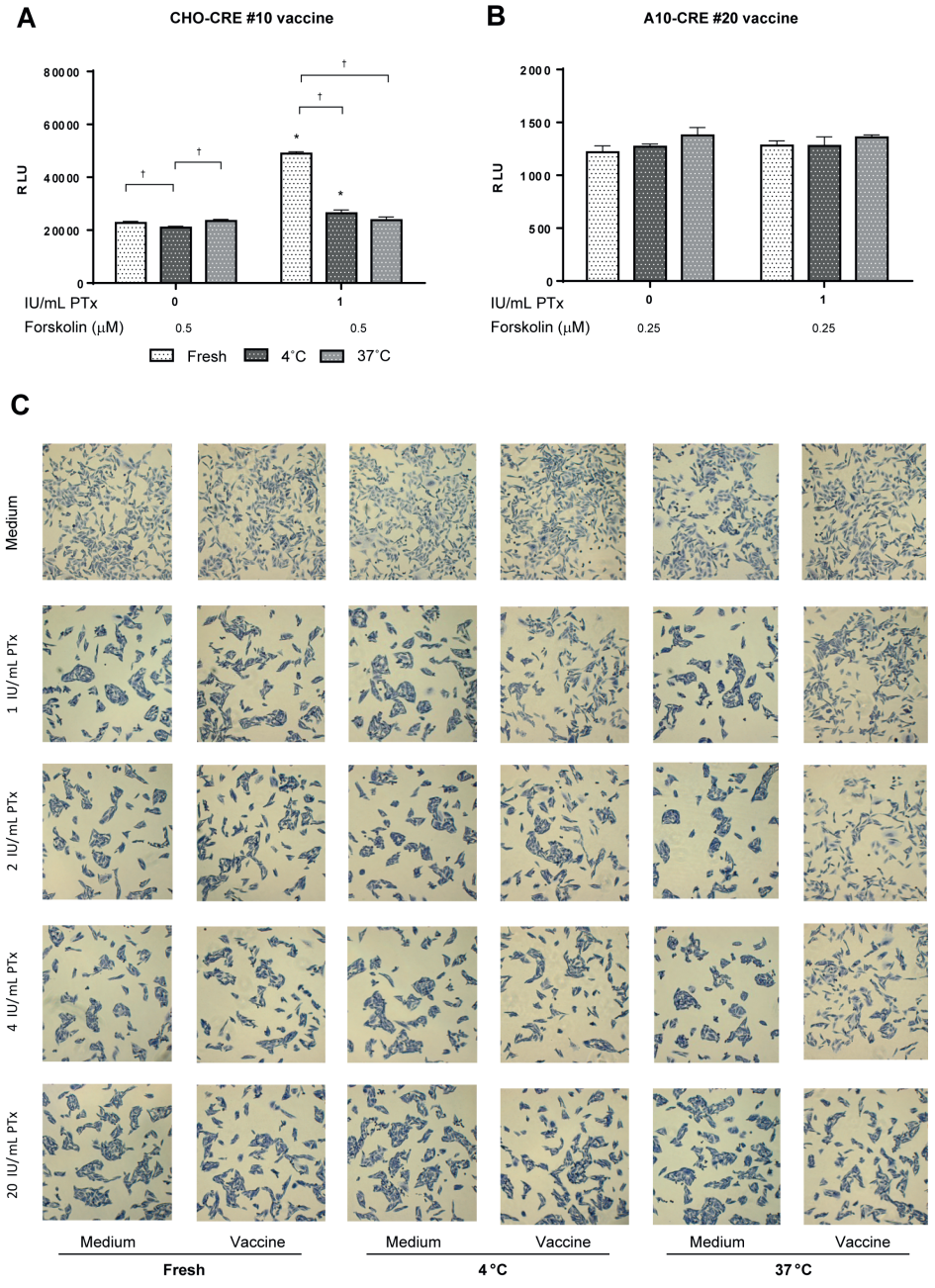


Figure S5 Effect of DTaP-IP vaccine on cAMP levels. CHO-CRE clones 10 and 17 were exposed to both vaccine or medium without PTx for 4 hours and subsequently stimulated with medium or forskolin [0.5 μM] for 6 hours (A). A10-CRE clones 20 and 28 were exposed to both plain vaccine or medium for 2 hours and stimulated with medium or forskolin [0.25 μM] for 4 hours (B). The responses are expressed as RLU. † significant difference between indicated samples for $p < 0.05$.

Figure S6 (right) Residual toxicity and reversion to toxicity in a DTaP-IP vaccine. Vaccines or medium were spiked with PTx (0, 1, 2 4 or 20 IU/mL) and stored at 4˚C and 37˚C for 4 weeks. The same samples were prepared freshly on the day of use. CHO-CRE clone 10 was exposed to the indicated samples for 4 hours and stimulated with forskolin for 6 hours (A). A10-CRE clone 20 was exposed to the indicated samples for 2 hours and stimulated with forskolin for 4 hours (B). The responses are expressed as the measured RLU. * $p < 0.05$, significantly different from corresponding medium exposed and forskolin stimulated cells. † significant difference between indicated samples for $p < 0.05$. Parental CHO cells were exposed to medium or vaccine spiked with PTx for 48 hours, fixed with methanol and stained with Giemsa stain (magnification 10x) (C).


A
CHO-CRE #10 vaccine
RLU
80000
60000
40000
20000
0
IU/mL PTx
Forskolin (μM)
0
0.5
1
0.5
Fresh
4°C
37°C
B
A10-CRE #20 vaccine
RLU
2000
1500
1000
500
0
IU/mL PTx
Forskolin (μM)
0
0.25
1
0.25
C
Medium
1 IU/mL PTx
2 IU/mL PTx
4 IU/mL PTx
20 IU/mL PTx
Medium
Vaccine
Medium
Vaccine
Medium
Vaccine
Fresh
4 °C
37 °C

References

1. Sato H, Ito A, Chiba J, Sato Y. Monoclonal antibody against pertussis toxin: effect on toxin activity and pertussis infections. Infection and immunity. 1984;46(2):422-8.
2. Black WJ, Munoz JJ, Peacock MG, Schad PA, Cowell JL, Burchall JJ, *et al.* ADP-ribosyltransferase activity of pertussis toxin and immunomodulation by *Bordetella pertussis*. Science. 1988;240(4852):656-9.
3. Kind LS. The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bacteriological reviews. 1958;22(3):173-82.
4. Bergman RK, Munoz J. Circulatory Collapse in Anaphylaxis and Histamine Toxicity in Mice. J Immunol. 1965;95:1-8.
5. Plaut RD, Carbonetti NH. Retrograde transport of pertussis toxin in the mammalian cell. Cellular microbiology. 2008;10(5):1130-9. Epub 2008/01/19.
6. Katada T, Tamura M, Ui M. The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Archives of biochemistry and biophysics. 1983;224(1):290-8. Epub 1983/07/01.
7. Gomez SR, Yuen CT, Asokanathan C, Douglas-Bardsley A, Corbel MJ, Coote JG, *et al.* ADP-ribosylation activity in pertussis vaccines and its relationship to the *in vivo* histamine-sensitisation test. Vaccine. 2007;25(17):3311-8. Epub 2007/02/09.
8. Isbrucker RA, Bliu A, Prior F. Modified binding assay for improved sensitivity and specificity in the detection of residual pertussis toxin in vaccine preparations. Vaccine. 2010;28(15):2687-92. Epub 2010/02/04.
9. Yuen CT, Horiuchi Y, Asokanathan C, Cook S, Douglas-Bardsley A, Ochiai M, *et al.* An *in vitro* assay system as a potential replacement for the histamine sensitisation test for acellular pertussis based combination vaccines. Vaccine. 2010;28(21):3714-21.
10. Xing D, Das RG, Newland P, Corbel M. Comparison of the bioactivity of reference preparations for assaying *Bordetella pertussis* toxin activity in vaccines by the histamine sensitisation and Chinese hamster ovary-cell tests: assessment of validity of expression of activity in terms of protein concentration. Vaccine. 2002;20(29-30):3535-42. Epub 2002/09/26.
11. Castro MG, McNamara U, Carbonetti NH. Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. Cellular microbiology. 2001;3(1):45-54. Epub 2001/02/24.
12. Hoonakker ME, Ruiterkamp N, Hendriksen CF. The cAMP assay: a functional *in vitro* alternative to the *in vivo* Histamine Sensitization test. Vaccine. 2010;28(5):1347-52. Epub 2009/11/28.
13. Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, *et al.* Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. ACS Chem Biol. 2012;7(11):1848-57.
14. Isbrucker R, Wagner L, Daas A, Costanzo A. Transferability study of CHO cell clustering assays for monitoring of pertussis toxin activity in acellular pertussis vaccines. . Pharmeuropa bio & scientific notes. 2016;2016-05:97-114.
15. Hochberg Y, Benjamini Y. More powerful procedures for multiple significance testing. Statistics in medicine. 1990;9(7):811-8.
16. Bache C, Hoonakker M, Hendriksen C, Buchheit KH, Spreitzer I, Montag T. Workshop on Animal free Detection of Pertussis Toxin in Vaccines--Alternatives to the Histamine Sensitisation Test. Biologicals: journal of the International Association of Biological Standardization. 2012;40(4):309-11. Epub 2012/05/23.

17. Baker JG. The selectivity of beta-adrenoceptor agonists at human beta1-, beta2- and beta3-adrenoceptors. British journal of pharmacology. 2010;160(5):1048-61.
18. Bentley PJ. The chemical structure, polymorphism, and evolution of hormones, Comparative Vertebrate Endocrinology (third ed.): Cambridge University 1998.
19. D'Allaire F, Atgie C, Mauriege P, Simard PM, Bukowiecki LJ. Characterization of beta 1- and beta 3-adrenoceptors in intact brown adipocytes of the rat. British journal of pharmacology. 1995;114(2):275-82.
20. Brawley L, Shaw AM, MacDonald A. Beta 1-, beta 2- and atypical beta-adrenoceptor-mediated relaxation in rat isolated aorta. British journal of pharmacology. 2000;129(4):637-44.
21. O'Donnell SR, Wanstall JC. Beta-1 and beta-2 adrenoceptor-mediated responses in preparations of pulmonary artery and aorta from young and aged rats. The Journal of pharmacology and experimental therapeutics. 1984;228(3):733-8.
22. Oh H, Kim BG, Nam KT, Hong SH, Ahn DH, Choi GS, *et al.* Characterization of the carbohydrate binding and ADP-ribosyltransferase activities of chemically detoxified pertussis toxins. Vaccine. 2013;31(29):2988-93.
23. WHO. Annex 4 Recommendations to assure the quality, safety and efficacy of acellular pertussis vaccines. Replacement of Annex 2 of WHO Technical Report Series. 2013;878.

8

Chapter 8

Safety testing of acellular pertussis vaccines: animal numbers and 3R alternatives

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Manuscript in preparation

Abstract

The current test of acellular *Bordetella pertussis* (aP) vaccines for residual pertussis toxin (PTx) is the Histamine Sensitization test (HIST), based on the empirical finding that PTx sensitizes mice to histamine. Although HIST has ensured the safety of aP vaccines for years, it is criticized for the limited understanding of how it works, its technical difficulty, and for animal welfare reasons. To identify the impact of HIST in terms of mice used worldwide, we surveyed major aP manufacturers and organizations performing, requiring, or recommending the test. The survey revealed marked regional differences in regulatory guidelines, including the number of animals used for a single test. Based on information provided by the parties surveyed, we estimated the worldwide number of mice used for testing to be 65,000 per year: ~48,000 by manufacturers and ~17,000 by national control laboratories. These animals covered the release of approximately 850 final lots and 250 bulks of aP vaccines yearly. Although there are several ways to refine and reduce the test, we question whether the efforts needed for validation and implementation of these interim alternatives are worthwhile, when there are several *in vitro* alternatives in various stages of development. We argue that upon implementation, one or more of these replacement alternatives can substantially reduce the number of animals currently used for the HIST, although careful evaluation of each alternative's mechanism and its suitable validation will be required before any of them can be implemented.

Introduction

Pertussis or whooping cough is a disease caused by a respiratory infection with the bacterium *Bordetella pertussis*. The disease is characterized by severe coughing, sometimes progressing to pneumonia, and can - on rare occasions and primarily in young infants - be fatal. The introduction of the first pertussis vaccines in the 1950s-60s led to a dramatic drop in pertussis incidence. These early vaccines were based on inactivated bacteria (wP vaccines). Downsides of these vaccines were their side effects and occasional but severe reactions temporally associated with their administration, which resulted in a search for safer vaccines. In the 1980's-90s, the acellular vaccines (aP) gradually replaced the wP vaccines in most industrialised countries (Table 1).[1,2] In non-industrialized countries, vaccination with wP vaccines has remained the preferred strategy, due to simpler manufacturing and consequent lower costs. aP vaccines consist of one or several purified proteins of the pathogen that are considered to contribute to protection.[3-5] Remarkably, the incidence of pertussis has increased since the 1980s, even in areas with high vaccination coverage.[6] Worldwide, the WHO estimated the number of pertussis cases to be about 16 million in 2008.[7,8] Because of the persistent circulation of *B. pertussis*, there will most likely be a continued need for aP and wP vaccines in the near future.

All aP vaccines include detoxified pertussis toxin (referred to as pertussis toxoid: PTd) and one or more of the following proteins: filamentous hemagglutinin, pertactin, fimbria type 2 and type 3. Since a certain level of activity of pertussis toxin (PTx) is considered to be of health concern for vaccine recipients, testing for the presence of residual activity and reversion of PTd to PTx is a regulatory requirement in all countries that use aP vaccines. For this purpose, the *in vivo* Histamine Sensitization test (HIST) is broadly applied, a procedure based on a discovery made in 1948 by Parfentjev and Goodline.[9] The test design consists of administering to groups of mice the vaccine lot under study, followed by a challenge with histamine 4-5 days post-vaccine injection. Originally the HIST was developed to test wP vaccines[10], but nowadays it is only used for testing of aP vaccines. In addition to its use for batch release testing, the HIST is also performed in-process, and included in stability programs and optimization protocols of the vaccine production processes. Although widely used, the HIST is criticized for its poor reproducibility and the high variability in the sensitivity of the mouse strains.[11] Additionally,

Table 1 Immunization programms*

Acellular pertussis vaccines	Whole cell pertussis vaccines	Mixed
North America	Africa	Eastern mediterranean
Europe	South-East Asia	Western Pacific

*[1][2]

the test can inflict severe pain and distress, especially for mice receiving a reference PTx or mice that receive a vaccine containing residual PTx. For these reasons, manufacturers, as well as regulators, are highly motivated towards the successful development and implementation of a reproducible, validated *in vitro* method to replace the HIST.[12]

This paper aims to identify the impact of the HIST in terms of animal numbers used worldwide. In addition, it discusses the potential of *in vitro* alternatives and the information that is required for implementation of such methods. To put the possibility and relevance of this change in context, we first provide some background information on the mechanism of PTx intoxication and the HIST. A preliminary version of the calculations summarized in Table 5 were presented at the international workshop: "Alternatives to HIST for Acellular Pertussis Vaccines: Progress and Challenges" held on August 24, 2014 in in Prague, Czech Republic.[13]

The mechanism of the HIST

PTx is an AB_5 toxin, composed of a B oligomer and an A subunit that are responsible for binding to the cell membrane and intracellular interference with the cAMP-PKA pathway, respectively (see Box 1 for a detailed description[12,14-25]). Since PTx by itself does not cause any noticeable acute effects in mice at the doses expected in aP vaccines, the HIST is based on the observation that PTx reduces the lethal dose of histamine about 30-300 fold.[10,26] Histamine is a compound involved in immunological responses, but can also act as a neurotransmitter.[27] It is typically released by mast cells upon activation by IgE antibodies and activates endothelial cells, increases blood vessel permeability and decreases blood pressure[28,29]. In the HIST, histamine administration causes a reduction in blood volume as a result of increased permeability of the blood vessels.[30] Bergman *et al.* have demonstrated that injection of compensatory volumes of saline into the bloodstream can prevent HIST-induced death.[26] Other studies have shown that PTx reduced the contractile properties of arteries, causing a hypersensitivity to histamine-induced vasodilation.[26,31] The histamine hypersensitivity is also observed in removal of adrenal glands and after blocking of β-adrenergic receptors of mice, suggesting that a common insensitivity to catecholamines is involved in both phenomena.[32] Taken together, these studies demonstrate that PTx affects essential properties of arteries and strongly suggest that these phenomena are responsible for PTx-induced sensitization to histamine.

HIST testing: regulatory requirements and test designs

aP vaccines were first manufactured and used in Japan in 1981.[33] Currently aP vaccines are also produced in Canada, Europe and China. These vaccines are widely used in the United States and Canada, the European region and the aforementioned Asian countries and are being gradually introduced in other parts of the world. Despite the widespread application of the HIST for aP vaccine testing, requirements for test performance are region-specific

Box 1. The cellular relevance of PTx in aP vaccines

PTx is an AB_5 toxin, composed of one A subunit (S1) and a B oligomer of 5 subunits (S2, S3, two copies of S4, and S5).[14] The B oligomer is of importance for binding of the toxin to glycoconjugate cell receptors[15], upon which the holotoxin enters the cell by endocytosis, followed by retrograde transport to the Golgi and the endoplasmic reticulum. Subsequently the S1 subunit is released into the cytosol.[16] Within the cytosol, the S1 subunit catalyzes the transfer of ADP-ribose from NAD^+ to the α-subunit of $G_{i/o}$ proteins[17], thereby preventing interaction of these proteins with their cognate receptors. ADP ribosylation fixes the α-subunit of the G-proteins in their inactive (ADP-bound) form and the α-subunit is therefore unable to inhibit its target enzyme, adenylate cyclase. The consequent accumulation of the second messenger (cAMP) interferes with cellular signaling. PTx-induced changes in cell signaling can generally be detected within a couple of hours.[18] The B oligomer binds to several target proteins on the membrane of cells and thereby PTx influences several cellular processes. The B oligomer by itself causes proliferation of T cells[19,20], results in glucose oxidation in adipocytes[19] and induces activation of TLR4.[21] These effects of the B oligomer appear 2-24 hours after exposure to PTx, while effects on intracellular cell signaling appear more rapidly[22,23]. Generally cellular effects of B oligomer require higher levels of PTx[24], i.e. 250ng/mL-2μg/mL[19-22], while ADP ribosylation by the S1 subunit are induced by 50ng/mL of PTx or lower concentrations[25] (Hoonakker *et al.*, submitted for publication). The levels necessary for B oligomer effects are substantially higher than the levels accepted in aP vaccines (approximately 14.8ng PTx/mL)[12]. The relevance of the direct effects of B oligomer for aP vaccines is therefore questionable, though B oligomer binding is essential for cell entry and therefore indirectly for S1 subunit functioning.

and vary with regard to mouse specifications (sex, age, etc.), inclusion of positive and/or negative control groups, test group sizes, time interval between vaccination and challenge, time interval between challenge and reading of the test outcome and histamine challenge dose. There are basically three versions of the test. The first version determines whether the level of PTx is at or below an acceptable threshold, by recording death of the mice after a histamine challenge. Similar to the first version, the second version is based on PTx-induced histamine sensitization; however, whether the level of PTx exceeds a specified threshold is determined by assessing a decrease in body temperature, rather than death of the mice.[34] The third version uses the same principle as the second version, but measures the actual level of PTx by comparing the associated decrease in body temperature caused by several doses of the vaccine on test, relative to a toxicity reference preparation. For the second and third versions,

Table 2 Global requirements for HIST

		WHO TRS 979	**EU** European Pharmacopeia	**US** License dosier	**Canada** Licence dossier	**China** Chinese Pharmacopeia	**Japan** Japanese Pharmacopeia
Vaccine	Final lot/bulk	Final bulk (one or more dilutions)	Lot	Lot or bulk*	Bulk	Bulk and final bulk*	Bulk and Lot
	Residual or reversion to toxiciy**	Residual and or reversion	Residual and reversion	Residual***	Residual	Residual and reversion	Residual and reversion
	Volume	1 or 2 HD (one or more dilutions)	1-2 HD	500 µL	1 HD	500 µL	500 µL
Controls	Positive	PTx (one or more dilutions)	PTx (one dilution)	PTx (one dilution)	PTx (one dilution)	PTx (several dilutions)	PTx (several dilutions)
	Negative	Diluent or none	Diluent	Diluent	Diluent	N.I.	N.I.
Mice	Number of animals per group	10 or appropriate number	10	20	16	10	10
	Number of groups	App. 5	4	App. 3	3	App. 5	App. 6
	Min. animal number per test	App. 50	40	App. 60	48	App. 50	App. 60
Challenge and reading	Histamine dose	Defined dose (usually 1 or 2 mg)	2 mg	1 mg	0.7 mg	2-4 mg	4 mg
	Interval sample - challenge	4-5 days	5 days	5 days	5 days	4 days	4 days
	Period of observation	30 minutes-24 hours	24 hours	24 hours	24 hours	30 minutes	30 minutes
	Parameter	Decrease in temperature or death	Death	Death	Death	Decrease in temperature	Decrease in temperature

* Lot or bulk means that the lot or the bulk is subjected to HIST, while bulk and lot/final bulk means both are

** Samples for residual toxicity are kept at 4˚C, samples for reversion to toxicity are kept at 37˚C for a specified period

*** Each manufacturer should put on stability testing at least one lot of each product per year, and it should be tested for HIST

HD = Human dose

N.I. = Not indicated

Table 3 Acceptance and validity criteria

	WHO TRS 979	**EU** European Pharmacopeia	**US** Licence dossier	**Canada** Licence dossier	**China** Chinese Pharmacopeia	**Japan** Japanese Pharmacopeia
Acceptance critaria	Residual activity of PT or the number of animals that die is not higher than specified by the NRA. If a vaccine lot fails in a single test, it should pass two additional test for release.	The vaccine complies with the test if in the group that receives the vaccine stored at 2-8°C or 37°C, there are no deaths or no more deaths than in the group that receives the reference vaccine. If one mouse dies in one or both of the vaccine groups, repetition is allowed with the same number of mice or more. The vaccine is accepted when overall death rate is 5% or less.	One undiluted single human dose of 0.5 mL sensitizes no more than 10% of mice injected. If the vaccine fails to meet the criterion in a first test, it should pass two additional tests.	The vaccine complies with the test if in the group that receives the vaccine, there is no more than one death. If more than one mouse dies in the negative control group or the vaccine group, repetition is allowed with the same number of mice or more. The vaccine is accepted when overall death rate is 6.25% or less.	The histamine-sensitizing toxicity of both test samples at 4°C and 37°C shall be no higher than 0.8 HSU/mL in mice upon statistical analysis.	The histamine-sensitizing toxicity of both test samples at 4°C and 37°C shall be no higher than 0.4 HSU/mL in mice upon statistical analysis.
Validity criteria	1. Validity criteria should be met. 2. Less than 5% deaths in the negative control group. 3. Demonstrated sensitivity of mice strain. 4. When linearity of log dose-response to PTx is demonstrated 1 positive control group suffice.	1. No mice die in the control group. 2. Sensitivity of the mice is demonstrated (e.g. 30% of the mice die in the positive control group). 3. A suitable mouse strain has a toxin LD50 between 6 IU and 50 IU.	1. PTx control group should show that mice used are sensitized by a dose of PTx below 100 ng, in terms of the HSD50. 2. No more than 10% of mice should die in the negative/ diluent group.	1. There are at least 16 mice challenged per group. 2. No more than one mouse dies in the negative control group. 3. Sensitivity of the mice is demonstrated, i.e. at least 7 mice die in the positive control group (= 43.75%, mice injected with 400 ng of PTx).	N.I.	N.I.

NRA = National regulatory authority

HSD = Histamine Sensitizing Dose

HSU = Histamine Sensitization Units

N.I. = Not indicated

body temperature can be monitored by a rectal probe or by infrared detection of the dermal temperature.[35] Although changes in body temperature and death are both dose-dependent, there doesn't seem to be a direct link between both parameters[35], and it is unclear whether both methods are based on the same mechanism.

Regional requirements and global guidelines (WHO) for the HIST, including relevant validity and acceptance criteria, are provided in Tables 2 and 3. Both Japan and China apply the third version and assess the PTx content of a new batch of aP vaccine relative to a toxicity reference to which histamine sensitization units (HSU) have been assigned. The accepted levels of PTx are 0.4 and 0.8 HSU, for Japan and China, respectively. In Europe, the US and Canada, safety testing of aP vaccine batches is primarily based on the HIST with a lethal endpoint, although the second version of the HIST is also approved in Europe. Though the general outline of the lethal and temperature versions of the HIST are similar, there are notable regional differences (Tables 2 and 3). Independent of the test design, testing is performed on the bulk product, the final formulation or both. Apart from the regulatory requirements, the final lay-out of the test is influenced by the testing stage, that is, whether the test is the first one performed, a repeat of the first test or a retest. Repetition of a HIST is required when the validity criteria of the test are not met, and test validity is primarily determined by the responses of the positive and negative control groups. Generally, for repeated testing the same design and number of animals are utilized. Retests are performed when a vaccine lot does not meet the acceptance criteria. For retesting, in general, twice the number of animals is required for the lethal endpoint method, while the same number of animals is required when the temperature method is utilized. Remarkably, repeated testing and retesting appears to occur more often for HIST protocols based on lethality as an endpoint, compared to the HIST with the body temperature as a readout parameter (as shown in Table 5 and addressed below). The reason could be the inherent higher sensitivity of the temperature method and the relative evaluation of residual PTx to a PTx reference that enables compensation of assay variation.[35]

The HIST and laboratory animal use

In order to identify the impact of the HIST in terms of animal numbers, a survey was conducted among manufacturers, national control laboratories (NCLs) and multinational organizations involved with vaccine quality. Sanofi Pasteur SA (France), Sanofi Pasteur Limited (Canada), GlaxoSmithKline Biologicals (Belgium) and Statens Serum Institut (Denmark) were asked to specify how many lots of vaccine containing an aP component were subjected to the HIST, either in the year 2012 or 2013; to indicate the specifications of the HIST performed and procedural details, including the number of animals used. Information on test design was combined with literature on aP vaccine production in Japan and China.[36,37] Manufacturers provided their consent for publication of the data.

Following safety testing by the manufacturers, NCLs may also subject the aP vaccine lot to the HIST. Eight NCLs/organizations (Pan American Health Organization (PAHO), US FDA, Health Canada (Canada), European Directorate for the Quality of Medicines & Health Care, Statens Serum Institut (Denmark), Indian Academy of Pediatrics (India), National Institute of Infectious Diseases (Japan), National Institutes for Food and Drug Control (China)) were queried for their testing strategies, or the testing strategies of the NCLs in the region. The organisations were given random code numbers herein, not related to the order above. Organizations 1-4 could provided complete information or as far as possible (for information see Table 4). Organizations 5 and 6 were unable to provide information, and therefore we assumed a testing regime in which 50% of the lots were tested. Precise information on testing by NCL's could not be provided by organization 7, while organisation 8 indicated that aP vaccines were not produced in that region. Since exact numbers were only partly available, some of the data were estimated as shown in Table 4. Although our calculations attempt to provide an estimation of the number of mice required for the HIST on a global scale, in reality they cover only the manufacturers and NCLs in the regions in which aP vaccines are mainly produced and used (Table 1). For example, one organization reported the purchase of aP-containing vaccines for 14 countries from three of the manufacturers surveyed, but data on additional HIST testing by NCL's at the local level, if any, were not available. The data are presented masked.

To calculate animal numbers used for the HIST, two assumptions were made, in consultation with the manufacturers and NCLs. Firstly, the percentages of repeated testing and retesting were known or estimated to occur between 0% and 16% and 0% and 7.5% of the cases (as indicated), respectively. Secondly, when information on testing by an NCL was not available, we assumed that 50% of the lots being released by these NCLs were tested, and that repeated testing and retesting frequencies were similar to those of the manufacturers. Evaluation on the HIST performance revealed substantial differences in the number of animals used for a single test, as well as group size (10 to 22 animals per group), and inclusion of positive and negative control groups (data not shown). Based on the evaluation we estimated that worldwide approximately 65,000 mice are used for the HIST each year, ~48,000 by the manufacturers and ~17,000 by the NCLs (Table 5). These tests covered the release of approximately 850 final lots and 250 bulks of aP vaccines each year for the protection of population of most industrialised countries and some emergent economies against pertussis.

The HIST and 3R alternatives: perspectives

The scientific and societal concerns associated with the HIST provide a strong incentive for the development and application of 3R methods. In general, replacement of an animal method by an animal-free method is a lengthy process, while a critical evaluation of existing animal models often reveals opportunities for refinement and reduction. In this context, we evaluated

Table 4 Origin of information on animal numbers

Manufacturer/ Organisation code	Year	Number of bulks	Number of final lots	Average number of lots/ bulks per test*	Average number of animals/ test	Total animal number	Percentage of animals used for first test	Percentage of animals used for repeated tests	Percentage of animals used for retesting
M1	2013	N.T.	Known	Estimated	Calculated	Known	Estimated	Estimated	Estimated
M2	2012	Known	N.T.	Known	Known	Calculated	Estimated	Estimated	Estimated
M3	2013	N.T.	Calculated	Estimated	Calculated	Known	Estimated	Estimated	Estimated
M4	2012	N.T.	Known	Known	Known	Known	Known	Known	Known
Or1	-	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or2	-	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or3	2012	N.T.	Known	Known	Known	Calculated	Known	Known	Known
Or4	2012	N.T.	Known	Known	Known	Calculated	Known	Known	Known
Or5	-	N.T.	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated
Or6	-	N.T.	Known	Estimated	Estimated	Estimated	Estimated	Estimated	Estimated
Or7	-	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.
Or8	-	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.

N.T. = Not tested, testing is not done or required by indicated organisation

M = Manufacturer

Or = Control, Regulatory or Guiding Organisation

Known: Numbers were provided

Estimated: Numbers were not available and therefore estimated in consulation with the organisation/manufacturer

Calculated: Numbers were calculated based on the known information

whether there were any possibilities to reduce animal numbers. Surprisingly, there were marked differences in group sizes used, primarily for the lethal version of the HIST, ranging from 10 to 22 animals per group. These differences in numbers are mainly associated with local regulatory requirements, although there might be a justification on the assay variability, as a consequence of the use of different mouse strains[11] or different vaccination-challenge schedules. There is no evident scientific justification for applying various group sizes, since the various performances of the HIST are based upon the same principle, and since each group size is accepted by one or more NCLs. Therefore, an obvious and straightforward approach to reduce animal numbers for the HIST would be using the smallest group size (i.e. 10 animals per group). Recalculations revealed that universal application of this group size would reduce total animal numbers by 10% (data not shown). However, application of such an approach entails international harmonization of regulatory requirements, which has proven to be a challenging process. In addition to reducing animal numbers, another option

Table 5 Annual number of animals used for HIST

		Total
Manufacturer	Number of final lots or bulks tested	1100
	Number of tests	640
	Number of animals per group	10-22
	Average number of animals per test*	40-132
	Total animal number	**47700**
	*Of which used for first test***	43800
	*Of which used for repeats****	1100
	Of which used for retesting§	2800
Con Or	**Total animal number**	**17100**
	Of which used for first test§§	15600
	Of which used for repeats§§§	400
	Of which used for retesting§	1100

Con Or = Control organisation

* Numbers depend on group size, inclusion of positive and negative controls and the number of lots/bulks tested

** Percentage of animals used for this purpose varied between 79-100%

*** Percentage of animals used for this purpose varied between 0-16%

§ Percentage of animals used for this purpose varied between 0-7.5%

§§ Percentage of animals used for this purpose varied between 90-100%

§§§Percentage of animals used for this purpose varied between 0-2.5%

is to minimize the distress inflicted to the animals. As mentioned before, the presence of PTx can be assessed in terms of changes in body temperature, a method primarily applied in Asia and by SSI in Denmark.[34] A drop in rectal or dermal temperature as a consequence of PTx is assessed within 30 minutes of histamine challenge. Application of this method might therefore reduce the duration of potential suffering.[34] However, the majority of the deaths occur within the 30 minutes after histamine injection[38], and these fatalities most likely happen among the animals that experience the highest level of suffering. Using body temperature as a readout parameter may only reduce suffering of animals that receive a borderline lethal level of PTx. In a study of the transferability of the non-lethal test for PTx in aP vaccines[39], the maximum decrease in temperature post-histamine challenge was achieved with a dose as low as 1.75 IU of PTx per mouse, a dose which already caused ≥ 50% death in groups of 10 mice, in 4/10 experiments. This represents a high level of lethality in a non-lethal alternative, if we consider that a collectively agreed suitable baseline HIST sensitivity is 2.0 IU of PTx per mL

of vaccine[12,40]. Taken together, possibilities to reduce and refine the HIST are available, but it is questionable whether the gain in animal welfare justifies the efforts needed for validation and implementation, considering the number of animals needed for validation. In addition, refinement and reduction alternatives still encompass the variation inherent to the principle of the HIST, as well as the fact that several promising *in vitro* methods are in the final stage of development.

In addition to refinement and reduction, several research groups have attempted to develop animal-free methods for replacement of the HIST. Generally, the acceptance of an alternative method is favoured if it is based on a principle which has similarity with pharmacological relevant mechanisms of PTx[41]. As discussed before, the mechanisms primarily involved in the HIST have been elucidated. However, the relationship between the mechanisms of the HIST and the pharmacological effects of PTx in humans are not fully elucidated. As mentioned in Box 1, theoretically B oligomer and S1 may have direct and independent effects on cell functioning. Nevertheless, the concentration of PTx that has raised concern for vaccine safety is one at which only S1 is able to induce cellular effects, while the direct activity of the B oligomer at these concentrations (see Box 1) has not been described. Noteworthy is that a functional B oligomer is necessary for cell binding and internalisation and therefore essential for S1 intracellular effects.

There has been debate on what the acceptable and clinically relevant level of PTx in aP vaccine is to guarantee vaccine safety.[12,42] Only indirect evidence is available to approach this question, since - for ethical reasons - it is not possible to carry out clinical studies to quantify toxic PTx effects in the clinic. Nonetheless, a study of the therapeutic effects of PTx for diabetes revealed that the intravenous injection of 1 µg/kg of the protein did not cause any pronounced adverse effects in a healthy volunteer.[43] Additionally, clinical evidence for a relevant level of PTx is provided by studies showing that the PTx content of whole cell vaccines is approximately 0.3 µg/mL[11,44], while these vaccines are generally considered to be safe and efficacious. Also, the HIST itself provides information by means of its sensitivity and acceptance criteria. According to the guidelines of the European Pharmacopeia, a mouse strain is generally considered suitable for the HIST if the LD_{50} for the BRP PTx is between 6 IU and 50 IU. A vaccine is accepted if not more than 5% of the immunized mice die when challenged with histamine[45]. Based on this information and data from a collaborative study organized by the EDQM[46], the LD_5 for the BRP1 PTx standard was estimated to be approximately 2.22 IU/ml[12] (corresponding to 14.8 ng/mL of BRP PTx).

There has been a range of initiatives aimed to replacing the HIST by suitable animal-free methods. One advanced alternative entails a combination of two analytical methods. The first of the two methods assesses the binding of PTx to a fetuin coated ELISA plate[47,48] and mimics B oligomer's binding to glycoproteins on the cell surface.[15] The second method is based on the HPLC measurement of the enzymatic activity of the A subunit, using a fluorescent peptide.[49-51] The combination of these assays reflects properties essential for PTx toxicity, although not simultaneously, and has proven to be a useful tool to study the inactivation procedures.[35] However, the assays do not cover the internalisation and translocation of the toxin from the cell membrane to the cytosol, nor is intracellular ADP-ribosylation or its effects measured. These latter aspects are an inherent part of the pharmacological effects of the toxin, and are covered by the three *in vitro* cell culture alternatives. The first of these methods is the Chinese Hamster Ovary cell (CHO) test, based on a PTx-induced clustered growth pattern.[52,53] A study in which genetically modified CHO cells constitutively expressed the S1 subunit demonstrated that this moiety was ultimately responsible of the cell clustering.[54] Nevertheless, under physiological conditions, B oligomer binding and internalisation of the toxin will be necessary for S1 to execute its effect in the cytosol. This CHO cell clustering test is only suitable for evaluation of pre-formulated and non-adjuvated products, due to inherent toxicity of the adjuvants for CHO cells[42], though recent studies have demonstrated that these cytotoxic effects can be overcome by dilution or the use of trans-well inserts.[55] Another hurdle is the visual reading of the cell clustering. Despite attempts of several research groups, automatic analysis reading is not yet possible. Nevertheless, the CHO-cell assay was deemed by the International Working Group on Alternatives to HIST at a recent workshop in London as deserving of further development/ validation (manuscript in preparation). The other two cell-based methods assess the intracellular effects of PTx on adenylate cyclase regulation, by measuring changes in hormone-stimulated cAMP or ATP levels.[25,42] Since toxin binding, internalisation and disturbance of the regulation of adenylate cyclase are intrinsic elements of the adverse outcome pathways, the methods cover all cellular aspects relevant for the safety of aP vaccines. Proof-of-principle for the cAMP-PTx assay has been positive and further development (pre-validation) of this quantitative methods has been supported.[42] In conclusion, there are various animal-free alternatives in differing stages of development, which reflect several distinct properties of PTx. A strategic, stepwise plan was proposed at the London meeting of the International Working Group on Alternatives to HIST, in which an alternative test would be adopted for release purposes first, and then, once sufficient confidence in its suitable performance has been gained, its use would be extended to stability testing. On the other hand, representatives of regulatory organizations present at the London meeting of the International Working Group on Alternatives to HIST agreed, in principle, that the waiving of the HIST testing requirement for stability might be entertained if manufacturers would be able to provide unequivocal evidence that the chemical treatment used to detoxify PTx yields irreversible toxoid. Such a waiver would obviously have a major

impact on the number of animals used for aP vaccine safety testing. It should be kept in mind that toxin inactivation by chemical procedures will continue being demonstrated in-process by the CHO-cell assay on every bulk of PTd before its adsorption to adjuvant.

Concluding remarks

A survey of vaccine manufacturers and regulatory bodies has allowed us to estimate the use of mice for safeguarding the safety of recipients of aP vaccines worldwide at about 65,000. Though the HIST has ensured the release of safe aP vaccines for many years, there are clear scientific and animal welfare reasons to change this testing regime. There are means to refine the test and reduce the animal number for the existing testing strategy. However, it is questionable whether the efforts needed for validation and implementation of these interim alternatives in the path to replacement are worthwhile, especially since various *in vitro* alternatives are under development. These *in vitro* alternatives have scientifically sound mechanistic substantiation. After appropriate validation, these alternatives have the potential to significantly reduce the numbers of animals that are currently used for the HIST, although a careful comparative evaluation of their mechanisms, as well as the sensitivity of each test, will be required before one of them is selected for implementation.

References

1. Libster, R. and K.M. Edwards, Re-emergence of pertussis: what are the solutions? Expert Rev Vaccines, 2012. 11(11): p. 1331-46.
2. WHO. WHO vaccine-preventable diseases: monitoring system. 2015 global summary. 2016 2015; Available from: http://apps.who.int/immunization_monitoring/globalsummary/schedules.
3. Robinson, A., *et al.*, Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. Vaccine, 1989. 7(4): p. 321-4.
4. de Gouw, D., *et al.*, Proteomics-identified Bvg-activated autotransporters protect against *Bordetella pertussis* in a mouse model. PLoS One, 2014. 9(8): p. e105011.
5. Mills, K.H., *et al.*, A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun, 1998. 66(2): p. 594-602.
6. Clark, T.A., N.E. Messonnier, and S.C. Hadler, Pertussis control: time for something new? Trends Microbiol, 2012. 20(5): p. 211-3.
7. Black, R.E., *et al.*, Global, regional, and national causes of child mortality in 2008: a systematic analysis. Lancet, 2010. 375(9730): p. 1969-87.
8. Pertussis vaccines: WHO position paper. Wkly Epidemiol Rec, 2010. 85(40): p. 385-400.
9. Parfentjev, I.A. and M.A. Goodline, Histamine shock in mice sensitized with Hemophilus pertussis vaccine. J Pharmacol Exp Ther, 1948. 92(4): p. 411-3.
10. Kind, L.S., The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bacteriol Rev, 1958. 22(3): p. 173-82.
11. van Straaten-van de Kappelle, I., *et al.*, Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biologicals, 1997. 25(1): p. 41-57.
12. Bache, C., *et al.*, Workshop on Animal free Detection of Pertussis Toxin in Vaccines--Alternatives to the Histamine Sensitisation Test. Biologicals, 2012. 40(4): p. 309-11.
13. Arciniega, J., *et al.*, Alternatives to HIST for acellular pertussis vaccines: progress and challenges in replacement. Report on an international workshop. . Pharmeur Bio Sci Notes, 2016. 2016-04: p. 82-96.
14. Tamura, M., *et al.*, Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry, 1982. 21(22): p. 5516-22.
15. Witvliet, M.H., *et al.*, Binding of pertussis toxin to eucaryotic cells and glycoproteins. Infect Immun, 1989. 57(11): p. 3324-30.
16. Plaut, R.D. and N.H. Carbonetti, Retrograde transport of pertussis toxin in the mammalian cell. Cell Microbiol, 2008. 10(5): p. 1130-9.
17. Katada, T., M. Tamura, and M. Ui, The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch Biochem Biophys, 1983. 224(1): p. 290-8.
18. el Baya, A., K. Bruckener, and M.A. Schmidt, Nonrestricted differential intoxication of cells by pertussis toxin. Infect Immun, 1999. 67(1): p. 433-5.
19. Tamura, M., *et al.*, A role of the B-oligomer moiety of islet-activating protein, pertussis toxin, in development of the biological effects on intact cells. J Biol Chem, 1983. 258(11): p. 6756-61.
20. Strnad, C.F. and R.A. Carchman, Human T lymphocyte mitogenesis in response to the B oligomer of pertussis toxin is associated with an early elevation in cytosolic calcium concentrations. FEBS Lett, 1987. 225(1-2): p. 16-20.

21. Wang, Z.Y., *et al.*, Induction of dendritic cell maturation by pertussis toxin and its B subunit differentially initiate Toll-like receptor 4-dependent signal transduction pathways. Exp Hematol, 2006. 34(8): p. 1115-24.
22. Schneider, O.D., A.A. Weiss, and W.E. Miller, Pertussis toxin utilizes proximal components of the T-cell receptor complex to initiate signal transduction events in T cells. Infect Immun, 2007. 75(8): p. 4040-9.
23. Nishida, M., *et al.*, Pertussis toxin up-regulates angiotensin type 1 receptors through Toll-like receptor 4-mediated Rac activation. J Biol Chem, 2010. 285(20): p. 15268-77.
24. Mangmool, S. and H. Kurose, G(i/o) protein-dependent and -independent actions of Pertussis Toxin (PTX). Toxins (Basel), 2011. 3(7): p. 884-99.
25. Hoonakker, M.E., N. Ruiterkamp, and C.F. Hendriksen, The cAMP assay: a functional *in vitro* alternative to the *in vivo* Histamine Sensitization test. Vaccine, 2010. 28(5): p. 1347-52.
26. Bergman, R.K. and J. Munoz, Circulatory Collapse in Anaphylaxis and Histamine Toxicity in Mice. J Immunol, 1965. 95: p. 1-8.
27. Cataldi, M., *et al.*, Histamine receptors and antihistamines: from discovery to clinical applications. Chem Immunol Allergy, 2014. 100: p. 214-26.
28. Bovet, D. and A. Carpi, Pharmacological aspects of peripheral circulation. Annu Rev Physiol, 1958. 20: p. 305-38.
29. Makabe-Kobayashi, Y., *et al.*, The control effect of histamine on body temperature and respiratory function in IgE-dependent systemic anaphylaxis. J Allergy Clin Immunol, 2002. 110(2): p. 298-303.
30. Munoz, J., Permeability changes produced in mice by *Bordetella pertussis*. J Immunol, 1961. 86: p. 618-26.
31. van Meijeren, C.E., *et al.*, *In vivo* pertussis toxin treatment reduces contraction of rat resistance arteries but not that of mouse trachea. Eur J Pharmacol, 2004. 488(1-3): p. 127-35.
32. Munoz, J. and R.K. Bergman, Histamine-sensitizing factors from microbial agents, with special reference to *Bordetella pertussis*. Bacteriol Rev, 1968. 32(2): p. 103-26.
33. Sato, Y. and H. Sato, Development of acellular pertussis vaccines. Biologicals, 1999. 27(2): p. 61-9.
34. Jensen, S.E., *et al.*, Specificity and detection limit of a dermal temperature histamine sensitization test for absence of residual pertussis toxin in vaccines. Biologicals, 2012. 40(1): p. 36-40.
35. Ochiai, M., *et al.*, Highly sensitive histamine-sensitization test for residual activity of pertussis toxin in acellular pertussis vaccine. Biologicals, 2007. 35(4): p. 259-64.
36. Watanabe, M. and M. Nagai, Acellular pertussis vaccines in Japan: past, present and future. Expert Rev Vaccines, 2005. 4(2): p. 173-84.
37. Wang, L., D. Lei, and S. Zhang, Acellular pertussis vaccines in China. Vaccine, 2012. 30(50): p. 7174-8.
38. Oishi, A., *et al.*, Regulation of RhoA signaling by the cAMP-dependent phosphorylation of RhoGDIalpha. J Biol Chem, 2012. 287(46): p. 38705-15.
39. Gaines-Das, R., *et al.*, Transferability of dermal temperature histamine sensitization test for estimation of pertussis toxin activity in vaccines. Hum Vaccin, 2009. 5(3): p. 166-71.
40. Arciniega, J., *et al.*, Alternatives to HIST for acellular pertussis vaccines: progress and challenges in replacement. Pharmeur Bio Sci Notes, 2016. 2016-04: p. 82-96.
41. Vaessen, S.F., *et al.*, Identification of biomarkers to detect residual pertussis toxin using microarray analysis of dendritic cells. Vaccine, 2013. 31(45): p. 5223-31.
42. Isbrucker, R., *et al.*, Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. Biologicals, 2014. 42(2): p. 114-22.

43. Toyota, T., *et al.*, Effects of islet-activating protein (IAP) on blood glucose and plasma insulin in healthy volunteers (phase 1 studies). Tohoku J Exp Med, 1980. 130(2): p. 105-16.
44. Ashworth, L.A., *et al.*, Antigens in whooping cough vaccine and antibody levels induced by vaccination of children. Lancet, 1983. 2(8355): p. 878-81.
45. European Pharmacopoeia, Residual pertussis toxin and irreversibility of pertussis toxoid. 2013. 8th edition: p. 224.
46. Xing, D., *et al.*, Collaborative study for the standardisation of the histamine sensitizing test in mice and the CHO cell-based assay for the residual toxicity testing of acellular pertussis vaccines. Pharmeur Bio Sci Notes, 2010. 2010(1): p. 51-63.
47. Isbrucker, R.A., A. Bliu, and F. Prior, Modified binding assay for improved sensitivity and specificity in the detection of residual pertussis toxin in vaccine preparations. Vaccine, 2010. 28(15): p. 2687-92.
48. Gomez, S.R., *et al.*, Development of a carbohydrate binding assay for the B-oligomer of pertussis toxin and toxoid. Anal Biochem, 2006. 356(2): p. 244-53.
49. Cyr, T., *et al.*, A quantitative analysis for the ADP-ribosylation activity of pertussis toxin: an enzymatic-HPLC coupled assay applicable to formulated whole cell and acellular pertussis vaccine products. Biologicals, 2001. 29(2): p. 81-95.
50. Yuen, C.T., *et al.*, Detection of residual pertussis toxin in vaccines using a modified ribosylation assay. Vaccine, 2002. 21(1-2): p. 44-52.
51. Yuen, C.T., *et al.*, An *in vitro* assay system as a potential replacement for the histamine sensitisation test for acellular pertussis based combination vaccines. Vaccine, 2010. 28(21): p. 3714-21.
52. Fujiwara, H. and S. Iwasa, The quantitative assay of the clustering activity of the lymphocytosis-promoting factor (pertussis toxin) of *Bordetella pertussis* on Chinese hamster ovary (CHO) cells. J Biol Stand, 1989. 17(1): p. 53-64.
53. Gillenius, P., *et al.*, The standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J Biol Stand, 1985. 13(1): p. 61-6.
54. Castro, M.G., U. McNamara, and N.H. Carbonetti, Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. Cell Microbiol, 2001. 3(1): p. 45-54.
55. Isbrucker, R., *et al.*, Transferability study of CHO cell clustering assays for monitoring of pertussis toxin activity in acellular pertussis vaccines. . Pharmeur Bio Sci Notes 2016. 2016-05: p. 97-114.

9

Chapter 9

Summarizing Discussion

Summarizing discussion

Historically, each final lot of vaccine was regarded as a unique product, due to the considerable inherent variation in the preceding biological production process. Consequently, each individual vaccine lot needed to be tested for safety and potency, frequently involving animal experiments. As discussed in **Chapter 1**, the animal models still in use are criticized because of a lack of scientific relevance, variability in test results and concerns regarding animal welfare. Most of these animal models have been developed in the '50s and '60s of the previous century, along with the production processes of the vaccines (e.g. diphtheria, tetanus and pertussis vaccines) and have not changed since. However, in the last decades many aspects of the production processes, as well as the abilities to monitor and control both the process and its products have improved significantly. A more contemporary strategy for vaccine lot release might therefore be to monitor vaccine quality and safety by ensuring consistency in the vaccine production processes, in combination with extensive characterization of the intermediate and final products, a strategy referred to as the Consistency Approach.[1] For evaluation of intermediate and final products, various methods are available that enable assessment of antigen composition, antigen conformation, antigen stability, antigen - adjuvant interaction, presence of (im)purities and sterility. These methods include physicochemical and immunochemical techniques such as gel electrophoresis, chromatography methods, mass spectrometry, ELISA, immunoblotting, circular dichroism, fluorescence and infrared spectroscopy. Such methods have been applied for the characterization of diphtheria and tetanus toxoid vaccines.[2,3]

In this thesis, various cellular *in vitro* methods were explored for their ability to complement physicochemical and immunochemical methods and for their capacity to monitor functional aspects of vaccines - i.e. relevant immunological and safety properties - using experimentally produced wP vaccines and routinely produced aP vaccines respectively. Along with this, the additional value of several existing physicochemical and immunochemical methods was investigated, since such techniques had not been evaluated for wP vaccines thus far. To examine the potential of these methods as indicators of vaccine quality, the *in vivo* potencies of our experimental wP vaccines were assessed, as well as the type and magnitude of innate and adaptive immune response induced by these vaccines.

9.1 Generation of wP vaccines of various qualities and their analytical evaluation

Currently, assessment of wP vaccine potency relies on the regulatory required mouse intracerebral challenge method, referred to as the Kendrick test or Mouse Protection Test. This test is criticised because of (a) lack of relevance considering the route of challenge, the type of immune response and pathology that is induced[4], (b) high variability within and between laboratories[5] and (c) its negative impact on animal welfare.[6] The development of alternative methods is therefore urgently needed. To determine whether analytical and functional *in vitro* methods are capable of monitoring wP vaccine quality, vaccines of varying quality were required. Such vaccines were experimentally produced by deliberate manipulation of the wP steady-state production process using $MgSO_4$. *B. pertussis* possesses a master regulatory system encoded by the BvgASR locus, which controls the expression of genes encoding for virulence proteins.[7] These proteins are involved in *B. pertussis* pathogenesis, and are also considered important for the induction of protective immunity.[8-11] $MgSO_4$ is known to affect the Bvg regulatory system, reducing the expression of the virulence proteins.[12] Since a Bvg-mediated repression of gene expression could also occur in the course of the production process (as a results of decreased nutrients availability[13,14]), $MgSO_4$ intervention mimics possible disturbances in the wP production process. During our *B. pertussis* culture, bacteria were harvested just before $MgSO_4$ addition and at several time points afterwards, resulting in a number of wP vaccines with varying amounts of virulence factors. These experimental vaccines were used for the evaluation of analytical techniques and *in vitro* methods.

In **Chapter 2** the production process of the experimental wP vaccines is described, as well as the evaluation of the vaccines by physicochemical and immunochemical methods, primarily focussing on virulence-associated genes and proteins. Monitoring of production parameters such as optical density, dissolved oxygen and gas inflows (air, O_2, N_2) and outflows (CO_2 out and O_2), revealed a high level of reproducibility, since only very slight differences between three runs of *B. pertussis* cultivation were found. As expected, addition of $MgSO_4$ substantially affected the expression of 177 genes and 151 proteins, of which 47 genes and 12 proteins were associated to virulence.[15] Five of the samples taken just before and at various times points after $MgSO_4$ addition were used to generate vaccines by heat and formaldehyde inactivation. These vaccines proved to contain gradually decreasing levels of the well-known virulence proteins Vag8, FIM2, FIM3, FHA and PRN (**Chapter 2** and **3**), while levels of PTd/PTx were marginal in all vaccines.

Since many of the virulence-associated proteins are considered important antigens that contribute to protection[8,10,11], they constitute the basis of aP vaccines. However, while the

role of PTd in induction of protection is apparent[16-18], the exact contribution of other virulence proteins to vaccine-induced protection is still unclear. For example, there are several studies in mice showing that administration of FHA or PRN alone failed to confer protection against intracerebral infection with *B. pertussis*[18,19], while others report that PRN[20,21] or a combination of FHA and PRN[22] was sufficient for protection against respiratory or intracerebral infection with *B. pertussis*, respectively. On the other hand, several studies in mice show that administration of FIM alone confers protection against intranasal infection[23,24]. Concurrently, clinical data show that a lower level of anti-FIM antibodies corresponds with a higher pertussis incidence in young children.[25] Additional evidence for the contribution of the virulence proteins comes from efficacy studies in humans. In general, these studies show that inclusion of more antigen components in aP vaccines is associated with a higher vaccine efficacy[26-28], although a comparison between a 2 and a 4-component vaccine revealed a slightly higher effectiveness for the 2-component vaccine.[29] Furthermore, studies demonstrate that wP vaccination results in superior protection[30] and durability of immunity compared to aP vaccination[31-33], suggesting a role in this behaviour for factors only present in wP vaccines or a broader immune response as a consequence of the complexity of the wP vaccine. This suggests that along with well-known virulence proteins, other proteins might be involved in the induction of protection. Results from our studies show that the levels of proteins associated with virulence, but also of several other proteins of *B. pertussis*, concurs with vaccine quality. Therefore a broad quantification of virulence- but also non-virulence associated proteins is important for evaluation of the consistency of vaccine production.

In addition to protein composition, activation of the PRR TLR4 by its canonical ligand LPS, is key to the initiation of wP vaccine-induced immunity.[34,35] However, high levels of LPS are also associated with the occasional reactogenicity of wP vaccines.[36] It has been shown that *B. pertussis* can substitute the phosphate groups of the lipid A moiety of its LPS with glucosamine (GlcN), a modification that leads to enhanced hTLR4 signalling and the secretion of pro-inflammatory cytokines.[37] The genes *lgmA*, *lgmB* and *lgmC* have recently been identified to encode the enzymes required for this GlcN modification of *B. pertussis* LPS.[37-39] Importantly, the expression of *lgmA* and *lgmB* was found to be regulated by the BvgASR master regulatory system.[40] Because of the importance of LPS for wP vaccine effectiveness in mice and its Bvg-dependent substitution with GlcN, we analysed the LPS quantity in our vaccines, its structure, and the genes encoding for enzymes responsible for LPS synthesis and modifications (**Chapter 3**). We could establish that *B. pertussis* cultures exposed to $MgSO_4$ had reduced expression of the *lgmA*, *lgmB* and *lgmC* genes, resulting in a gradual decrease in GlcN modification of lipid A. It should be noted that the lipid A structures substituted with GlcN represented a minor LPS species, though it was the only species in our vaccines for which the quantity was associated with culture time in the presence of $MgSO_4$. LPS-induced TLR4 signalling plays

a key role in steering immunity toward Th1/Th17 response[35], a type of response central to protection against *B. pertussis*.[35,41-43] In contrast to wP vaccines, aP vaccines induce a Th2 type of response.[44-47] The discrepancy between aP and wP vaccine induced differentiation of Th cells, could be explained by the presence of PAMP like LPS in wP vaccines and their absence in aP vaccines. Recent studied in mice have shown that addition of non-toxic LPS derivative (of Neisseria meningitidis) to aP vaccines causes skewing of the immune response towards Th1/Th17[48] and improved the protective properties of the vaccine.[49] The key role of TLR4 signalling is further supported by a study of Errea *et al.*, showing that co-administration of *B. pertussis* bacteria with LPS of various organisms impaired colonization in the lungs of mice.[50] Because of the importance of LPS and TLR4 signalling in pertussis vaccine induced immunity and the association between LPS structure and bacterial virulence, analysis of LPS structure and quantity is crucial for guaranteeing consistent wP vaccine quality.

9.2 Cellular *in vitro* methods for evaluation of wP vaccine quality

Physicochemical and immunochemical methods provide information on a wP vaccines' protein and LPS composition. However, appropriate APC activation is critical for the initiation of the adaptive immune response[51] and consequently both immune components are key to the effectiveness of vaccination. Although *B. pertussis* induces APC activation largely through TLR2 and TLR4[52,53], it has been reported that in mice, only TLR4 contributes to the early innate immune response, antibody response, T cell induction[34] and ultimately to protection[35] (as addressed in section 9.1). In **Chapter 3** the abilities of our experimental wP vaccines to induce TLR2 and TLR4 signalling were assessed using NF-κB/SEAP HEK-Blue reporter cell lines expressing human TLR4/MD-2/CD14 or human TLR2/CD14. We found that modulation of the Bvg system by $MgSO_4$ resulted in a gradually decreasing hTLR4 activating capacity of our vaccines, while the modulation had no clear effect on TLR2 activation. Besides hTLR2 and hTLR4, other PRRs may be activated by wP vaccines. Since APC express multiple PRRs and are essential for initiation and steering of adaptive immune responses, the capacities of four APC platforms to discriminate between vaccines derived from culture at various stages of $MgSO_4$ modulation were analysed in **Chapter 4**. The platforms were all of human origin, and included primary, monocyte-derived DC (moDC) and primary monocytes, as two clinically relevant models, and two cell lines, a DC like cell line (MUTZ-3) and the monocytic cell line MonoMac6 (MM6). The evaluation revealed that MUTZ-3 cells were inappropriate for the intended purpose because of their low sensitivity for LPS. Primary monocytes were not selected either, due to high intra-assay variability. On the contrary, the MM6 cells and the moDC proved appropriate platforms that discriminated between the wP vaccine derived from the non-modulated condition (Bvg^+ phase; vaccine A) and the wP vaccine derived from the $MgSO_4$-modulated condition (Bvg^- phase; vaccine E), but

could not distinguish between the vaccines derived from the non-modulated condition and the intermediate stage of modulation ($Bvg^{\pm}$ phase; vaccine C). Blocking of either hTLR2 or hTLR4 on these APC platforms showed that hTLR4- and not hTLR2-mediated signalling was primarily responsible for the observed vaccine quality-dependent responses of MM6 cells and moDC (**Chapter 3**). Together these findings indicate that the MM6 cells, the moDC and the hTLR4-expressing HEK-Blue cell line are capable of discriminating between wP vaccines derived from the extremes of the manipulated production process (Bvg^{+} and Bvg^{-} phase), but not between the wP vaccines derived from the non-modulated condition (Bvg^{+} phase) and the wP vaccine derived from the intermediate stage of modulation ($Bvg^{\pm}$ phase). Because of the importance of hTLR4 and APC activation for the effectiveness of vaccination, the selected APC platforms and the hTLR4-expressing cell line constitute relevant tools that can assess the quality of our wP vaccines.

In these studies, the wP vaccine was used as a model. However, the APC platforms and PRR-expressing cell lines might also represent a valuable tool for lot release testing of other vaccines that currently rely on animal models, such as diphtheria-, polio- and tetanus vaccines. Most of these vaccines (as well as wP vaccines) are not administered alone, but are formulated as multivalent vaccines and are tested for potency using *in vivo* models. Testing of final formulations entails a challenge for *in vitro* methods for two reasons. Firstly, these vaccines frequently contain an adjuvant, often an aluminium-salt, since these products enhance the adaptive immune response toward otherwise poorly immunogenic proteins, such as diphtheria and tetanus toxoid.[54] However, due to their strong binding capacity, adjuvants may hamper the availability of the antigens for cells in culture. In addition, adjuvants such as aluminium-salts are quite often cytotoxic[55], though several studies have used *in vitro* cultured DCs[54,56,57], monocytes and macrophages[57-59] to evaluate effects of aluminium-salts. The strong binding between antigen and adjuvants and possible cytotoxic effects of these products for cells *in vitro*, are aspects that need to be considered for *in vitro* evaluation of adjuvant-containing products. Secondly, APC platforms and PRR models measure the activation induced by the entire multivalent vaccine, reflecting what happens *in vivo*. However, *in vitro* models are not capable of discriminating between the qualities (i.e. innate immune activation capacities) of individual components (e.g. tetanus and pertussis) within the final formulation. Evaluation of the individual components before formulation in combination with assessment of antigen-adjuvant interaction after formulation, can circumvent this issue. Further studies will be needed to evaluate these aspects and eventually adapt the *in vitro* methods, e.g. by using commercially available semi-permeable transwell-inserts for culture microplates, to prevent direct contact between the cells and the adjuvants.

9.3 *In vitro* methods for evaluating adaptive immune responses

In the studies described in **Chapter 3** and **4** we showed that *in vitro* methods based on innate immune cell activation might allow us to predict the quality of wP vaccines. Since vaccine-induced protection also involves adaptive immunity, *in vitro* assessment of adaptive immune responses might be explored to complement *in vitro* tests for innate immunity. Emerging innovative techniques, particularly miniaturized *in vitro* (multi-)organ systems - such as organ-on-a-chip or human-on-a-chip - may be tools for studying parameters indicative for adaptive immunity. Potential methods will be discussed in more detail below.

Although it will be difficult to mimic a full *in vivo* immune response *in vitro*, there are multiple approaches that assess a single or several of the properties that contribute to adaptive immune cell activation, such as crosstalk between APC and T cells, or the capacity of APCs to direct the Th cell response and production of antigen-specific antibodies. The most straightforward method to study adaptive immunity *in vitro* is cultivation of human donor-derived PBMC - containing APCs (monocytes, DCs and B cells) and cytotoxic T cells and Th cells - with the pathogen or antigen under study. This may trigger T cells to proliferate and secrete cytokines[60], and B cells to produce antibodies. Responses of these cells can be analysed by methods such as ELISA, flow cytometry, multiplex immunoassay and ELISPOT.

The second type of method consists of culturing APCs together with T cells derived from the same (autologous setting) or a different donor (allogeneic setting). Autologous co-cultures can be applied to monitor the presentation of vaccine or protein specific epitopes by APCs and the induction of skewing toward a specific type of Th cell response. This approach has been applied for investigating yellow fever[61] and influenza[62] vaccines. The low frequency of vaccine or protein-specific T cells in the blood represents a problem for this approach, which in particular situations can be solved by using T cells of previously vaccinated donors or by depletion of regulatory T cells.[63] In allogeneic co-cultures, the T cells respond to a combination of “foreign” MHC-presented peptides (referred to as allo-antigens)[64] and the cytokine environment created by the APC.[65,66] As such, this approach allows the evaluation of the T-cell activating and skewing capacity of the APC and not the activation of antigen-specific T cells. Allogeneic co-culture approaches have been used to study, for instance *Bordetella pertussis*[52], Human Papilloma virus[67] and Mycobacterium tuberculosis[68] T-cell responses. Another tool to analyse peptide presentation is the use of T cell hybridomas. These hybridomas are generated by the fusion of T cell clones with T cell lymphomas. Upon recognition of MHC-bound peptides (presented by allogeneic APC), T cell hybridoma secrete IL-2, allowing the evaluation of protein degradation and presentation of a specific peptide. A disadvantage of using hybridomas is that some T cell-specific characteristics are lost, due to the immortalized nature of the

lymphomas.[69] Nevertheless, T cell hybridomas proved to be a useful tool for the analysis of diphtheria toxin- and tetanus toxin-derived peptides.[70] Taken together, there are several examples of approaches that can be used to study pathogen or vaccine-induced activation of adaptive immune response *in vitro*, though these approaches come with intrinsic limitations.

The co-culture approaches addressed above are based on well plates, essentially having a 2D structure, while immune cells naturally operate in a 3D matrix of fibers and in the context of stromal cells (section 1.4, Figure 4). Several research groups have attempted to develop 3D cell culture matrices that better represent the natural environment of immune cells. Several of these models utilized rodent (immune) cells[71-73] and are described in more detail in two recent reviews.[74,75] Developing a human 3D cell culture matrix is challenging, because of the low number of naïve immune cells in human blood.[76] There are two approaches in an advanced stage of development. Firstly, a miniaturized bioreactor system referred to as the Human Artificial Lymph Node, developed by Giese *et al.*, which consists of a hydrogel matrix that is loaded with moDCs and lymphocytes, and has a constant flow of medium.[77] This system has been tested using Hepatitis A vaccine, which induced T cell-specific cytokines and IgM production.[78] The system, however, does not fully allow germinal centre formation, Ig class switching and affinity maturation. An alternative approach - the MIMIC system - has been established by the company VaxDesign and contains three modules that each simulate a part of the immune response. It contains: an innate module, an adaptive module and a module in which cellular responses are measured. Distinctive of this model, compared to other approaches, is that differentiation of APC's is not initiated by growth factors, but by migration of the APCs through HUVEC cells into a collagen matrix, which more closely resembles the *de novo* generation of mature APCs *in vivo*.[79] Using this system, the magnitude of *in vitro* tetanus- and influenza-specific antibody responses of donor cells proved comparable to the response profiles in human volunteers.[80,81]

An immune response does not occur as an event limited to the lymph node, but takes place in the context of multiple interacting organ systems. Therefore, the next generation of emerging approaches are referred to as "organs-on-a-chip" and "human-on-a-chip", as reviewed by others.[82-84] These approaches are based on a micro-system for culturing various cell types of multiple organs on a single chip. The organs can interact via microvascular beds, which also enable traffic of immune cells. These are very promising techniques, but they are still in an infant state of development. Nevertheless, progress within this field should be closely monitored, because such techniques might be valuable for evaluation of complex responses as those involved in immunity and eventually for vaccine quality control.

9.4 wP vaccine potency determined by the Kendrick test

Along with the development of the first wP vaccines in the 1940-50s, these was an urgent need for suitable means to determine the potency of these vaccines. In 1947, Kendrick *et al.*[85] were the first to describe that the wP vaccine could protect mice against an intracerebral challenge with virulent *B. pertussis*. After demonstrating that the potency determined by this model correlated with protection in children[86,87], the Kendrick test became the standard regulatory test for assessing wP vaccine potency. Mice are normally not susceptible to a natural respiratory infection with *B. pertussis*, whereas they do become infected when the bacterium is administered intracerebrally. Several studies described the immunological mechanisms and events occurring after intracerebral injection with this pathogen. An early study in 1960[88] reported that intracerebral injection with *B. pertussis* leads to multiplication of the bacterium in the ventricles and causes increased permeabilisation of the blood-brain barrier. The study also showed the infiltration of lymphocytes, plasma cells and some large mononuclear cells to the site of infection. The reported clinical signs included meningitis, haemorrhages and necrosis of nerve cells.[88] Subsequent studies demonstrated that wP specific-antibodies could reduce the bacterial load shortly after infection and later during infection.[89,90] The contribution of lymphocytes and primarily T cells to protection is less clear, since administration of lymphocytes alone (derived from spleens or lymph-nodes of vaccinated donor mice) resulted in limited or no protection, while in combination with antibodies, lymphocytes do improve protection.[89,91] Since *B. pertussis* is a pathogen that affects the respiratory tract rather than the neural system, it is not likely that the pathology and immunity induced by the intracerebral challenge accurately reflect the situation during a human respiratory infection. In addition to the questionable immunological relevance of this animal model, the relevance of the challenge strain used (18323) can be disputed, since it has many genetic similarities to isolates of *B. bronchiseptica* and *B. parapertussis*.[92] However, due to a lack of alternatives, the Kendrick test remains the only potency test for wP vaccines that is used worldwide. Consequently, determination of the potencies of our vaccines was considered important for the interpretation of the results of our *in vitro* studies. Evaluation of the vaccines in the Kendrick test (**Chapter 2**) revealed that the potencies of vaccines A (non-modulated conditions; Bvg^+ phase), C (intermediate $MgSO_4$-modulated condition; $Bvg^{\pm}$ phase) and E ($MgSO_4$-modulated conditions; Bvg^- phase) were 7 IU/ml (95% confidence interval of 2 – 27 IU/ml), 5 IU/ml (2 – 10 IU/ml) and 1 IU/ml (0 – 3 IU/ml), respectively. Both the WHO and the European Pharmacopoeia require the potency to be at least 4 IU/mL and a lower limit of the 95% interval of 2 IU/mL.[93,94] The potencies of vaccines A and C fulfilled these criteria, whereas the potency of vaccine E did not. Based on these results, the quality of vaccine A was considered good, the quality of vaccine E was considered poor and the quality of vaccine C was considered intermediate. The potencies of the vaccines compared well with the results of the *in vitro* experiments, i.e. the decreasing amounts of virulence proteins (**Chapter 2**) in vaccines A-E

and the diminished degree of hTLR4 and APC activation induced by these vaccines (**Chapter 3** and **4**) matched a gradual decrease in vaccine potency. Moreover, the decision on the acceptance or rejection of vaccines A, C or E would have been the same when based on the mouse potency data or on the data from the *in vitro* human TLR4 cells and APC platforms (**Chapter 3** and **4**).

9.5 The advanced pertussis serological potency test (PSPT)

The questionable immunological relevance of the Kendrick test, along with ethical issues of the lethal challenge procedures, provide a strong incentive for the development of alternative methods or strategies for potency testing of wP vaccines. Since the establishment and implementation of animal-free methods proved to be a lengthy process, an *in vivo* alternative was developed as a bridging method between the Kendrick test and animal-free approaches. The pertussis serological potency test (PSPT) was first described in the 1990s and assesses the total amount of wP vaccine-induced IgG antibodies directed against surface antigens of *B. pertussis*.[95] Antibodies directed against *B. pertussis* are considered to function by (a) neutralizing bacterial toxins, (b) reducing the binding of bacteria to the respiratory epithelial cells or (c) by enhancing the phagocytosis of the bacteria by neutrophils and macrophages.[96] Nevertheless, the evidence for the exact contribution of antibodies to protection is unclear. Although humans generate pertussis-specific antibodies[46,95] and memory B cells[97] 3-4 weeks after wP vaccination, respiratory exposure of mice to the bacterium (using a nebulizer) does not result in detectable antibodies until 6 weeks post-infection.[42] Since bacteria are almost cleared from the lungs after 6 weeks, these data suggest that other immune components such as B cell[98] and or T cell responses are involved in the clearance of the bacterium.[48] Indeed, adoptive transfer experiments demonstrate that B cells play an antibody-independent role in protection.[42,99] Such antibody-independent B cell mechanisms were not studied in detail, but possibly include antigen presentation, production of cytokines and chemokines and interaction with other immune cells, such as macrophages or natural killer cells.

Initial studies into the T cell arm of adaptive immunity showed that *B. pertussis*-specific $CD4^+$ T cells, but not $CD8^+$ T cells, were essential for clearance of the bacterium.[42] Subsequent studies in baboons and mice demonstrated that *B. pertussis* infection induces IL-1 and IL-23 driven-Th cells secreting IL-17.[43,100] In mice, these cells appear critical for the clearance of bacteria from the lungs[43], mediated by the recruitment of macrophages[35] and neutrophils[101] and subsequent killing of bacteria. Similar to natural infection, immunization with wP vaccines triggers IFN-γ and IL-17-producing Th cells in both baboons[102] and mice.[35,44] In mice, these Th17 cells appear to be important for vaccine-induced protective immunity, since IL-17 deficiency, as well as inhibition of IL-17 by blocking antibodies, reduces the rate of *B. pertussis* clearance after wP

vaccination.[35,103] Despite the importance of Th17 cells for protection, Th17 cells have also been associated with autoimmunity and are believed to contribute to pathology associated with *B. pertussis* infection.[96] The hypoxemia and pulmonary hypertension, common in fatal cases of pertussis[104] and the release of IL-23 by human DCs-exposed *in vitro* to *B. pertussis*[53] point indeed toward detrimental effect of Th17 cells.[105] However, additional studies are required to gain insight into the positive and negative effects of Th17 cell responses during *B. pertussis* infection.

The conflicting effects of Th17 cells are indicative of the complexity of the immune responses involved in pertussis vaccine-induced protection. The PSPT measures only one of these components, but also provides the opportunity to include additional immunological parameters. Therefore, we studied our wP vaccines in the PSPT (**Chapter 5**), evaluating both the total IgG antibody levels directed against the entire bacterium, but also the levels of early circulating pro-inflammatory cytokines, antibody specificity and T helper cell responses (the "advanced" PSPT). These experiments revealed that there was not a clear difference between these vaccines with regard to the early serum pro-inflammatory cytokine levels (IL-6 and IP10) following immunization, neither were there differences between the induced levels of IgG1 and IgG2A antibodies directed against the entire bacterium. In contrast, the specificities of the IgG antibodies were unique for each vaccine and the levels of antibodies directed against particular proteins (e.g. Vag8, BP3561 and argC) corresponded with the quality of the vaccine. Furthermore, *ex vivo* stimulation of cells of immunised mice showed that vaccination with reference wP vaccine (vaccine A, non-modulated conditions; Bvg^+ phase) had primarily resulted in splenocytes with mixed Th1 and Th17 phenotypes, although low levels of Th2 cytokines were detected as well. Evaluation of the wP vaccines of intermediate (C; intermediate $MgSO_4$ modulated condition; $Bvg^{\pm}$ phase) and low quality (E; $MgSO_4$ modulated conditions; Bvg^- phase) revealed that especially the levels of IL-17, but also the magnitude of IL-4 and IFN-γ responses, decreased in parallel to decreasing vaccine quality. The differences in magnitude of the Th cell responses could be the result of different levels of PAMPs or proteins in the vaccines. Although our vaccines contain various LPS forms, this most likely did not influence the differentiation of the Th cells, since an earlier study showed that, in contrast to human TLR4, signalling through murine TLR4 is not affected by glucosamine modification of the lipid A.[37] In agreement with this, we confirmed that Bvg modulation of our *B. pertussis* culture had not resulted in differences between the vaccines with regard to *in vitro* activation of murine TLR4 (**Chapter 3**). Furthermore, there were no significant differences in the levels of *in vivo* inflammatory cytokines induced, between vaccines A, C and E (**Chapter 5**). This suggests that the capacity of our vaccines to induce innate immune responses in mice did not differ significantly. Therefore, the levels of immunogenic proteins in our vaccines, as discussed in section 9.1, might have played a role in the vaccine-specific magnitude of Th responses. Apart from their immunogenic

properties, these proteins frequently exhibit immune modulatory - i.e. activating or inhibitory – properties. One of the known immune modulatory virulence proteins is FHA, that mediates phagocytosis of *B. pertussis* by macrophages[106], but also suppresses the secretion of IL-12 and induces the secretion of IL-10 by macrophages and DCs.[107,108] This causes the generation of IL-10-producing Treg cells that inhibit IFN-γ production, and thereby suppress the generation of Th1 cells.[107,108] PTx, PRN and adenylate cyclase toxin are other examples of proteins with immune-modulatory properties.[109-120] Since the virulence proteins and mRNA levels in our wP vaccines decreased along with the Th1 and Th17 responses, the levels of these proteins and their immune-modulatory properties might have contributed to vaccine quality-dependent Th cell response. The role of individual proteins has been investigated in both respiratory and intracerebral challenge models, as discussed in section 9.1. Moreover, the contribution of the entire set of virulence factors has been investigated by vaccines derived from a bacteria harvested after the logarithmic growth, or vaccines derived from a culture modulated by nicotinic acid, both causing changes in the Bvg regulatory system similar to those observed for $MgSO_4$.[12] Both the longitudinal culture and nicotinic acid modulated culture of *B. pertussis* have been associated with reduced protection against intracerebral infection.[121,122]

The results from the PSPT experiments shown in this thesis demonstrate that the conventional readout parameter (IgG antibodies directed against the entire bacterium) cannot discriminate between our wP vaccines of various qualities, while analysis of Th1/Th17 responses, as well as serum antibody-specificity (Vag8, BP3561 and argC), did enable such discrimination. Since B and T cell responses contribute to wP vaccine-induced protection, we think that inclusion of the analysis of the Th17 response and antibodies directed against specific proteins might increase the relevance of the PSPT model and might facilitate its implementation in wP vaccine lot release testing. However manufacturer-specific evaluation, optimisation and validation of such an advanced PSTP model will be required.

Furthermore, the results of the PSPT enable a comparison between immunological responses induced by the vaccines and the protective properties of the vaccine in the Kendrick test, suggesting that Th1/Th17 and Th2 cytokines might play a role in wP vaccine-induced protection against an intracerebral challenge as well. However, the contribution of subsets of Th cells have only been studied in respiratory infection animal models, and not yet in the Kendrick test or in humans. There are marked differences in resident innate immune cells[123-125] and immunological mechanisms in- and outside the central nervous system.[126,127] So, although it is tempting to speculate that pertussis-specific Th1/Th17 cells and the same protein-specific antibodies play a role in the protective properties of these vaccines against the intracerebral infection, further investigation will be required to elucidate whether and how these immune components contribute to protection in the Kendrick test.

9.6 A functional *in vitro* method for detection of PTx in aP vaccines

Commercial aP vaccines are diverse in their composition, but all contain PTd, optionally supplemented with one or more additional purified proteins of *B. pertussis*, including FHA, PRN and FIM2/FIM3. PTd is obtained by chemical inactivation of PTx with formaldehyde, glutaraldehyde or a combination of the two[128], resulting in a stable nontoxic protein. Despite careful monitoring of the inactivation procedure, low levels of residual PTx may remain in the vaccine. Since small amounts of PTx are considered potentially harmful, regulatory authorities require testing for residual toxicity and for reversion to toxicity by the *in vivo* Histamine Sensitization test (HIST). This *in vivo* test is based on the empirical finding that PTx reduces the lethal dose of histamine up to 30-300 fold.[129,130] The test is difficult to standardize and is known to exhibit high inter- and intra-laboratory variability[5] and occasional false positive results. These scientific disadvantages, together with the potential impact of the test on animal welfare, have fuelled research into alternative strategies to monitor residual PTx in aP vaccines. Alternative strategies are generally based on the mechanism of PTx toxicity as described in several in-depth publications.[131-133] In earlier studies, we demonstrated that reduced contractile properties of arteries are part of histamine sensitization pathophysiology.[131,133] Based on this finding, we established an *in vitro* assay using a vascular smooth muscle cell line (A10) (**Chapter 6**). Since PTx ADP-ribosylates G_i proteins, cells exposed to this toxin accumulate higher levels of cAMP after hormonal stimulation. We used this endpoint for assay development as described in **Chapter 6**.[134] We showed that PTx exposure of A10 cells causes a dose-dependent enhancement of isoprenaline-induced cAMP accumulation, with sensitivity equal to the LD_{50} of the HIST. Since the reproducibility and the sensitivity of the original assay needed optimization, we evaluated various alternative cAMP-quantification methods (data not shown), none of which reduced the variability of the results. Consequently, we examined the possibility to express a reporter construct in A10 cells and also in CHO cells, another cell line known to be PTx sensitive.[135,136] By stable transfection of these cell lines with a commercially available luciferase construct that is controlled by a cAMP responsive element, reporter cell lines were generated (**Chapter 7** and Figure 1). After evaluation of optimal assay conditions, we demonstrated that the resulting A10-CRE and CHO-CRE reporter cells enabled detection of PTx in a dose-dependent manner. New insights based on the data of a collaborative study, revealed that the level of PTx acceptable in aP vaccines is approximately 2.22 IU/mL[137,138], which is the LD_5 of the HIST. Both CHO-CRE and A10-CRE reporter cell lines detected at least 0.2 IU/mL, which is a concentration of PTx well below the LD_5 of the HIST. Since commercially available multivalent vaccines can have cytotoxic effects on CHO and A10 cells (EDQM collaborative study BSP114 phase 1)[21], most likely caused by the aluminium-salts in the vaccine, we used transwell-inserts to prevent the direct contact between aluminium-salts and cells. Using this approach, we

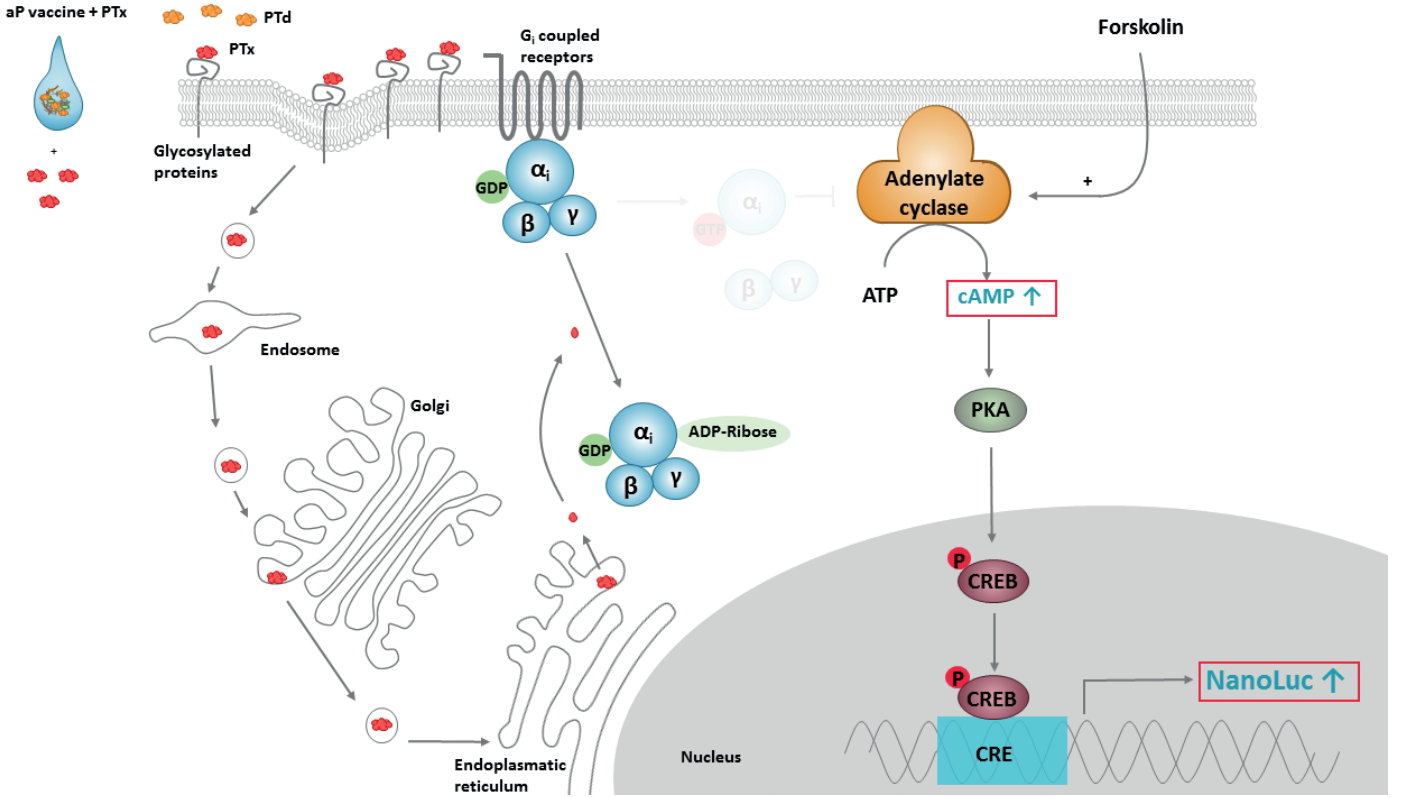

aP vaccine + PTx
PTd
PTx
Glycosylated proteins
Gi coupled receptors
Forskolin
Adenylate cyclase
ATP
cAMP ↑
PKA
Endosome
Golgi
GDP
ADP-Ribose
P
CREB
NanoLuc ↑
CRE
Nucleus
Endoplasmatic reticulum

Figure 1 The mechanisms of the cAMP reporter assay. The B oligomer of PTx binds to glycoconjugate proteins on the cell surface, upon which the holotoxin enters the cell by endocytosis, followed by retrograde transport to endosome, the Golgi and the endoplasmatic reticulum. Subsequently the S1 subunit is released into the cytosol. Within the cytosol, the S1 subunit catalyses the transfer of ADP-ribose from NAD^+ a to the α-subunit of G_i proteins, thereby preventing interaction of these proteins with their cognate receptors. ADP ribosylation fixes the α-subunit of the G-proteins in their inactive (ADP-bound) form and the α-subunit is therefore unable to inhibit its target enzyme; adenylate cyclase. Forskolin stimulation of adenylate cyclase therefore results in unrestricted conversion of ATP into cAMP. This second messenger binds to and activates protein kinase A (PKA), which is responsible for phosphorylation of the cAMP response element-binding protein (CREB), which binds to the DNA sequences cAMP response elements (CRE) and thereby increases the transcription of the gene encoding NanoLuc. Inactivation of PTx most likely limits or halts binding to glycosylated cell membrane proteins and if PTd is internalised, its enzymatic activity is restricted or absent.

demonstrated that the sensitivity of CHO-CRE reporter cell lines was sufficient (1 IU/mL PTx spiked into vaccine), while the sensitivity of the A10-CRE cells insufficient (4 IU/mL PTx spiked into vaccine). The data demonstrated proof-of-principle that the CHO-CRE cell reporter system is a relevant, sensitive and reproducible assay to analyse PTx alone or in the context of a multivalent aP vaccine.

To evaluate what the impact of implementation of an alternative such as our CHO-CRE reporter cell line can be in terms of animal reduction, global HIST batch release testing was evaluated by a survey among manufacturers, national control laboratories (NCL's) and other organisations involved in the quality control of vaccines. These organization were queried for their testing strategies, or the testing strategies of the NCLs in the region of interest of the organization (**Chapter 8**). This evaluation revealed marked differences in regulatory requirements, including readout parameter, test group sizes, positive and negative control groups, amount of vaccine and acceptance criteria. Based on the information and data provided by the contacted institutes, the worldwide number of animals used for HIST testing was estimated to be about 65,000 mice per year, ~48,000 by the manufacturers and ~17,000 by the national control laboratories. Furthermore, various manners to reduce numbers of animals in the HIST or to refine animal use were identified and discussed, though it was questioned whether the efforts needed for validation and implementation are worthwhile. *In vitro* methods, such as our reporter assays, have a scientific sound mechanistic substantiation. It was therefore concluded that *in vitro* methods are favourable over current HIST or reduction and refinement alternatives and have the potential to significantly reduce the numbers of animals that are currently used for the HIST testing.

9.7 New vaccine approaches highlight the need for appropriate *in vitro* and *in vivo* models

Despite intensive vaccination programs using aP or wP vaccines or a combination of both, *B. pertussis* keeps circulating in the environment. In some parts of the world the incidence of pertussis disease has even increased in the last few decades[139-142], possibly as a result of 1) genetic changes in the circulating *B. pertussis* strains in the population, resulting in an antigenic mismatch with the vaccine strains, 2) improved diagnostic tests that enable more sensitive and accurate detection of pertussis, resulting in increased number of reported cases, and 3) waning immunity as a result of the introduction of aP vaccines.[33,143]

Recognition of the shortcomings of the current pertussis vaccine has led to multiple initiatives for new or improved vaccine strategies. Firstly, it has been proposed to replace the

conventional aluminium-salt adjuvants in aP vaccines by TLR agonists, such as CpG or a non-toxic LPS derivative, aiming to steer the immune response toward a Th1/Th17 type of immune response.[43,48,144] Secondly, adding new antigens has been suggested in order to improve the breath of the immune response. Potential candidate proteins include ACT, BrkA, and IRP-1[145], since these are considered to exhibit protective properties. A third approach is the generation of a live-attenuated and less reactogenic *B. pertussis* strain, which can be administered intranasally, mimicking the natural route of infection. In an *in vitro* model using human DCs and T cells, this vaccine approach promoted a Th1/Th17 type of immune response[146], while a study in mice revealed long lasting immunity.[147] The fourth approach consists of vaccines based on outer membrane vesicles containing various immunogenic antigens, thereby more closely resembling the protein composition of wP vaccines. This type of vaccines has shown to be safe and protective in mouse models.[148]

Along with the need to continue with current vaccination programs and the development of new pertussis vaccines, animal models will be required to examine vaccines' immunological properties and their safety. As such, the novel *in vitro* methods and adaptation of current *in vivo* methods as presented in this thesis might be valuable, not only to improve the capacity to monitor vaccine safety and potency, but also to improve the understanding of the immunological properties of these vaccines.

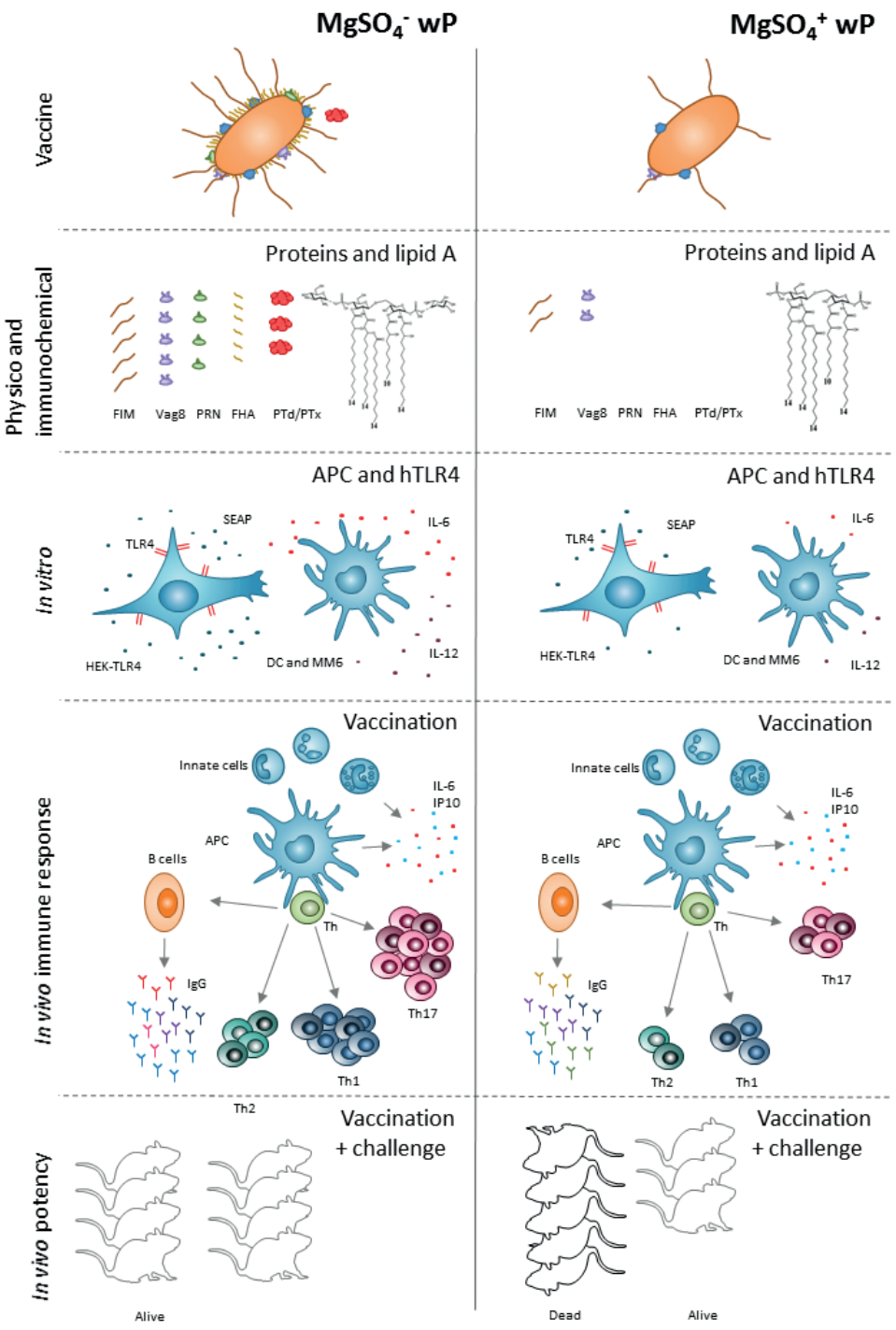

MgSO4- wP
MgSO4+ wP
Vaccine
Physico and immunochemical
Proteins and lipid A
FIM
Vag8
PRN
FHA
PTd/PTx
In vitro
APC and hTLR4
SEAP
TLR4
IL-6
HEK-TLR4
DC and MM6
IL-12
In vivo immune response
Vaccination
Innate cells
IL-6
IP10
APC
B cells
Th
IgG
Th17
Th1
Th2
In vivo potency
Vaccination + challenge
Alive
Dead

Figure 2 Physicochemical and immunochemical, *in vitro* and *in vivo* characterization of experimental wP vaccines derived from $MgSO_4$-non-modulated and $MgSO_4$-modulated bacterial culture. The physicochemical and immunochemical characteristics of the vaccines, as well as their production process, are described in **Chapters 2** and **3**. The *in vitro* responses induced by the vaccines of APC platforms and a HEK cell line expressing human TLR4 were evaluated in **Chapters 3** and **4**. Furthermore, the innate and the adaptive immune response, as result of immunization, were investigated in mice in **Chapter 5**. The *in vivo* potencies of the vaccines were determined by the Kendrick test described in **Chapter 2**.

9.8 Concluding remarks and future perspectives

Animal-based lot release testing of vaccines accounts for a substantial number of animal experiments on an annual basis. Due to ethical concerns and the questionable relevance of several of the *in vivo* models used, we aimed to develop and evaluate *in vitro* methods for their potential to assess functional characteristics of vaccine-induced immune responses or toxicological responses in the context of the Consistency Approach. To this end, we used experimental wP and commercial aP vaccines as model vaccines. For the wP vaccines used in this study, the production process was thoroughly monitored using a combination of specific probes, mass spectrometry of O_2, N_2 and CO_2 and regular characterization of samples.

The studies in this thesis provide examples of *in vitro* cell-based assays and physicochemical tools, such as HB-hTLR4, APC platforms, cAMP reporter cell lines and mass spectrometry-based analysis that can monitor the consistency of pertussis vaccine quality. In addition to the evaluation of these *in vitro* models, we studied the protective properties and immune responses induced by our experimental wP vaccines. The findings of the studies with these experimental wP vaccines are summarized in Figure 2, while Figure 1 represents the mechanisms of the reporter assay developed for aP vaccines. Although we provided proof-of-principle for the individual components of the Consistency Approach, it will be necessary to define a strategy for integrating information gathered from the production process and the intermediate and final products. Other aspects that have to be addressed are validation of each of the methods and the Consistency Approach as such, including the assessment of aspects such as specificity, sensitivity and reproducibility. For some of the assays described in this study, these aspects have been addressed, but additional studies will be required for thorough characterization and (pre-) validation of these models. Validation is historically assessed by comparing the performance of the alternative test with the original *in vivo* test[149], an approach that can be applied for rather simple *in vivo* tests, such as the HIST. For methods that are integrated in a Consistency-based Approach, classical criteria are insufficient, because data originate from multiple

sources, with a large variation in their nature (cellular, physicochemical, immunochemical and in-process parameters). Therefore, fit-to-use criteria need to be established for a robust and comprehensive validation of this approach. These criteria will help to meet regulatory expectations and ultimately pave the way for implementation.

In this thesis, we used *Bordetella pertussis* vaccines as a model with the primary aim to evaluate the possibilities and value of cellular *in vitro* methods. However, the methods evaluated might also be applied to the quality testing of other vaccines that yet rely on animal models for lot release. These include for example vaccines directed against diphtheria, tetanus, rabies, and polio, but also vaccines with a smaller target group such as tick-borne encephalitis and anthrax. The ability to apply *in vitro* methods and physicochemical and immunochemical methods for these vaccines will depend on the nature of the vaccine, that is the presence of PAMPs, adjuvants and other vaccine components (as discussed in section 9.2). In addition, the character of the method will influence the breath of application; i.e. APC platform and PRR-expressing cell lines will generally be important for characterization of vaccine-induced innate immune cell activation and might have a broader application than models for vaccine safety, which often concern specific toxic components.

Since the analytical, *in vitro* and *in vivo* methods described in this thesis assess aspects of vaccine quality that are fundamental for product safety and efficacy, we anticipate that these methods will contribute to the policy to reduce, refine and replace the use of animals in lot release testing of pertussis vaccines and might make the implementation of the Consistency Approach advance.

References

1. Hendriksen, C.F., Replacement, reduction and refinement alternatives to animal use in vaccine potency measurement. Expert Rev Vaccines, 2009. 8(3): p. 313-22.
2. Regnier, M., *et al.*, Structural perturbation of diphtheria toxoid upon adsorption to aluminium hydroxide adjuvant. Vaccine, 2012. 30(48): p. 6783-8.
3. Metz, B., *et al.*, Physicochemical and immunochemical assays for monitoring consistent production of tetanus toxoid. Biologicals, 2013. 41(4): p. 231-7.
4. van der Ark, A.A., *et al.*, Resurgence of pertussis calls for re-evaluation of pertussis animal models. Expert Rev Vaccines, 2012. 11(9): p. 1121-37.
5. van Straaten-van de Kappelle, I., *et al.*, Collaborative study on test systems to assess toxicity of whole cell pertussis vaccine. Biologicals, 1997. 25(1): p. 41-57.
6. Hendriksen, C.F. and B. Steen, Refinement of vaccine potency testing with the use of humane endpoints. ILAR J, 2000. 41(2): p. 105-13.
7. Decker, K.B., *et al.*, The *Bordetella pertussis* model of exquisite gene control by the global transcription factor BvgA. Microbiology, 2012. 158(Pt 7): p. 1665-76.
8. Robinson, A., *et al.*, Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. Vaccine, 1989. 7(4): p. 321-4.
9. de Gouw, D., *et al.*, Differentially Expressed Genes in *Bordetella pertussis* Strains Belonging to a Lineage Which Recently Spread Globally. PLoS One, 2014. 9(1): p. e84523.
10. Mills, K.H., *et al.*, A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infect Immun, 1998. 66(2): p. 594-602.
11. Roberts, M., *et al.*, Recombinant P.69/pertactin: immunogenicity and protection of mice against *Bordetella pertussis* infection. Vaccine, 1992. 10(1): p. 43-8.
12. de Gouw, D., *et al.*, Pertussis: a matter of immune modulation. FEMS Microbiol Rev, 2011. 35(3): p. 441-74.
13. van de Waterbeemd, B., *et al.*, Gene-expression-based quality scores indicate optimal harvest point in *Bordetella pertussis* cultivation for vaccine production. Biotechnol Bioeng, 2009. 103(5): p. 900-8.
14. Nakamura, M.M., *et al.*, Growth phase- and nutrient limitation-associated transcript abundance regulation in *Bordetella pertussis*. Infect Immun, 2006. 74(10): p. 5537-48.
15. Streefland, M., *et al.*, PAT for vaccines: the first stage of PAT implementation for development of a well-defined whole-cell vaccine against whooping cough disease. Vaccine, 2007. 25(16): p. 2994-3000.
16. Hviid, A., *et al.*, Impact of routine vaccination with a pertussis toxoid vaccine in Denmark. Vaccine, 2004. 22(27-28): p. 3530-4.
17. Trollfors, B., *et al.*, A placebo-controlled trial of a pertussis-toxoid vaccine. N Engl J Med, 1995. 333(16): p. 1045-50.
18. Robbins, J.B., *et al.*, Pertussis vaccine: a critique. Pediatr Infect Dis J, 2009. 28(3): p. 237-41.
19. Sato, H. and Y. Sato, *Bordetella pertussis* infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. Infect Immun, 1984. 46(2): p. 415-21.
20. Shahin, R.D., *et al.*, Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of *Bordetella pertussis*. J Exp Med, 1990. 171(1): p. 63-73.

21. Charles, I., *et al.*, Expression of P.69/pertactin from *Bordetella pertussis* in a baculovirus/insect cell expression system: protective properties of the recombinant protein. Res Microbiol, 1993. 144(9): p. 681-90.
22. Novotny, P., *et al.*, Biologic and protective properties of the 69-kDa outer membrane protein of *Bordetella pertussis*: a novel formulation for an acellular pertussis vaccine. J Infect Dis, 1991. 164(1): p. 114-22.
23. Jones, D.H., *et al.*, Protection of mice from *Bordetella pertussis* respiratory infection using microencapsulated pertussis fimbriae. Vaccine, 1995. 13(7): p. 675-81.
24. Jones, D.H., *et al.*, Orally administered microencapsulated *Bordetella pertussis* fimbriae protect mice from *B. pertussis* respiratory infection. Infect Immun, 1996. 64(2): p. 489-94.
25. Fadugba, O.O., *et al.*, Immune responses to pertussis antigens in infants and toddlers after immunization with multicomponent acellular pertussis vaccine. Clin Vaccine Immunol, 2014. 21(12): p. 1613-9.
26. Cherry, J.D., *et al.*, A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. Vaccine, 1998. 16(20): p. 1901-6.
27. Storsaeter, J., *et al.*, Low levels of antipertussis antibodies plus lack of history of pertussis correlate with susceptibility after household exposure to *Bordetella pertussis*. Vaccine, 2003. 21(25-26): p. 3542-9.
28. Storsaeter, J., *et al.*, Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. Vaccine, 1998. 16(20): p. 1907-16.
29. Bisgard, K.M., *et al.*, Pertussis vaccine effectiveness among children 6 to 59 months of age in the United States, 1998-2001. Pediatrics, 2005. 116(2): p. e285-94.
30. Fulton, T.R., *et al.*, Protective Effect of Contemporary Pertussis Vaccines: A Systematic Review and Meta-analysis. Clin Infect Dis, 2016. 62(9): p. 1100-10.
31. Sheridan, S.L., *et al.*, Number and order of whole cell pertussis vaccines in infancy and disease protection. JAMA, 2012. 308(5): p. 454-6.
32. Wendelboe, A.M., *et al.*, Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J, 2005. 24(5 Suppl): p. S58-61.
33. Liko, J., S.G. Robison, and P.R. Cieslak, Priming with whole-cell versus acellular pertussis vaccine. N Engl J Med, 2013. 368(6): p. 581-2.
34. Fransen, F., *et al.*, Differential effect of TLR2 and TLR4 on the immune response after immunization with a vaccine against Neisseria meningitidis or *Bordetella pertussis*. PLoS One, 2010. 5(12): p. e15692.
35. Higgins, S.C., *et al.*, TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. J Immunol, 2006. 177(11): p. 7980-9.
36. Dias, W.O., *et al.*, An improved whole cell pertussis vaccine with reduced content of endotoxin. Hum Vaccin Immunother, 2013. 9(2): p. 339-48.
37. Marr, N., *et al.*, Substitution of the *Bordetella pertussis* lipid A phosphate groups with glucosamine is required for robust NF-kappaB activation and release of proinflammatory cytokines in cells expressing human but not murine Toll-like receptor 4-MD-2-CD14. Infect Immun, 2010. 78(5): p. 2060-9.
38. Geurtsen, J., *et al.*, A novel secondary acyl chain in the lipopolysaccharide of *Bordetella pertussis* required for efficient infection of human macrophages. J Biol Chem, 2007. 282(52): p. 37875-84.
39. Shah, N.R., *et al.*, Minor modifications to the phosphate groups and the C3' acyl chain length of lipid A in two *Bordetella pertussis* strains, BP338 and 18-323, independently affect Toll-like receptor 4 protein activation. J Biol Chem, 2013. 288(17): p. 11751-60.
40. Marr, N., *et al.*, Glucosamine found as a substituent of both phosphate groups in Bordetella lipid A backbones: role of a BvgAS-activated ArnT ortholog. J Bacteriol, 2008. 190(12): p. 4281-90.

41. Leef, M., *et al.*, Protective immunity to *Bordetella pertussis* requires both B cells and CD4(+) T cells for key functions other than specific antibody production. J Exp Med, 2000. 191(11): p. 1841-52.
42. Mills, K.H., *et al.*, Cell-mediated immunity to *Bordetella pertussis*: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infect Immun, 1993. 61(2): p. 399-410.
43. Ross, P.J., *et al.*, Relative contribution of Th1 and Th17 cells in adaptive immunity to *Bordetella pertussis*: towards the rational design of an improved acellular pertussis vaccine. PLoS Pathog, 2013. 9(4): p. e1003264.
44. Redhead, K., *et al.*, Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infect Immun, 1993. 61(8): p. 3190-8.
45. Vermeulen, F., *et al.*, Cellular immune responses of preterm infants after vaccination with whole-cell or acellular pertussis vaccines. Clin Vaccine Immunol, 2010. 17(2): p. 258-62.
46. Hendrikx, L.H., *et al.*, Different IgG-subclass distributions after whole-cell and acellular pertussis infant primary vaccinations in healthy and pertussis infected children. Vaccine, 2011. 29(40): p. 6874-80.
47. Mascart, F., *et al.*, Modulation of the infant immune responses by the first pertussis vaccine administrations. Vaccine, 2007. 25(2): p. 391-8.
48. Brummelman, J., *et al.*, Modulation of the CD4(+) T cell response after acellular pertussis vaccination in the presence of TLR4 ligation. Vaccine, 2015. 33(12): p. 1483-91.
49. Geurtsen, J., *et al.*, Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccine and reduce type I hypersensitivity in mice. Clin Vaccine Immunol, 2007. 14(7): p. 821-9.
50. Errea, A., *et al.*, Mucosal innate response stimulation induced by lipopolysaccharide protects against *Bordetella pertussis* colonization. Med Microbiol Immunol, 2010. 199(2): p. 103-8.
51. Iwasaki, A. and R. Medzhitov, Toll-like receptor control of the adaptive immune responses. Nat Immunol, 2004. 5(10): p. 987-95.
52. Fedele, G., *et al.*, *Bordetella pertussis* commits human dendritic cells to promote a Th1/Th17 response through the activity of adenylate cyclase toxin and MAPK-pathways. PLoS One, 2010. 5(1): p. e8734.
53. Brummelman, J., *et al.*, *Bordetella pertussis* naturally occurring isolates with altered lipooligosaccharide structure fail to fully mature human dendritic cells. Infect Immun, 2015. 83(1): p. 227-38.
54. Eisenbarth, S.C., *et al.*, Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature, 2008. 453(7198): p. 1122-6.
55. Isbrucker, R., *et al.*, Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. Biologicals, 2014. 42(2): p. 114-22.
56. Franchi, L. and G. Nunez, The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol, 2008. 38(8): p. 2085-9.
57. Seubert, A., *et al.*, The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells. J Immunol, 2008. 180(8): p. 5402-12.
58. Rimaniol, A.C., *et al.*, Aluminum hydroxide adjuvant induces macrophage differentiation towards a specialized antigen-presenting cell type. Vaccine, 2004. 22(23-24): p. 3127-35.
59. Ulanova, M., *et al.*, The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. Infect Immun, 2001. 69(2): p. 1151-9.
60. Baker, M.P., *et al.*, Immunogenicity of protein therapeutics: The key causes, consequences and challenges. Self Nonself, 2010. 1(4): p. 314-322.

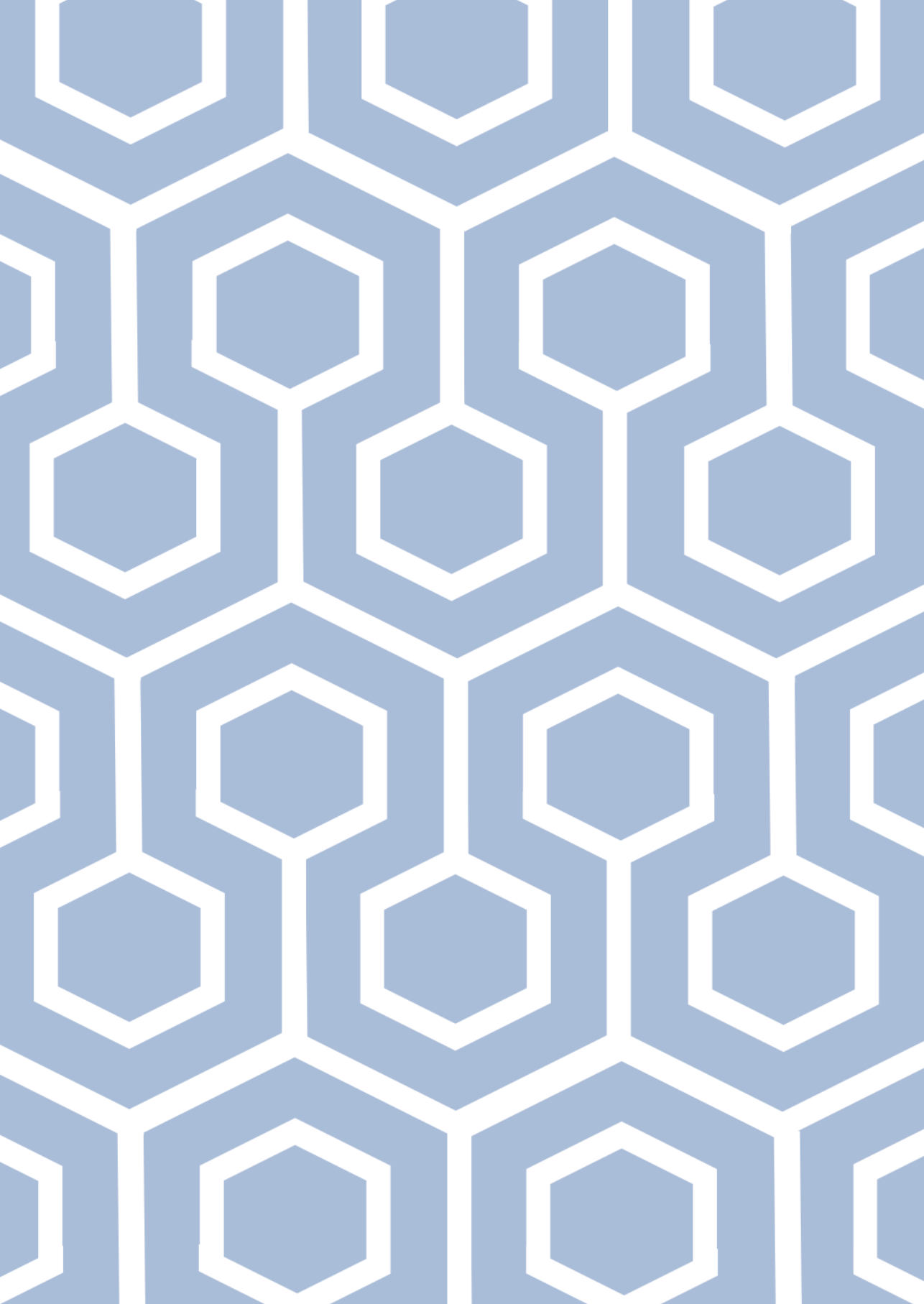
61. Moser, J.M., *et al.*, Optimization of a dendritic cell-based assay for the *in vitro* priming of naive human CD4+ T cells. J Immunol Methods, 2010. 353(1-2): p. 8-19.
62. Song, H., *et al.*, *In vitro* stimulation of human influenza-specific CD8+ T cells by dendritic cells pulsed with an influenza virus-like particle (VLP) vaccine. Vaccine, 2010. 28(34): p. 5524-32.
63. Vocanson, M., *et al.*, Depletion of human peripheral blood lymphocytes in CD25+ cells allows for the sensitive *in vitro* screening of contact allergens. J Invest Dermatol, 2008. 128(8): p. 2119-22.
64. Sherman, L.A. and S. Chattopadhyay, The molecular basis of allorecognition. Annu Rev Immunol, 1993. 11: p. 385-402.
65. Wallgren, A.C., *et al.*, Direct allorecognition promotes activation of bystander dendritic cells and licenses them for Th1 priming: a functional link between direct and indirect allosensitization. Scand J Immunol, 2005. 62(3): p. 234-42.
66. de Haan, A., *et al.*, Activation of alloreactive T cells by allogeneic nonprofessional antigen-presenting cells and interleukin-12 from bystander autologous professional antigen-presenting cells. Transplantation, 2000. 69(8): p. 1637-44.
67. Ramanathan, P., *et al.*, Dendritic cells primed with HPV positive cervical tumor lysate are superior to unprimed DCs in migratory capacity and induce a potent Th1 response. Hum Immunol, 2014. 75(12): p. 1216-24.
68. Kim, T.H., *et al.*, Critical role of TRIF and MyD88 in Mycobacterium tuberculosis Hsp70-mediated activation of dendritic cells. Cytokine, 2015. 71(2): p. 139-44.
69. Kubota, K. and K. Iwabuchi, Phenotypic changes in growth-arrested T cell hybrids: a possible avenue to produce functional T cell hybridoma. Front Immunol, 2014. 5: p. 229.
70. Canaday, D.H., *et al.*, T-cell hybridomas from HLA-transgenic mice as tools for analysis of human antigen processing. J Immunol Methods, 2003. 281(1-2): p. 129-42.
71. Seifert, M., *et al.*, Crosstalk between immune cells and mesenchymal stromal cells in a 3D bioreactor system. Int J Artif Organs, 2012. 35(11): p. 986-95.
72. Okamoto, N., *et al.*, Artificial lymph nodes induce potent secondary immune responses in naive and immunodeficient mice. J Clin Invest, 2007. 117(4): p. 997-1007.
73. Tomei, A.A., *et al.*, Fluid flow regulates stromal cell organization and CCL21 expression in a tissue-engineered lymph node microenvironment. J Immunol, 2009. 183(7): p. 4273-83.
74. Kobayashi, Y., K. Kato, and T. Watanabe, Synthesis of functional artificial lymphoid tissues. Discov Med, 2011. 12(65): p. 351-62.
75. Cupedo, T., A. Stroock, and M. Coles, Application of tissue engineering to the immune system: development of artificial lymph nodes. Front Immunol, 2012. 3: p. 343.
76. Giese, C. and U. Marx, Human immunity *in vitro* - solving immunogenicity and more. Adv Drug Deliv Rev, 2014. 69-70: p. 103-22.
77. Giese, C., *et al.*, A human lymph node *in vitro*--challenges and progress. Artif Organs, 2006. 30(10): p. 803-8.
78. Giese, C., *et al.*, Immunological substance testing on human lymphatic micro-organoids *in vitro*. J Biotechnol, 2010. 148(1): p. 38-45.
79. Sallusto, F. and A. Lanzavecchia, Monocytes join the dendritic cell family. Cell, 2010. 143(3): p. 339-40.
80. Drake, D.R., *et al.*, *In vitro* Biomimetic Model of the Human Immune System for Predictive Vaccine Assessments. Disruptive science and technology, 2012. 1(1): p. 28-40.
81. Byers, A.M., *et al.*, *In vitro* antibody response to tetanus in the MIMIC system is a representative measure of vaccine immunogenicity. Biologicals, 2009. 37(3): p. 148-51.

82. Shuler, M.L., Modeling life. Ann Biomed Eng, 2012. 40(7): p. 1399-407.
83. Marx, U., *et al.*, 'Human-on-a-chip' developments: a translational cutting-edge alternative to systemic safety assessment and efficiency evaluation of substances in laboratory animals and man? Altern Lab Anim, 2012. 40(5): p. 235-57.
84. Huh, D., G.A. Hamilton, and D.E. Ingber, From 3D cell culture to organs-on-chips. Trends Cell Biol, 2011. 21(12): p. 745-54.
85. Kendrick, P.L., *et al.*, Mouse Protection Tests in the Study of Pertussis Vaccine: A Comparative Series Using the Intracerebral Route for Challenge. Am J Public Health Nations Health, 1947. 37(7): p. 803-10.
86. VACCINATION against whooping-cough; the final report to the Whooping-Cough Immunization Committee of the Medical Research Council and to the medical officers of health for Battersea and Wandsworth, Bradford, Liverpool, and Newcastle. Br Med J, 1959. 1(5128): p. 994-1000.
87. Standfast, A.F., The comparison between field trials and mouse protection tests against intranasal and intracerebral challenges with *Bordetella pertussis*. Immunology, 1958. 1(2): p. 135-43.
88. Berenbaum, M.C., J. Ungar, and W.K. Stevens, Intracranial infection of mice with *Bordetella pertussis*. J Gen Microbiol, 1960. 22: p. 313-22.
89. Dolby, J.M., D.E. Dolby, and C.J. Bronne-Shanbury, The effects of humoral, cellular and non-specific immunity on intracerebral *Bordetella pertussis* infections in mice. J Hyg (Lond), 1975. 74(1): p. 85-102.
90. Dolby, J.M., Passive protection of mice against intracerebral infections with *Bordetella pertussis*. J Hyg (Lond), 1972. 70(4): p. 707-18.
91. Wiertz, E.J., *et al.*, Acellular and whole cell pertussis vaccines protect against the lethal effects of intracerebral challenge by two different T-cell dependent humoral routes. Biologicals, 1990. 18(3): p. 173-80.
92. Musser, J.M., *et al.*, Genetic diversity and relationships in populations of Bordetella spp. J Bacteriol, 1986. 166(1): p. 230-7.
93. WHO, Recommendations for whole-cell pertussis vaccine. WHO Technical Report Series 2007. 941, Geneva, Switzerland: p. 301-33.
94. European Pharmacopoeia, Assay of pertussis vaccine (whole cell). 2013. 8th edition: p. 242.
95. van der Ark, A., *et al.*, Development of pertussis serological potency test. Serological assessment of antibody response induced by whole cell vaccine as an alternative to mouse protection in an intracerebral challenge model. Biologicals, 1994. 22(3): p. 233-42.
96. Higgs, R., *et al.*, Immunity to the respiratory pathogen *Bordetella pertussis*. Mucosal Immunol, 2012. 5(5): p. 485-500.
97. Hendrikx, L.H., *et al.*, Identifying long-term memory B-cells in vaccinated children despite waning antibody levels specific for *Bordetella pertussis* proteins. Vaccine, 2011. 29(7): p. 1431-7.
98. Hendrikx, L.H., *et al.*, Enhanced memory B-cell immune responses after a second acellular pertussis booster vaccination in children 9 years of age. Vaccine, 2011. 30(1): p. 51-8.
99. Mahon, B.P., M.T. Brady, and K.H. Mills, Protection against *Bordetella pertussis* in mice in the absence of detectable circulating antibody: implications for long-term immunity in children. J Infect Dis, 2000. 181(6): p. 2087-91.
100. Warfel, J.M. and T.J. Merkel, *Bordetella pertussis* infection induces a mucosal IL-17 response and long-lived Th17 and Th1 immune memory cells in nonhuman primates. Mucosal Immunol, 2013. 6(4): p. 787-96.
101. Kirimanjeswara, G.S., *et al.*, Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. J Clin Invest, 2005. 115(12): p. 3594-601.

102. Warfel, J.M., L.I. Zimmerman, and T.J. Merkel, Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. Proc Natl Acad Sci U S A, 2014. 111(2): p. 787-92.
103. Fennelly, N.K., *et al.*, *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. Infect Immun, 2008. 76(3): p. 1257-66.
104. Paddock, C.D., *et al.*, Pathology and pathogenesis of fatal *Bordetella pertussis* infection in infants. Clin Infect Dis, 2008. 47(3): p. 328-38.
105. Wang, C., *et al.*, CD5L/AIM Regulates Lipid Biosynthesis and Restrains Th17 Cell Pathogenicity. Cell, 2015. 163(6): p. 1413-27.
106. Mobberley-Schuman, P.S., B. Connelly, and A.A. Weiss, Phagocytosis of *Bordetella pertussis* incubated with convalescent serum. J Infect Dis, 2003. 187(10): p. 1646-53.
107. McGuirk, P., C. McCann, and K.H. Mills, Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J Exp Med, 2002. 195(2): p. 221-31.
108. McGuirk, P. and K.H. Mills, Direct anti-inflammatory effect of a bacterial virulence factor: IL-10-dependent suppression of IL-12 production by filamentous hemagglutinin from *Bordetella pertussis*. Eur J Immunol, 2000. 30(2): p. 415-22.
109. Carbonetti, N.H., Pertussis toxin and adenylate cyclase toxin: key virulence factors of *Bordetella pertussis* and cell biology tools. Future Microbiol, 2010. 5(3): p. 455-69.
110. Andreasen, C., D.A. Powell, and N.H. Carbonetti, Pertussis toxin stimulates IL-17 production in response to *Bordetella pertussis* infection in mice. PLoS One, 2009. 4(9): p. e7079.
111. Schellenberg, A.E., *et al.*, Blood-brain barrier disruption in CCL2 transgenic mice during pertussis toxin-induced brain inflammation. Fluids Barriers CNS, 2012. 9(1): p. 10.
112. Charles, I.G., *et al.*, Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. Proc Natl Acad Sci U S A, 1989. 86(10): p. 3554-8.
113. Everest, P., *et al.*, Role of the *Bordetella pertussis* P.69/pertactin protein and the P.69/pertactin RGD motif in the adherence to and invasion of mammalian cells. Microbiology, 1996. 142 (Pt 11): p. 3261-8.
114. Bagley, K.C., *et al.*, Pertussis toxin and the adenylate cyclase toxin from *Bordetella pertussis* activate human monocyte-derived dendritic cells and dominantly inhibit cytokine production through a cAMP-dependent pathway. J Leukoc Biol, 2002. 72(5): p. 962-9.
115. Kamanova, J., *et al.*, Adenylate cyclase toxin subverts phagocyte function by RhoA inhibition and unproductive ruffling. J Immunol, 2008. 181(8): p. 5587-97.
116. Gray, M.C. and E.L. Hewlett, Cell cycle arrest induced by the bacterial adenylate cyclase toxins from Bacillus anthracis and *Bordetella pertussis*. Cell Microbiol, 2011. 13(1): p. 123-34.
117. Khelef, N. and N. Guiso, Induction of macrophage apoptosis by *Bordetella pertussis* adenylate cyclase-hemolysin. FEMS Microbiol Lett, 1995. 134(1): p. 27-32.
118. Boyd, A.P., *et al.*, *Bordetella pertussis* adenylate cyclase toxin modulates innate and adaptive immune responses: distinct roles for acylation and enzymatic activity in immunomodulation and cell death. J Immunol, 2005. 175(2): p. 730-8.
119. Ross, P.J., *et al.*, Adenylate cyclase toxin from *Bordetella pertussis* synergizes with lipopolysaccharide to promote innate interleukin-10 production and enhances the induction of Th2 and regulatory T cells. Infect Immun, 2004. 72(3): p. 1568-79.

120. Dunne, A., *et al.*, Inflammasome activation by adenylate cyclase toxin directs Th17 responses and protection against *Bordetella pertussis*. J Immunol, 2010. 185(3): p. 1711-9.
121. Bellalou, J. and E.H. Relyveld, Studies on culture conditions of *Bordetella pertussis* and relationship to immunogenicity of vaccines. Ann Microbiol (Paris), 1984. 135B(1): p. 101-10.
122. Dolby, J.M., J.P. Ackers, and D.E. Dolby, The effect of the antigen which elicits the bactericidal antibody and of the mouse-protective antigen on the growth of *Bordetella pertussis* in the mouse brain. J Hyg (Lond), 1975. 74(1): p. 71-83.
123. Ginhoux, F., *et al.*, Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science, 2010. 330(6005): p. 841-5.
124. Gosselin, D., *et al.*, Environment drives selection and function of enhancers controlling tissue-specific macrophage identities. Cell, 2014. 159(6): p. 1327-40.
125. Lavin, Y., *et al.*, Tissue-resident macrophage enhancer landscapes are shaped by the local microenvironment. Cell, 2014. 159(6): p. 1312-26.
126. Libbey, J.E. and R.S. Fujinami, Adaptive immune response to viral infections in the central nervous system. Handb Clin Neurol, 2014. 123: p. 225-47.
127. Barichello, T., *et al.*, Pathophysiology of bacterial infection of the central nervous system and its putative role in the pathogenesis of behavioral changes. Rev Bras Psiquiatr, 2013. 35(1): p. 81-7.
128. Oh, H., *et al.*, Characterization of the carbohydrate binding and ADP-ribosyltransferase activities of chemically detoxified pertussis toxins. Vaccine, 2013. 31(29): p. 2988-93.
129. Kind, L.S., The altered reactivity of mice after inoculation with *Bordetella pertussis* vaccine. Bacteriol Rev, 1958. 22(3): p. 173-82.
130. Parfentjev, I.A. and M.A. Goodline, Histamine shock in mice sensitized with Hemophilus pertussis vaccine. J Pharmacol Exp Ther, 1948. 92(4): p. 411-3.
131. van Meijeren, C.E., *et al.*, Pertussis toxin relaxes small arteries with no vascular lesions or vascular smooth muscle cell injury. Exp Toxicol Pathol, 2004. 56(3): p. 139-43.
132. van Meijeren, C.E., *et al.*, Pertussis toxin-induced histamine sensitisation: an aspecific phenomenon independent from the nitric oxide system? Eur J Pharmacol, 2004. 493(1-3): p. 139-50.
133. van Meijeren, C.E., *et al.*, *In vivo* pertussis toxin treatment reduces contraction of rat resistance arteries but not that of mouse trachea. Eur J Pharmacol, 2004. 488(1-3): p. 127-35.
134. Hoonakker, M.E., N. Ruiterkamp, and C.F. Hendriksen, The cAMP assay: a functional *in vitro* alternative to the *in vivo* Histamine Sensitization test. Vaccine, 2010. 28(5): p. 1347-52.
135. Castro, M.G., U. McNamara, and N.H. Carbonetti, Expression, activity and cytotoxicity of pertussis toxin S1 subunit in transfected mammalian cells. Cell Microbiol, 2001. 3(1): p. 45-54.
136. Hewlett, E.L., *et al.*, Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect Immun, 1983. 40(3): p. 1198-203.
137. Xing, D., *et al.*, Collaborative study for the standardisation of the histamine sensitizing test in mice and the CHO cell-based assay for the residual toxicity testing of acellular pertussis vaccines. Pharmeur Bio Sci Notes, 2010. 2010(1): p. 51-63.
138. Bache, C., *et al.*, Workshop on Animal free Detection of Pertussis Toxin in Vaccines--Alternatives to the Histamine Sensitisation Test. Biologicals, 2012. 40(4): p. 309-11.
139. Lam, C., *et al.*, Rapid increase in pertactin-deficient *Bordetella pertussis* isolates, Australia. Emerg Infect Dis, 2014. 20(4): p. 626-33.
140. Billingsley, M., Pregnant women in UK are offered whooping cough vaccine to protect newborns. BMJ, 2012. 345: p. e6594.

141. Clark, T.A., Changing pertussis epidemiology: everything old is new again. J Infect Dis, 2014. 209(7): p. 978-81.
142. Klein, N.P., *et al.*, Waning protection after fifth dose of acellular pertussis vaccine in children. N Engl J Med, 2012. 367(11): p. 1012-9.
143. Klein, N.P., *et al.*, Comparative effectiveness of acellular versus whole-cell pertussis vaccines in teenagers. Pediatrics, 2013. 131(6): p. e1716-22.
144. Asokanathan, C., M. Corbel, and D. Xing, A CpG-containing oligodeoxynucleotide adjuvant for acellular pertussis vaccine improves the protective response against *Bordetella pertussis*. Hum Vaccin Immunother, 2013. 9(2): p. 325-31.
145. Locht, C. and N. Mielcarek, New pertussis vaccination approaches: en route to protect newborns? FEMS Immunol Med Microbiol, 2012. 66(2): p. 121-33.
146. Fedele, G., *et al.*, Attenuated *Bordetella pertussis* vaccine candidate BPZE1 promotes human dendritic cell CCL21-induced migration and drives a Th1/Th17 response. J Immunol, 2011. 186(9): p. 5388-96.
147. Feunou, P.F., *et al.*, Long-term immunity against pertussis induced by a single nasal administration of live attenuated *B. pertussis* BPZE1. Vaccine, 2010. 28(43): p. 7047-53.
148. Bottero, D., *et al.*, Characterization of the immune response induced by pertussis OMVs-based vaccine. Vaccine, 2016.
149. European Pharmacopoeia, General notices. 2014. 8th edition: p. 3897-3904.



Appendix

Nederlandse samenvatting
Curriculum vitae
List of publications
Dankwoord

Nederlandse samenvatting

Cursief en dikgedrukte woorden zijn opgenomen in een verklarende woordenlijst.

Het immuun systeem en vaccinatie

Het immuun system van zoogdieren heeft als doel het individu te beschermen tegen ***pathogenen*** zoals bacteriën, virussen, schimmels en parasieten. De eerste linie is het niet specifieke of ***aangeboren immuun systeem***, gevormd door cellen die een breed scala aan pathogenen herkennen en snel opruimen. Door middel van specifieke receptoren zijn deze cellen in staat bepaalde geconserveerde structuren te herkennen die alleen pathogenen bezitten, zoals bijvoorbeeld ***lipopolysaccharide***. Indien het aangeboren immuun systeem niet in staat is het pathogeen snel te verwijderen, wordt de tweede linie - het ***adaptieve immuun systeem*** - in werking gesteld. Het adaptieve immuun systeem bestaat uit cellen die in staat zijn specifieke onderdelen van een pathogeen te identificeren en onschadelijk te maken. Hierbij zijn ***T helper cellen***, ***cytotoxische T cellen*** en ***B cellen*** (en ***antilichamen***) betrokken. Om effectief te zijn, moeten de cellen van het adaptieve immuun systeem zich klonaal vermenigvuldigen en daarom duurt het enige tijd voordat een adaptieve immuun respons zich voldoende ontwikkeld heeft. Een belangrijke eigenschap van het adaptieve immuun systeem is de ontwikkeling van een immunologisch geheugen tegen al herkende pathogenen. Hierdoor kan sneller een specifieke afweer ontwikkeld worden bij herhaalde blootstelling aan hetzelfde pathogeen. Dit kenmerk wordt gebruikt bij vaccinatie, waarbij het immuun systeem blootgesteld wordt aan een onschadelijk gemaakt pathogeen of aan onderdelen van het pathogeen. Hiermee wordt een infectie nagebootst met als doel een immunologisch geheugen op te bouwen tegen dit pathogeen.

Vaccins, proefdierkundig onderzoek en de Consistency Benadering

Vaccins en dierexperimenten zijn onlosmakelijk met elkaar verbonden. Tijdens hun ontwikkeling worden vaccins uitvoerig getest op hun capaciteit om een beschermende immuun respons (werkzaamheid) te induceren en op mogelijke schadelijke effecten (veiligheid). Echter ook iedere geproduceerde partij vaccin wordt getest op nagenoeg dezelfde aspecten. Veelal wordt hiervoor gebruik gemaakt van diertesten, met name voor vaccins als difterie-, tetanus-, kinkhoest- en polio-vaccins. Deze diertesten zijn vaak gestandaardiseerd en worden uitgevoerd volgens specifieke richtlijnen beschreven in wet- en regelgeving. Deze diertesten staan tegenwoordig ter discussie omdat er veelal grote spreiding zit in de testresultaten, maar ook vanwege de hoge mate van ongerief voor de proefdieren en de immunologische verschillen tussen het proefdier en de mens of – in geval van veterinaire vaccins - het proefdier en het doeldier in het geval dat deze van elkaar verschillen. Vanwege de complexiteit van een immuun respons en de biologische aard van een vaccin, is het moeilijk met één proefdiervrije

methode te volstaan. Een andere benadering is gebaseerd op het leveren van bewijs dat een vaccin op consistente wijze wordt geproduceerd en niet verschilt van een voorgaande partij met bewezen kwaliteit. Dit kan door aannemelijk te maken dat het productieproces reproduceerbaar verloopt (procesinformatie), én door met een aantal analytische en *in vitro* technieken een "vingerafdruk" van het product te maken (productinformatie). Voor deze ***Consisteny Benadering*** zijn een aantal fysisch-chemische en immuno-chemische technieken ontwikkeld of in ontwikkeling die toegepast kunnen worden voor de karakterisatie van difterie en tetanus vaccins. Alhoewel deze technieken inzicht verschaffen in de samenstelling, vorm en structuur van de eiwitten en andere moleculen in een vaccin, geven ze geen informatie over functionele aspecten, dat wil zeggen de immuun stimulerende capaciteit van een vaccin. In dit proefschrift is onderzocht of het met cellulaire methoden mogelijk is om dergelijke functionele aspecten te meten.

Kinkhoest vaccins en diertesten

Voor de evaluatie van de cellulaire modellen zijn ***kinkhoest*** vaccins als model gebruikt, omdat a) er een substantieel aantal proefdieren ingezet wordt voor de bepaling van de werkzaamheid en veiligheid van deze vaccins, b) de diermodellen die op dit moment gebruikt worden, gepaard kunnen gaan met een hoge mate van ongerief, c) er veel variatie is in de resultaten van deze dierexperimenten, d) kinkhoest een ernstige ziekte is die blijft circuleren waardoor vaccinatie noodzakelijk is, en e) het vaccin aantrekkelijk is vanuit een wetenschappelijk oogpunt, zoals hieronder bediscussieerd.

Er zijn twee verschillende soorten kinkhoest vaccins. Het whole cell kinkhoest vaccin (wP) is in de jaren 1950-1960 geïntroduceerd en is gebaseerd op ***virulente kinkhoest bacteriën*** die onschadelijk gemaakt worden door verhitting en behandeling met ***formaldehyde***. Deze vaccins bevatten veel verschillende eiwitten en andere bacterie fragmenten (tezamen antigenen genoemd) die een bijdrage leveren aan de beschermende werking. Alhoewel het vaccin effectief is, gaan inentingen met dit vaccin regelmatig gepaard met bijwerkingen zoals zwelling en roodheid op de plek van injectie en koorts. De zoektocht naar veiliger vaccins heeft geresulteerd in de ontwikkeling van acellulaire kinkhoest vaccins (aP vaccins). aP vaccins bevatten het geïnactiveerde ***pertussis toxine*** (PTx), pertussis toxoid genaamd, aangevuld met één tot vier andere gezuiverde eiwitten van de kinkhoestbacterie. aP vaccins worden met name toegepast in westerse landen (zie **Hoofdstuk 8** voor een overzicht), terwijl ontwikkelingslanden meestal gebruik maken van wP vaccins, omdat de kosten voor wP vaccins relatief laag zijn. In Nederlands is tot 2005 het wP vaccin toegepast, waarna het vervangen is door een aP vaccin gebaseerd op drie eiwitten. Alhoewel aP vaccins effectief zijn, heeft recent onderzoek uitgewezen dat de duur van de bescherming geïnduceerd door aP vaccins minder lang is dan geïnduceerd door wP vaccins. Dit heeft de ontwikkeling van nieuwe vaccins gestimuleerd. Een

van de nieuwe vaccinconcepten is gebaseerd op genetisch verzwakte kinkhoest bacteriën. Toediening van dit vaccin leidt tot een immuun respons, maar resulteert niet in ziekte. Een ander vaccinconcept is gebaseerd op zogenaamde Outer Membrane Vesicles, blaasjes die van de bacteriewand afgescheiden worden en eiwitten bevatten die ook in de bacteriewand zitten. Andere nieuwe vaccin concepten worden besproken in **Hoofdstuk 9**.

Voordat een nieuwe partij wP of aP vaccin toegepast mag worden in de mens, wordt de werkzaamheid bepaald door middel van gestandaardiseerde diertesten. De werkzaamheid van wP vaccins wordt bepaald met behulp van de zogenaamde Kendrick test, ook wel de muis beschermingstest genoemd. Muizen worden gevaccineerd met verschillende concentraties van het vaccin. Vervolgens wordt de mate van bescherming bepaald door de hersenen te infecteren met virulente kinkhoest bacteriën. Indien de muizen niet of onvolledig beschermd zijn zullen ze overlijden als gevolg van de infectie, met als gevolg dat deze dieren een hoge mate van ongerief ondervinden. De werkzaamheid van aP vaccins wordt bepaald door dieren te vaccineren met verschillende concentraties van het vaccin. Daarna wordt aan de hand van de antilichaam niveaus in het bloed bepaald of het vaccin voldoende effectief is.

Naast de werkzaamheid, dient ook de veiligheid van kinkhoest vaccins bepaald te worden door middel van gestandaardiseerde diertesten. Voor wP vaccins wordt de zogenaamde Mouse Weight Gain test voorgeschreven. In deze test worden muizen geïnjecteerd met het te onderzoeken vaccin en vervolgens wordt het gewicht van de dieren nauwkeurig gemonitord. Een vertraagde groei duidt op de aanwezigheid van toxines of andere ongewenste componenten. De relatief eenvoudige samenstelling van aP vaccins maakt dat voor dit vaccin alleen eventuele resten niet geïnactiveerd PTx bepaald hoeven te worden, omdat gedacht wordt dat dit toxine de belangrijkste oorzaak is van bijwerkingen. De voorgeschreven diertest is gebaseerd op het mechanisme dat PTx muizen gevoeliger maakt voor ***histamine***; dat wil zeggen dat een te hoge dosis PTx (in een aP vaccin) gevolgd door een blootstelling aan histamine letaal is. Deze diertest wordt de Histamine Sensitisatie test genoemd en kan gepaard gaan met een hoge mate van ongerief. Daarnaast wordt de test bekritiseerd vanwege de grote variatie in testresultaten en het beperkte begrip van het mechanisme dat te grondslag ligt aan de toxine geïnduceerde gevoeligheid voor histamine.

Dit proefschrift

In dit proefschrift hebben we onderzocht of cellulaire methoden in staat zijn om de kwaliteit en veiligheid van kinkhoest vaccins te meten en een functionele aanvulling kunnen vormen voor de Consistency Benadering. Voor de volledigheid zijn de gebruikte vaccins ook door middel van enkele fysisch-chemische en immuno-chemische technieken geëvalueerd.

Om te bepalen of cellulaire methoden in staat zijn om een functionele indicatie te geven van vaccin kwaliteit is het noodzakelijk te beschikken over vaccins van verschillende kwaliteiten, immers een test moet een onderscheid kunnen maken tussen een vaccin van een goede en van een slechte kwaliteit. Omdat dergelijke vaccins niet commercieel verkrijgbaar zijn, hebben we gebruik gemaakt van experimentele wP vaccins, geproduceerd door ons instituut, waarvan het productieproces doelbewust gemanipuleerd is met **$MgSO_4$**. Na toevoeging van $MgSO_4$ zijn verscheidenen monsters afgenomen en verwerkt tot vaccins. $MgSO_4$ leidt tot veranderingen waardoor de kinkhoestbacterie bepaalde eiwitten - de ***virulente eiwitten*** - minder gaat produceren. Dit wordt de avirulente staat genoemd. Gedacht wordt dat veel van de virulente eiwitten een bijdrage leveren aan de beschermende werking van het vaccin.

In **Hoofdstuk 2 en Hoofdstuk 3** is door middel van fysisch- en immuno-chemische technieken, zoals massa spectrometry en ELISA, de samenstelling van de zojuist beschreven vaccins onderzocht. Met deze technieken hebben we laten zien dat manipulatie van het productieproces met $MgSO_4$ resulteert in een geleidelijke vermindering van de hoeveelheid virulente eiwitten, en effect heeft op de vorm van het lipopolysaccharide in de vaccins. Om deze resultaten in perspectief te kunnen plaatsen is in **Hoofdstuk 2** onderzocht wat de werkzaamheid van drie van onze experimentele wP vaccins is door middel van de muis beschermingstest. Deze diertest heeft uitgewezen dat het vaccin met het hoogste niveau aan virulente eiwitten in staat is om muizen te beschermen en dat dit vaccin voldoet aan de regulatoir gestelde eisen voor werkzaamheid, terwijl het vaccin met het laagste niveau aan virulente eiwitten in mindere mate bescherming biedt en niet voldoet aan de wettelijk gestelde eisen. Voor het vaccin met het tussenliggende niveau aan virulente eiwitten, voldeed de werkzaamheid net aan de eisen. Op basis van deze resultaten hebben we geconcludeerd dat deze vaccins een kwaliteit hebben die stapsgewijs verloopt van goed naar slecht.

In **Hoofdstuk 3** en **Hoofdstuk 4** zijn verschillende cellulaire modellen onder de loep genomen en is bestudeerd of en in hoeverre dergelijke methoden in staat zijn vaccins van verschillende kwaliteit van elkaar te onderscheiden. In totaal zijn zes verschillende cellulaire modellen onderzocht die mogelijk een indicatie zouden kunnen geven van activatie van de aangeboren immuun respons of een gedeelte daarvan. Van deze zes modellen zijn er twee ***cellijnen*** die elk één type receptor tot expressie brengen (of humaan TLR2 of humaan TLR4). Dit zijn receptoren die betrokken zijn bij de herkenning van geconserveerde structuren in de kinkhoestbacterie. De vier andere celtypes zijn ***antigeen presenterende cellen***. Dit zijn cellen die betrokken zijn bij de aangeboren immuun respons. Ze presenteren delen van een pathogeen (zogenaamde antigenen) aan het adaptieve immuun systeem. Twee van de onderzochte celtypes hebben we geïsoleerd uit het bloed van donoren (zogenaamde primaire cellen), de andere twee zijn cellijnen die geïsoleerd zijn uit patiënten met een vorm van bloedkanker. Cellijnen zijn veelal

makkelijk in stand te houden in het laboratorium. Alhoewel alle zes modellen reageerden op de wP vaccins, waren drie van de zes modellen in staat het wP vaccin van een goede kwaliteit te onderscheiden van het wP vaccin van een slechte kwaliteit. Deze drie cel modellen - één van de twee cellijnen met een receptor (humaan TLR4), één van de twee primaire celtypes (monocyte derived dendritic cells) en één van de twee bloedkanker cellijnen (MonoMac 6) - reageerden sterker op het vaccin van een goede kwaliteit in vergelijking met het vaccin van een slechte kwaliteit. De reactie op het vaccin van een middelmatige kwaliteit verschilde niet significant van de reactie op het goede vaccin en een onderscheid tussen deze twee vaccins kon dus niet gemaakt worden met behulp van deze modellen. Zoals eerder beschreven kan op basis van de werkzaamheid ook geen onderscheid gemaakt worden tussen het vaccin van een goede en een tussenliggende kwaliteit. Deze resultaten geven dus aan dat het onderscheidend vermogen van de muis beschermingstest en de drie celtypen vergelijkbaar is en de drie cel modellen een veelbelovende en functionele aanvulling kunnen zijn voor de kwaliteitsbepaling van wP vaccins.

In **Hoofdstuk 5** is vervolgens de immuun respons die onze wP vaccins induceren onderzocht in muizen. Hiertoe zijn muizen geïmmuniseerd met dezelfde drie vaccins als eerder onderzocht in de muis beschermingstest, echter zijn de muizen niet geïnfecteerd met virulente kinkhoest bacteriën. Deze studie heeft uitgewezen dat de vaccin kwaliteit geen meetbaar effect heeft op de aangeboren immuun respons in muizen, maar wel effect heeft op de adaptieve immune respons. Immunisatie met het vaccin van een goede kwaliteit heeft in het algemeen een hogere T helper cel respons tot gevolg van een type (Th17/Th1) in vergelijking met de andere twee vaccins. Dit type T helper cel respons is in muizen en in mensen geassocieerd is met een goede bescherming. Naast T cellen, hebben we ook de wP vaccin geïnduceerde antilichaam responsen bestudeerd. Opmerkelijk is dat de kwaliteit van het vaccin geen effect heeft op het antilichaam niveau gericht tegen de hele bacterie, maar wel op het antilichaam niveau gericht tegen bepaalde eiwitten. Ook het patroon aan eiwitten herkend door antilichamen is afhankelijk van het vaccin waarmee geïmmuniseerd is. Beide parameters, T helper cel respons en antilichaam responsen gericht tegen specifieke eiwitten, bieden de mogelijkheid om in een muismodel vaccin kwaliteit te bepalen, zonder dat infectie van de muis en het daarmee gepaarde gaande hoge ongerief (zoals het geval in de muis beschermingstest) noodzakelijk is.

Naast werkzaamheid, wordt ook de veiligheid van vaccins bepaald met behulp van gestandaardiseerde diertesten. Omdat er een substantieel aantal proefdieren omgaat in dit type onderzoek, is in dit proefschrift onderzocht of het mogelijk is een functioneel en proefdiervrij alternatief te ontwikkelen voor één van deze veiligheidstesten, de Histamine Sensitisatie test, uitgevoerd voor het bepalen van PTx in aP vaccins. Eerder onderzoek naar het mechanisme van de Histamine Sensitisatie test heeft uitgewezen dat PTx effect heeft op

gladde spiercellen van bloedvaten, dusdanig dat deze cellen minder goed in staat zijn om samen te trekken indien dat nodig is. Op basis van dit en ander onderzoek, wordt gedacht dat gladde spiercellen een belangrijke rol spelen bij PTx geïnduceerde gevoeligheid voor histamine. **Hoofdstuk 6** beschrijft de ontwikkeling van een proefdiervrije methode gebaseerd op A10 cellen (een gladde spiercellijn), waarbij het effect van PTx gemeten wordt als een verhoging van het molecuul cAMP in de cel. Deze methode is in staat PTx te meten, met een gevoeligheid die vergelijkbaar is met muizen. Vanwege de variatie in resultaten, maar ook vanwege technische en logistieke problemen met de cAMP detectie technieken, is er gezocht naar een alternatieve manier om de hoeveelheid cAMP te bepalen. Hiertoe hebben we in **Hoofdstuk 7** de A10 cellijn, maar ook een tweede PTx gevoelige cellijn (de CHO cellijn) genetisch gemodificeerd. De genetische modificatie heeft als doel dat de vorming van cAMP in de cel, leidt tot een automatisch productie van een bepaald enzym in diezelfde cel. Dit enzym kan vervolgens gemakkelijk gemeten worden. In **Hoofdstuk 7** laten we zien dat deze genetisch gemodificeerde cellen en met name de CHO cellen, erg gevoelig zijn voor PTx en in staat zijn residuen van PTx aan te tonen in aP vaccins, met een gevoeligheid die gelijk is of hoger is dan de Histamine Sensitisatie test. Met name de genetisch gemodificeerde CHO cellen zijn daarmee een veel belovend alternatief voor de huidige diertest. Om te bepalen wat de impact zou zijn indien een dergelijk alternatief geïmplementeerd wordt, is onderzocht hoeveel Histamine Sensitisatie testen er jaarlijks op mondiale schaal uitgevoerd worden en is berekend hoeveel muizen hiervoor ongeveer gebruikt worden (**Hoofdstuk 8**). Daartoe zijn de voornaamste fabrikanten van aP vaccin en betrokken controle autoriteiten benaderd. Deze inventarisatie laat zien dat ongeveer 640 Histamine Sensitisatie testen op jaarlijkse basis uitgevoerd worden, waarbij ongeveer 65000 muizen worden gebruikt. Alhoewel de uitvoering van deze testen gegarandeerd heeft dat een groot deel van de wereld populatie gevaccineerd is met een veilig aP vaccin, maakt de inventarisatie duidelijk dat implementatie van een alternatieve methode voor de Histamine Sensitisatie test een substantiële reductie in dierproeven teweeg kan brengen.

Conclusie en vooruitblik

In dit proefschrift is onderzocht of celkweek methoden ons in staat stellen de werkzaamheid en veiligheid van vaccins te bepalen en of dergelijk methoden een functionele aanvulling kunnen zijn voor de Consistency Benadering. Met wP als model, hebben we laten zien dat – naast fysisch- en immuno-chemische technieken – verscheidene cellulaire methoden een duidelijk onderscheid kunnen maken tussen een vaccin van een goede en een slechte kwaliteit. Dergelijk methoden kunnen daarmee een indicatie geven van vaccin geïnduceerde aangeboren immuun responsen. Alhoewel de activatie van een aangeboren immuun respons essentieel is voor het induceren van een adaptieve immuun respons, is de vorming van adequate adaptieve immuun responsen (d.w.z. een immunologisch geheugen) het uiteindelijke doel bij vaccinatie.

Toekomstig onderzoek dient zich dan ook te richten op cellulaire methoden die indicatief zijn voor adaptieve immuun responsen. Ook zou verder onderzoek betrekking kunnen hebben op de ontwikkeling en evaluatie van dergelijke methoden voor andere vaccins.

Daarnaast hebben we ons bezig gehouden met de ontwikkeling van cellulaire methoden om de veiligheid van aP vaccins te bepalen. We hebben aangetoond dat A10 cellen een gevoeligheid hebben voor PTx die in ieder geval vergelijkbaar is met de gevoeligheid van muizen. In geval van genetisch gemodificeerde CHO cellen is de gevoeligheid zelfs nog hoger dan de diertest. Met name CHO cellen zijn dan ook geschrikt voor het aantonen van PTx in aP vaccins. Additioneel onderzoek beschreven in dit proefschrift laat zien dat met wereldwijd uitgevoerde Histamine Sensitisatie testen, ongeveer 65000 muizen per jaar gebruikt worden voor het bepalen van de veiligheid van aP vaccins. Vervolgonderzoek is dan ook van belang om de genoemde A10 en CHO testen te valideren en – indien relevant – te implementeren.

Dit werk levert een belangrijke aanwijzing dat functionele cellulaire methoden in staat zijn om zowel de kwaliteit als de veiligheid van kinkhoest vaccins te bepalen. Mogelijk zijn deze methoden een goede aanvulling voor de Consistency Benadering. Omdat verwacht wordt dat kinkhoest vaccinatie noodzakelijk blijft, is het van belang om deze benadering in het algemeen en functionele cellulair methoden in het bijzonder verder te ontwikkelen en te valideren zodat het proefdiergebruik voor de kwaliteitscontrole van deze vaccins kan worden afgebouwd.

Verklarende woordenlijst

Aangeboren immuun systeem	Deel van het immuun systeem dat bepaalde geconserveerde structuren van ziekteverwekkers herkend. Het aangeboren immuun systeem reageert snel, maar is vaak aspecifiek.
Adaptieve immuun systeem	Dit deel van het immuun systeem is specifiek en ontwikkelt zich in de loop van de tijd. Door blootstelling van de naïeve cellen van dit systeem aan onderdelen van pathogenen, ontwikkelen de cellen zich op een dusdanige manier dat ze bij een tweede blootstelling op een specifieke wijze snel reageren.
Antigeen presenterende cellen	Cellen van het aangeboren immuun systeem die pathogenen of eiwitten opnemen, verwerken en in stukjes presenteren op hun cel membraan, om de cellen van het adaptieve systeem in staat te stellen het pathogeen te herkennen.
Antilichamen	Dit zijn eiwitten geproduceerd door B cellen die in staat zijn een deel van een ander eiwit te herkennen. Antilichamen kunnen een eiwit of pathogeen onschadelijk maken of beter herkenbaar maken voor cellen van het aangeboren immuun systeem.
B cellen	Immunologische cellen van het verworven immuun systeem die antilichamen produceren.
Cellijnen	Een celcultuur van identieke cellen die buiten het lichaam in kweek gehouden kunnen worden en delen.
Consistency Benadering	Een benadering waarin vaccin kwaliteit bepaald wordt door een combinatie van informatie verkregen uit het productieproces en karakterisatie van tussen- en eindproduct. Met deze benadering wordt gestreefd naar proefdiervrije vrijgifte van een partij vaccin.

Cytotoxische T cellen	Cellen van het adaptieve immuun systeem die herkennen wanneer een cel geïnfecteerd is en zorgen dat deze cellen opgeruimd worden.
Formaldehyde	Molecuul met de formule CH_2O, die in oplossing gebruikt wordt om preparaten te fixeren met als gevolg dat deze preparaten lang houdbaar worden.
Histamine	Dit is een eiwit dat opgeslagen ligt in blaasjes in cellen en vrijkomt tijdens een allergische reactie.
Kinkhoest	Bacterie die de luchtwegen van mensen infecteert. De infectie veroorzaakt kenmerkende hoestbuien en kan gevaarlijk zijn, met name voor jonge kinderen.
$MgSO_4$	Magnesiumzout van zwavelzuur, waarvan proefondervindelijk aangetoond is dat het de genexpressie van kinkhoest bacteriën op een dusdanige manier beïnvloed wordt, dat ze minder virulente eiwitten tot expressie brengen.
Lipopolysaccharide	Grote moleculen die bestaan uit een vetachtig deel en meervoudige koolhydraten. Ze komen voor in de buitenmembraan van veel bacteriën, waaronder de kinkhoest bacterie en hebben vaak een sterk activerende werking op het aangeboren immuun systeem.
Pathogenen	Ziekteverwekker van biologische oorsprong, zoals bacteriën, virussen, parasieten en schimmels.
Pertussis toxine	Een van de toxines die de kinkhoest bacterie produceert. Alhoewel aangenomen wordt dat dit toxine bijdraagt aan de bijwerkingen van wP vaccins, is het ook een eiwit dat belangrijk is voor het induceren van een beschermende immuun respons.

T helper cellen	Cellen van het adaptieve immuun systeem die de stukjes van pathogenen herkennen die door antigeen presenterende cellen gepresenteerd worden. Deze cellen helpen cytotoxische T cellen en B cellen, maar kunnen ook cellen van het aangeboren immuun systeem aantrekken. De T helper cellen zijn onder te verdelen in verschillende subcategorieën (Th1/ Th2/ Th17) die specifieke eiwitten produceren en zo in belangrijke mate de immuun respons beïnvloeden.
Virulente eiwitten	Eiwitten die van belang zijn voor binding van de bacterie aan cellen in de luchtwegen en die bijdragen aan de symptomen van kinkhoest. De eiwitten zijn ook van belang voor het induceren van een (beschermende) immuun respons.
Virulente kinkhoest bacteriën	Kinkhoest bacteriën die in een staat verkeren waarin ze virulente eiwitten produceren.

Curriculum Vitae

Marieke Hoonakker was born on July 2nd, 1985 in Spijkenisse, The Netherlands. After graduating from Stad en Esch (Gymnasium) in Meppel in 2003, she started her study Biomedical Sciences at the Utrecht University. During this part of her study she performed an internship in the Zoo Antwerpen in Belgium on the behaviour and welfare of Spectacled bears. In 2007 she obtained her Bachelors' degree and she continued her study with the Master's programme Behavioural Neuroscience. During her first internship at the Boudewijn Seapark (dolphinarium) in Bruges (Belgium) she studied the general behaviour, welfare and anticipatory behaviour of Bottlenose dolphins. She performed her second internship at the Netherlands Vaccine Institute (NVI), which later became part of the Institute for Translational Vaccinology. During the internship she studied the behaviour of cotton rats and started with the development of the cAMP PTx assay (described in this thesis). In 2009 she obtained her Master's degree. In the same year she started a survey at the NVI on the current status of laboratory animal use and availability and use of 3R methods in vaccine R&D and lot release testing. The results were published in 2011 in a report entitled "Vaccines, Animal experiments and Alternatives". In 2011 she started her PhD study at Utrecht University, but seconded to NVI/Intravacc under the supervision dr. Wanda Han, dr. Arjen Sloots, prof. dr. Coenraad Hendriksen and prof. dr. Willem van Eden, which resulted in this thesis. Besides her PhD position, she is working as a projectmanager at Intravacc, the secretary of the Animal Ethics Committee at Intravacc (since 2014) and member of the Intravacc's animal welfare body (since 2015).

List of publications

Metz B, **Hoonakker ME**, Uittenbogaard JP, Weyts M, Mommen GPM, Meiring HD, Tilstra W, Pennings JLA, Van der Pol LA, Kuipers B, Sloots A, Van den IJssel J, Van de Waterbeemd B, Van der Ark A. Proteome analysis is a powerful tool to evaluate antigen expression during upstream processing of whole-cell pertussis vaccines. Submitted to Journal of Proteome Research.

Hoonakker ME, Verhagen LM, Pupo E, de Haan A, Metz B, Hendriksen CF, Han WG, Sloots A. Vaccine-Mediated Activation of Human TLR4 Is Affected by Modulation of Culture Conditions during Whole-Cell Pertussis Vaccine Preparation PLoS One. 2016 Aug 22;11(8):e0161428.

Hoonakker ME, Verhagen LM, Hendriksen CF, van Els CA, Vandebriel RJ, Sloots A, Han WG. *In vitro* innate immune cell based models to assess whole cell *Bordetella pertussis* vaccine quality: a proof of principle. Biologicals. 2015 Mar;43(2):100-9.

Hoonakker ME, Verhagen LM, van der Maas L, Metz B, Uittenbogaard JP, van de Waterbeemd B, van Els CA, van Eden W, Hendriksen CF, Sloots A, Han WG. Adaptive immune response to whole cell pertussis vaccine reflects vaccine quality: a possible complementation to the Pertussis Serological Potency test. Vaccine. 2016 Aug 17;34(37):4429-36.

Hoonakker ME, Ruiterkamp N, Hendriksen CF. The cAMP assay: a functional *in vitro* alternative to the *in vivo* Histamine Sensitization test. Vaccine. 2010 Feb 3;28(5):1347-52.

Hoonakker ME, Verhagen LM, Van der Maas L, Sloots A, Hendriksen CFM. Reporter cell lines for detection of pertussis toxin in acellular pertussis vaccines as a functional animal-free alternative to the *in vivo* Histamine Sensitization test. Submitted for publication in Vaccine.

Hoonakker ME, Hendriksen CFM. Safety testing of acellular pertussis vaccines: animal numbers and 3Rs Alternatives. Manuscript in preparation.

Bache C, **Hoonakker M**, Hendriksen C, Buchheit KH, Spreitzer I, Montag T. Workshop on Animal free Detection of Pertussis Toxin in Vaccines--Alternatives to the Histamine Sensitisation Test. Biologicals. 2012 Jul;40(4):309-11.

Isbrucker R, Arciniega J, McFarland R, Chapsal JM, Xing D, Bache C, Nelson S, Costanzo A, **Hoonakker M**, Castiaux A, Halder M, Casey W, Johnson N, Jones B, Doelling V, Sprankle C, Rinckel L, Stokes W. Report on the international workshop on alternatives to the murine histamine sensitization test (HIST) for acellular pertussis vaccines: state of the science and the path forward. Biologicals. 2014 Mar;42(2):114-22.

Arciniega J, Wagner L, Prymula R, Sebo P, Isbrucker R, Descampe B, Chapsal JM, Costanzo A, Hendriksen C, **Hoonakker M**, Nelson S, Lidster K, Casey W, Allen D. Alternatives to HIST for acellular pertussis vaccines: progress and challenges in replacement. Report on an international workshop. . Pharmeur Bio Sci Notes, 2016. 2016-04: p. 82-96.

Dankwoord

Het heeft ,iets, langer geduurd dan oorspronkelijk het plan was, maar mijn proefschrift is nu toch echt af! Vijf jaar pipetteren, schrijven en discussiëren hebben geleid tot dit boekje en ik ben erg blij met het eindresultaat. Mijn promotietraject is leerzaam geweest en ik ben blij dat ik de mogelijkheid heb gekregen om deze weg in te slaan. Dit proefschrift was er echter niet geweest zonder jullie steun, input en energie!

Coenraad, allereerst wil ik jou bedanken. In de afgelopen 9 jaar hebben we aan verschillende projecten gewerkt en hebben we elkaar aardig goed leren kennen. Bedankt dat je me de mogelijkheid hebt gegeven om dit promotietraject in te gaan. Je hebt mij in die 9 jaar met raad en daad bijgestaan en ik heb heel veel van je mogen leren. Ik ben erg blij dat je me veel vrijheid hebt gegeven om dit project naar eigen inzicht in te vullen. Alhoewel we niet altijd dezelfde keuzes maakten, heb je me wel altijd gesteund en geholpen en geadviseerd over de meest uiteenlopende onderwerpen. Bedankt voor deze zeer leerzame en leuke periode en je begeleiding!

Lisa, zonder jou was dit alles niet goed gekomen! Je hebt zoveel werk verzet en zo goed ook, dat ik niet goed uit kan drukken hoe blij ik daarmee ben. Naast dat, wist en weet je me soms te overtuigen dat we niet alles uit hoeven te zoeken, zodat experimenten qua omvang nog een beetje behapbaar blijven. Dank je wel voor je inzet en steun! Ik ben erg blij dat je mijn paranimf wil zijn.

En dan mijn copromotoren ***Wanda*** en ***Arjen***. ***Wanda*** bedankt voor je optimisme en kritische blik en je onvoorwaardelijke steun. Ook al waren de resultaten soms moeilijk te duiden, jij wist er altijd iets uit te halen en bleef vertrouwen houden. En ik kon altijd bij je terecht voor vragen of problemen. Bedankt ook voor je kritische blik op onze manuscripten; ik heb op dat gebied veel van je geleerd. ***Arjen***, je rol tijdens mijn promotie is steeds groter geworden. Eerst heb je me wegwijs gemaakt op het lab, terwijl je aan het eind vooral veel tijd en energie hebt gestoken in het verbeteren en schrijven van onze manuscripten. Onze schrijfstijlen verschillen, maar volgens mij is het goed gelukt om daar een middenweg in te vinden en om een aantal mooie manuscripten op papier te zetten. We werken nu nog nauw samen en ik hoop dat dat in de toekomst tot nog meer mooie resultaten zal leiden.

Willem, je bent pas in het laatste jaar betrokken geraakt bij mijn promotie, maar desondanks heb ik veel gehad aan je commentaar en suggesties tijdens de besprekingen en ook aan je kritische blik op de manuscripten en mijn thesis. Dank daarvoor!

Dorothee, we kennen elkaar al vanaf de eerste dag van onze studie, dus dat is al 13 jaar. Ik vind het heel erg leuk dat je op deze dag naast me wil staan als mijn paranimf. Ook wil ik je bedanken voor je steun in de afgelopen maanden en relativeringsvermogen (4 geaccepteerde artikelen is toch genoeg?!?!). Ik ben super blij voor je dat het zo goed gaat met jullie kindje ***Tobias*** en hoop dat ***Tobias***, jij en ***Arne*** het naar jullie zin gaan hebben in Sierra Leone, maar ik ga jullie wel missen!

Lida, mijn steun en toeverlaat als het op de DEC en de IvD aankomt. Ik ben erg blij met onze samenwerking en waardeer je inzet enorm, maar ik ben je ook erg dankbaar voor je luisterend oor en je bereidheid om me te helpen. Bedankt daarvoor! ***Jolanda R.*** en ***Miriam***, samen met ***Lida*** vormen we sinds twee jaar de IvD van Intravacc. Bedankt voor de erg leuke een leerzame samenwerking! Alhoewel de DEC niet direct gelinkt is aan mijn promotie, wil ik toch ook de ***leden van de DEC*** bedanken voor jullie begrip voor mijn dubbele functies en de leuke en prettige DEC vergaderingen. Ook wil ik de collega's van het ARC - de biotechnici, ***Charlotte***, ***Mathijs*** en ***Piet*** - bedanken voor jullie advies tijdens de studies.

Daarnaast wil ik een aantal oud-collega's van het voormalige NVI bedanken. ***Johan*** bedankt voor je advies, steun, maar vooral je geloof en de inzet om alternatieven - op wat voor manier dan ook - een stap verder te brengen. Bedankt ook voor je oneindige optimisme en de vele uitvoerige discussies en gesprekken! ***Marco***, ***Evelien***, ***Mervin***, ***Karlijn*** en ***Frank*** bedankt voor de gezelligheid en ***Evelien*** en ***Mervin*** voor jullie inzet tijdens de CHO cell clustering/PSPT studies! ***Nicole***, toen ik in 2007 via ***Coenraad*** stage kwam lopen bij het NVI was je mijn stage begeleidster, maar we konden het ook buiten het werk goed met elkaar vinden. Bedankt voor de gezelligheid en voor je begeleiding; ik heb heel veel van je mogen leren. Ook wil ik ***Ineke*** en ***Dirk*** bedanken voor de hulp en het leveren van materialen. ***Jeroen de B.***, het was erg leuk en leerzaam om jou te begeleiden.

Tijdens mijn AIO periode heb ik op verschillende afdelingen gezeten en ben ik ook vaak van kamer gewisseld. Ik wil dan ook mijn kamergenoten ***Lisa, Naomi, Marco***, ***Tamar***, ***Jolanda B.***, ***Elise*** en ***Daantje*** bedanken voor de gezelligheid en leuke sfeer. ***Inonge***, succes nog even met de laatste loodjes! ***René***, ***Peter*** en ***Afshin*** bedankt voor jullie gezelligheid en tips en tricks voor het afronden van dit proefschrift. En natuurlijke Coenraads andere angels, ***Marie-Jeanne*** en ***Marsha***, bedankt voor de leuke samenwerking. Daarnaast wil ik ook een aantal andere collega's van het RIVM (afdeling IIV), ***Sietske, Kina***, ***Jeroen H.***, ***Marion***, ***Josien***, ***Harry***, ***Teun*** en ***Jörgen*** bedanken voor jullie hulp, advies en de gezelligheid. ***Ronald*** en ***Jacqueline*** bedankt voor jullie FACS kennis en hulp. ***Betsy*** bedankt voor de vele discussies, je kennis over kinkhoest, de materialen en de tijd die je altijd vrij wist te maken om te helpen. ***Hendrik-Jan*** en ***Elder***, bedankt voor het werk dat jullie hebben verzet voor de LPS analyse en ***Alex*** voor de analyse

van de vetzuren. ***Jeroen P.***, zonder jou was het onmogelijk geweest de microarrays analyse uit te voeren, wat een hele mooie aanvulling is geweest voor één van de artikelen. Bedankt daarvoor! ***Larissa***, dank je wel voor het werk dat je hebt verzet voor 2D elektroforese, maar ook voor je kritische blik op de data en de discussies over de Western blots. ***Wichard*** bedankt voor alle hand en spandiensten! ***Joost*** bedankt voor je flexibiliteit en het uitvoeren van de massa spectometrie analyse. ***Coen***, leuk dat je ons de afgelopen maanden hebt versterkt en bedankt voor je positieve energie tijdens je stage! Ook wil ik Intravacc als organisatie bedanken voor het faciliteren van dit promotieonderzoek. In het bijzonder wil ik ***Jelle***, ***Danielle*** en ***Rob*** bedanken voor hun ondersteuning. Daarnaast wil ik ook ***Arno*** bedanken voor het delen van zijn kennis over kinkhoest en de vele discussies over zowel kinkhoest vaccins als de daarvoor gebruikte diermodellen. ***Elly***, bedankt voor je positieve energie, je steun en je hulp bij het schrijven van 'PTx-cAMP reporter cel' manuscript.

Bas, dat je vaccins nog zo lang en intensief gebruikt zouden worden, had je waarschijnlijk niet gedacht (ik in ieder geval niet!). Die vaccins zijn cruciaal geweest voor bijna alle studies in dit boekje. Dank je wel daarvoor, maar daarnaast ook voor je hulp, ook nadat je niet meer bij het NVI/Intravacc werkte. De vaccins zijn echter niet oneindig houdbaar gebleken, dus toen we aan het einde van mijn studies nieuwe vaccins nodig hadden kwam ***Tim*** in beeld. ***Tim*** heeft me ingewijd in de wereld van de schudflesculturen, helaas niet helemaal met het resultaat wat we hadden verwacht, maar het was wel een leuke en leerzame ervaring. ***Tim*** bedankt voor je inzet en hulp!

Hoe had dit project eruit gezien zonder de klankbordgroep? Ja de klankbordgroep ☺. Het klinkt als iets uit de vorige eeuw, maar het is een select groepje mensen uit verschillende hoeken die mijn promotietraject hebben begeleid en bijgestuurd en die de manuscripten hebben voorzien van kritisch commentaar. Alhoewel we niet zo heel vaak bij elkaar kwamen, waren de vergaderingen een goed moment om even uit te zoomen en de data in een breder perspectief te plaatsen. Bedankt ***Cecile***, ***Arnoud***, ***Bernard***, ***Rob***, en ***Gideon*** voor jullie advies.

During my PhD, Intravacc has participated in a working group called "The pertussis spiked working group", which aims to facilitate the development of alternatives to the HIST. I want to thank all the working group members, but in particular ***Sue***, ***Jean-Michel***, ***Angele***, ***Dave***, ***Leslie***, and ***Johan D***. for the collaboration. Furthermore, ***Juan*** thank you for your criticism and for reviewing the introduction and discussion of this thesis. And of course I also want to thank the colleagues from the Paul Erhlich Institute, ***Cristina***, ***Björn***, ***Jürgen***, ***Ingo*** and ***Thomas***. I enjoyed our collaboration and hope we will keep the same track in the future.

Ondanks dat ik een heel groot gedeelte van mijn PhD door heb gebracht bij het NVI/Intravacc

waren ook de uren buiten werktijd erg belangrijk voor de totstandkoming van dit proefschrift. Bedankt ***Vincent***, ***Nina***, ***Jojanneke***, ***Josje***, ***Prisca***, ***Derek***, ***Bernard***, ***Sophie*** en ***Freek*** voor de gezellige etentjes, feestjes en borrels. Ook dank aan ***Marjo*** voor de welkome sportieve afwisseling, volgens mij ben ik je nog heel veel chocolade repen verschuldigd voor al die keren dat ik te laat kwam voor ons klimavondje omdat ik nog iets af moest maken (of dacht te moeten maken). Sorry dat ik zo weinig tijd voor jullie heb gehad de laatste tijd, ik hoop dat er nu weer wat meer ruimte in mijn agenda komt!

Lief broertje en lieve zusjes. ***Thijs***, naast onze gemeenschappelijke liefde voor lekker eten, zijn we ook allebei verliefd geworden op het boulderen. Hopelijk kunnen we heel lang, heel veel samen boulderen, kamperen, biertjes drinken, lachen en BBQ-en en lekker kissebissen over of een route nu wel of niet makkelijk is als je lang bent. ***Branna*** (=mijn zusje ***Anna*** en haar vriend ***Bram***) bedankt voor alle spelletjesavonden, etentjes, het op ons huis passen als we er niet zijn en ***Anna***, gewoon voor het zijn van mijn kleine/grote zusje. ***Iris***, we zien elkaar niet zo vaak, maar als we elkaar zien -zowel met als zonder ***Tygo***, ***Manuel*** en ***Nick***- is het altijd gezellig. Maar het meeste heb ik misschien nog wel genoten van de momenten dat we allemaal bij elkaar zijn, lekker door elkaar heen praten, eten en lachen.

Yvonne en ***Ibrahim***. Ik bof met zulke lieve schoonouders, die oprecht geïnteresseerd zijn, zonder wie ons huis er echt heel anders uit had gezien en die altijd - met liefde - even heen en weer rijden naar Utrecht. Ik wil jullie vooral ook bedanken voor jullie geduld, hulp en de gezellige middagen en avonden. Ock nu mina föräldrar. Den är färdig, min doktorsavhandling. Jag hoppas at ni tycker om det och at jag lyckas at klargöra var det handlar om. ***Mamma***, tack så hemskt mycket för din ovillkorlig bistånd, kärlek och at jag när som helst kan ringa dej. ***Pappa***, du också, tack så hemskt mycket för din tålamod, hjälp ock kärlek. ***Oma***, dank je wel voor je begrip dat ik zo weinig bij jou en ***opa*** ben geweest de afgelopen jaren. Helaas kan ***opa*** er niet bij zijn, maar desondanks vind ik het heel erg leuk dat jij er op deze speciale dag wel bij bent.

En dan tot slot… Lieve lieve ***Jeffrey***, zonder jou was ik niet zover gekomen. Er zijn zoveel dingen op deze wereld die we allebei leuk vinden om te doen en te zien en we lijden allebei aan het probleem dat we alles willen doen. Met als gevolg dat er permanent te weinig tijd lijkt te zijn…. De afgelopen jaren zijn niet altijd makkelijk voor ons geweest, maar ik ben heel erg blij dat het ons gelukt is om samen een weg te vinden en vooral om samen ook heel veel plezier te hebben. Dank je wel voor je geduld, je porren in m'n zij, je grapjes en je liefde. Je bent echt de allerliefste.