

From THE DEPARTMENT OF MICROBIOLOGY, TUMOR
AND CELL BIOLOGY
Karolinska Institutet, Stockholm, Sweden

B-CELL RESPONSES AFTER PERTUSSIS VACCINATION

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“For myself I am an optimist, it does not seem to be much use being anything else.”

Sir Winston Churchill



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B-cell responses after pertussis vaccination

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ABSTRACT

Despite years of vaccination efforts, whooping cough, or pertussis, is not under control. Although immunization has greatly reduced the disease incidence, thousands of children die of whooping cough each year. Therefore, there is an urgent need to improve the available vaccines as well as our understanding of the immunological mechanisms required for optimal protection against pertussis. The focus of this thesis was on B-cell responses after pertussis vaccination within the scope of two clinical trials.

The B-cell ELISpot protocol was optimized to ensure sensitive detection of B-cell responses. In paper I, the superiority of the novel protocol was demonstrated with regard to B-cell activation, detection sensitivity, antigen consumption, and assay time in comparison to an established protocol.

The first trial evaluated the safety and immunogenicity of a novel live, attenuated, whole-cell vaccine named BPZE1 in a phase 1 clinical setting (reported in papers II and III). This vaccine strain is genetically modified and is designed for intranasal administration and a subsequent nonpathologic infection in the immunized subjects. The second trial was a phase 4 booster study of a fifth consecutive dose of an acellular pertussis vaccine in adolescents. Memory B-cell and serological responses are reported in paper IV. In both studies, strong serological and B-cell responses were detected with ELISA and ELISpot. Colonization was crucial for the BPZE1 study, whereas both antigen content and concentration influenced the responses in the booster study.

Immunity against pertussis is still not fully understood. Evaluating several parameters of the immune response will give a better understanding of the immunological activities following pertussis infection or vaccination. More knowledge will enable better vaccines and contribute to the control of pertussis.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Kikhosta är en sjukdom som kännetecknas av frekventa och svåra hostanfall som avslutas med en kiknande inandning. Kikhosta kallas även för pertussis, vilket betyder svår hosta på latin (*per* - svår, *tussis* - hosta). Sjukdomen orsakas av bakterien *Bordetella pertussis* och drabbar alla åldrar, dock är ovaccinerade spädbarn värst drabbade.

Vaccination är ett effektivt skydd mot kikhosta. Den globala introduktionen av kikhostevaccinet på 1950-talet ledde till en dramatisk minskning av sjukdomen. Det vaccin som användes var ett så kallat helcellsvaccin och bestod av hela avdödade *B. pertussis* bakterier. På 1980-talet utvecklades en annan typ av vaccin, det acellulära, som endast består av enstaka komponenter av bakterien och ger mindre biverkningar.

Människans skydd mot kikhosta är kortvarigt, varken naturlig infektion eller vaccination ger ett livslångt skydd mot kikhosta. En naturlig infektion anses dock ge längre skydd än ett vaccin och helcellsvacciner anses generellt vara mer effektiva än acellulära vacciner. Individer som har genomgått en infektion eller som har vaccinerats får vanligtvis inte svår kikhosta om de smittas utan en mildare form av sjukdomen som kanske inte ens diagnostiseras eller uppmärksammas. Detta leder till att infekterade personer inte är medvetna om sin smittsamhet, vilket blir problematiskt om de har nära kontakt med ovaccinerade barn. Att öka skyddet för spädbarn och att öka vaccineffektiviteten har därför hög prioritet för att minska fallen av allvarlig och dödlig kikhosta.

Trots hög vaccinationstäckning har man de senaste åren internationellt sett en ökning av kikhosta med flera dödsfall som följd. Det verkar därför som om de acellulära vaccinerna vi idag använder inte fungerar tillfredsställande. En effektivisering av nuvarande vacciner och vaccinationsprogram är därför efterfrågad. För att bättre förstå hur detta ska göras måste kunskapen om immunförsvarets roll ökas. För att få en bättre bild av detta har vi utvärderat svaret, som en viss typ av immunceller (så kallade B-celler) ger efter vaccination mot kikhosta. B-celler skyddar mot infektioner genom att utsöndra antikroppar. När antikropparna binder till mikroorganismen så neutraliseras den, det vill säga mikroorganismen hindras från att orsaka infektion.

I avhandlingen är två kliniska studier av kikhostevaccin inkluderade. Först utvecklade och utvärderade vi ett metodprotokoll för att mäta antalet B-celler i blod, en så kallad B-cells ELISpot, för att försäkra oss om att vi skulle få pålitliga resultat. Vårt nya protokoll visade sig vara snabbare och bättre på att påvisa B-celler än ett redan etablerat protokoll, så i fortsättningen användes bara det nya protokollet.

Den första kliniska studien som genomfördes innehöll ett nytt vaccin vid namn BPZE1. I studien prövades vaccinet för första gången på människor och vaccinets säkerhet och förmåga att aktivera immunförsvaret studerades. Den andra kliniska studien utvärderade B-cellssvaret efter en femte dos av två olika typer av acellulärt kikhostevaccin i ungdomar för att mäta dess effektivitet och säkerhet. Skillnaden mellan de två vaccinerna var antalet bakteriekomponenter och deras koncentration. En grupp fick ett vaccin med en komponent medan den andra gruppen fick fem komponenter i sitt vaccin. I båda studierna kunde vi se ett starkt B-cellssvar, vilket visar på att B-cellerna aktivt bidrar till det immunsvar som ses efter vaccination.

Trots år av vaccination är kikhostans spridning inte under kontroll och runt 200 000 barn i världen dör av sjukdomen varje år. Effektivare vaccin och vaccinationsprogram kan ge befolkningen bättre skydd och minska smittorisken för spädbarn. Ett annat tillvägagångssätt är att vaccinera spädbarnen och på så sätt mer direkt skydda dem mot smitta. Det nya vaccinet BPZE1 är tänkt att så småningom ges till spädbarn vid födseln för att så tidigt som möjligt kunna skydda dem mot kikhosta. Det kommer att dröja innan vi har full förståelse för hur vi bäst kan skydda oss mot kikhosta, men med tålamod och forskning kommer vår insikt om hur vi bäst löser problemet öka.

LIST OF SCIENTIFIC PAPERS

- I. **Maja Jahnmatz**, Gun Kesa, Eva Netterlid, Anne-Marie Buisman, Rigmor Thorstensson, and Niklas Ahlborg
Optimization of a human IgG B-cell ELISpot assay for the analysis of vaccine-induced B-cell responses.
Journal of Immunological Methods 391(2013) 50-59
- II. Rigmor Thorstensson, Birger Trollfors, Nabil Al-Tawil, **Maja Jahnmatz**, Jakob Bergström, Margaretha Ljungman, Anna Törner, Lena Wehlin, Annie Van Broekhoven, Fons Bosman, Anne-Sophie Debie, Nathalie Mielcarek, and Camille Locht
A phase I clinical study of a live attenuated Bordetella pertussis vaccine – BPZE1; A single centre, double-blind, placebo-controlled, dose-escalating study of BPZE1 given intranasally to healthy adult male volunteers.
PLOS ONE 2014 Jan 8;9(1):e83449
- III. **Maja Jahnmatz**, Sylvie Amu, Margaretha Ljungman, Lena Wehlin, Francesca Chiodi, Nathalie Mielcarek, Camille Locht, and Rigmor Thorstensson
B-cell responses after intranasal vaccination with the novel attenuated Bordetella pertussis vaccine strain BPZE1 in a randomized phase I clinical trial.
Submitted manuscript.
- IV. **Maja Jahnmatz**, Margaretha Ljungman, Eva Netterlid, Maria C Jenmalm, Lennart Nilsson, and Rigmor Thorstensson
Pertussis-specific memory B-cell and humoral IgG responses in adolescents after a fifth consecutive dose of acellular pertussis vaccine.
Manuscript in preparation.

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LIST OF ABBREVIATIONS

ACT	Adenylate cyclase toxin
APC	Antigen-presenting cell
ASC	Antibody-secreting cell
BCR	B-cell receptor
cfu	Colony-forming unit
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DTaP	Diphtheria, tetanus, and acellular pertussis vaccine
DTwP	Diphtheria, tetanus, and whole-cell pertussis vaccine
FHA	Filamentous hemagglutinin
Fim2/3	Fimbriae serotypes 2 and 3
GC	Germinal centre
IDMC	Independent data monitoring committee
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
LLPC	Long-lived plasma cell
MHCII	Major histocompatibility complex class II
MMR	Measles, mumps, and rubella vaccine
Pa	Acellular pertussis
PAMP	Pathogen-associated molecular pattern
PRN	Pertactin
PRR	Pattern recognition receptor
PT	Pertussis toxin
Pw	Whole-cell pertussis
SLO	Secondary lymphoid organ
Tdap	Full-dose tetanus and low dose diphtheria and pertussis vaccine
T _{FH}	Follicular helper T cell
T _H	Helper T cell
TLR	Toll-like receptor

1 AIMS

The general aim of this thesis was to investigate the impact of pertussis vaccines on B-cell responses. The studies mainly focused on memory B cells, but plasmablasts and serological antibody levels were also evaluated.

The specific aims of the papers were as follows:

1. To optimize and validate a novel B-cell ELISpot protocol for high-sensitivity B-cell detection (paper I).
2. To evaluate the immunogenicity of the novel live, attenuated *Bordetella pertussis* vaccine strain BPZE1 in a phase 1 clinical trial with regard to serological, memory B-cell, and plasmablast responses (papers II and III).
3. To assess the impact of a fifth consecutive dose of an acellular pertussis (Pa) vaccine on memory B-cell and serological responses in adolescents (paper IV).

2 STUDIES INCLUDED – AN OVERVIEW

To facilitate the reading of the thesis, the studies included are summarized here.

Optimization of a novel B-cell ELISpot assay (paper I)

The sensitivity of a novel IgG B-cell ELISpot protocol was evaluated to ensure that an optimal B-cell ELISpot protocol was used for this research. This project was undertaken in collaboration with the Swedish biotech company Mabtech AB.

The activating cocktail included in the novel protocol (R848 and interleukin [IL] 2) had previously been described but not directly compared to some of the well-established B-cell activators. To assess the performance of the assay, the novel protocol and an established protocol were run in parallel to detect the antigen-specific response in eight subjects. Because these two protocols utilize two different detection systems – monoclonal antibodies for the novel protocol and polyclonal antibodies for the established protocol – a separate evaluation of this parameter was also performed. The functionality of the novel assay was established by detection of several different antigen-specific responses as well as by an evaluation of an alternative, less antigen-consuming detection system.

It was shown that the R848 and IL-2 cocktail was more efficient at activating B-cells compared to the other activators tested. The novel protocol displayed a significantly better detection sensitivity compared to the established protocol, despite shortened B-cell activation time and reduced antigen consumption. Moreover, the novel protocol was easily adapted to five antigens, confirming its functionality.

B-cell responses in a phase 1 clinical trial of the novel live, attenuated vaccine strain BPZE1 (papers II and III)

The novel live, attenuated *Bordetella pertussis* (*B. pertussis*) vaccine strain BPZE1, developed by a French group, was evaluated for the first time in humans in a phase 1 clinical trial performed at the Swedish Institute for Communicable Disease Control (now the Public Health Agency of Sweden) in collaboration with the Karolinska Trial Alliance (KTA) at Karolinska University Hospital in Huddinge. The primary aim of the study was safety, and the secondary aim was immunogenicity.

The study was designed as a placebo-controlled, dose-escalating, double-blind, randomized trial that included 48 subjects divided into three groups. The vaccine was given intranasally in droplets with 10^3 , 10^5 , and 10^7 colony-forming units (cfu) of BPZE1 per dose. Each group included 12 subjects that received the active vaccine (low, medium, or high dosage) and 4 subjects that received a placebo. None of the subjects had received a previous pertussis vaccination, but they were not considered naïve due to pertussis circulation in the population.

An extensive safety follow-up was performed, and nasopharynx samples from all subjects were cultured for BPZE1 to determine culture positivity and colonization time. Blood samples were collected on days 0, 7, 14, and 28 and at 5-6 months after vaccination for the determination of immunological responses. Serum IgG levels and number of IgG-secreting plasmablasts and memory B cells were evaluated.

The first-in-man study of BPZE1 showed that intranasal administration of a live, attenuated *B. pertussis* vaccine strain was safe for human use. Colonization was dose-dependent and strong pertussis-specific antibody and B-cell responses were detected in subjects colonized by the bacteria.

Memory B-cell and serological responses after a fifth consecutive dose of acellular (Pa) pertussis vaccine in adolescents (paper IV)

In an adolescent booster study, the immunogenicity of a fifth consecutive dose of a Pa vaccine was evaluated. The study was an open-label, randomized, multicentre study with blinded analysis. Adolescents aged 14–15 years who had previously participated in a Pa vaccine clinical trial during the 1990s as well as a trial evaluating the safety and efficacy of a Pa booster at 5½ years of age were recruited to the study [5, 6]. All had received a Pa vaccine consisting of five components of the bacteria at all four previous occasions.

In total, 230 subjects were recruited to the study and randomized into groups that received a Pa vaccine consisting of either one or five components of the bacteria. Of the 230 subjects, 34 had available samples for memory B-cell analysis; 18 of these subjects had received the 1-component vaccine and 16 had received the 5-component vaccine. Blood samples were collected on two occasions (day 0 and day 28–42), and three antigen-specific memory B-cell responses were evaluated.

A fifth dose of an acellular pertussis vaccine induced significant increases of pertussis-specific antibody levels as well as memory B-cell levels in the 34 included adolescents. The antigen-content and concentration did however influence the vaccine-induced response.

3 VACCINATION – A BRIEF INTRODUCTION

The first vaccine was developed more than 200 years ago [7], and since its discovery vaccines have saved millions of lives. Immunization prevents approximately 6 million deaths each year [8] and is one of the most effective methods we have for disease prevention.

The principle of immunization is to induce a strong immunological memory without producing a harmful and potentially fatal infection in the individual. During an infection, the immune system forms a pathogen-specific immunological memory that will protect against subsequent infections. Depending on the pathogen, this protective memory can last from years to a lifetime. Vaccination utilizes this immunologic capacity and introduces the host immune system to the pathogen under controlled, nonpathologic circumstances. This enables the immune system to evoke a protective memory without the host being afflicted with the disease.

3.1 VACCINES

Today, vaccines against a wide array of pathogens are available. Vaccines are classified into different types: live, attenuated; inactivated or killed; toxoid; subunit or acellular; and conjugate (for examples, see Table 1).

Live, attenuated vaccines consist of weakened (attenuated) forms of the pathogens that is able to replicate without causing disease. *Inactivated vaccines* contain the killed, non-replicating form of the pathogen. These two types are known as whole-cell vaccines. Inactivation is usually obtained by chemical treatment or irradiation to create a non-replicating pathogen with an intact surface structure that can interact with the immune system. Attenuation is generally obtained using serial passage in cell lines, but the development of molecular techniques has allowed more controlled genetic mutations and deletions to be used to attenuate pathogens.

Toxoid vaccines consist of a detoxified toxin (toxoid) that is used against bacteria whose toxin is the sole cause of illness. Detoxification is usually done through chemical treatment that does not affect the ability of the toxoid to interact with the immune system. The toxoid vaccine induces an immune response specific for the naturally occurring toxin.

Subunit, or acellular, vaccines are similar to the toxoid vaccines in that they only contain purified parts of the pathogen and not the entire organism. Adhesion proteins or

other surface structures and toxoids are common antigens included in such vaccines. Acellular vaccine components are poorly immunogenic by themselves, and the vaccines must include adjuvants to evoke a strong pathogen-specific immunity.

Conjugate vaccines are used to vaccinate small children against polysaccharide-coated bacteria. The immature immune system of children does not respond well to these types of bacteria. Therefore, the polysaccharide is conjugated to a protein to induce a strong immune response.

Table 1. Different vaccine types and examples of available vaccines.

Vaccine type	Includes	Available vaccines
Live, attenuated	Whole organism	Measles, mumps, rubella, polio (oral polio vaccine), influenza (intranasal), varicella, rotavirus, tuberculosis (BCG)
Inactivated	Whole organism	Polio (inactivated polio vaccine), hepatitis A, pertussis (whole cell)
Toxoid	Detoxified toxin	Diphtheria, tetanus
Subunit/acellular	Components of the pathogen	Hepatitis B, pertussis (acellular), HPV ^a , influenza (parenteral)
Conjugate	Protein-conjugated polysaccharide	Hib ^b , Streptococcus pneumoniae, Neisseria meningitidis

^aHuman papillomavirus, ^b*Haemophilus influenzae* type b

3.2 CHILDHOOD IMMUNIZATION PROGRAM

Currently, the World Health Organization (WHO) recommends a global routine childhood immunization program that includes vaccines against 12 different pathogens [9]. Depending on the nation-specific disease burden, this program is adapted to provide optimal protection in each country. In Sweden, nine of these vaccines are included in the general childhood vaccination program (see Table 2). Protection against mumps is also offered to all children in combination with measles and rubella (the MMR vaccine). The Swedish vaccination program also offers tuberculosis (BCG), hepatitis B, and influenza vaccines to children in high-risk groups. Rotavirus vaccination will also be implemented in the near future in some counties. The adherence to the national childhood vaccination program in Sweden is excellent, and more than 98% of the children are vaccinated [10].

Globally, vaccines are well accepted, and vaccination coverage is around 80% in the world today [11]. High vaccination coverage is vital for maintaining herd immunity in the population, which offers protection to unvaccinated individuals. Some individuals cannot be immunized due to medical conditions such as immunodeficiency, but there are also some people that choose not to be vaccinated due to various beliefs. Anti-vaccinationists have existed since the invention of vaccines. A cautionary example is the Wakefield study [12] in which MMR vaccination was falsely connected to autism. Although this article was retracted by the journal [13], it led to a widespread decrease of MMR vaccination in children and subsequent measles outbreaks due to loss of immunity in the population as seen, for example, in Ireland [14]. This demonstrates the fragility of our protection against vaccine-preventable diseases and is a reminder that many of the vaccine-targeted pathogens are still a threat. Therefore, it is crucial to maintain high vaccination coverage because this also protects those who cannot be vaccinated.

Table 2. Comparison of the general childhood vaccination schedule in Sweden with WHO recommendations.

Vaccine against	Recommended by		The general Swedish childhood vaccination schedule (by vaccination age) ^e							
	WHO	Sweden	3 m	5 m	12 m	18 m	5-6 y	6-8 y	10-12 y	14-16 y
Tuberculosis	Yes	To high-risk groups								
DTP ^a	Yes	Yes	I	II	III		IV			V ^f
Polio	Yes	Yes	I	II	III		IV			
Hib ^b	Yes	Yes	I	II	III					
Pneumococci	Yes	Yes	I	II	III					
MMR ^c	Only measles and rubella	Yes				I		II		
HPV ^d	To girls only	To girls only							I+II+III	
Rotavirus	Yes	In some counties								
Influenza	Certain immunization programs	To high-risk groups								
Hepatitis B	Yes	To high-risk groups								

^a Diphtheria, tetanus, and pertussis; ^b *Haemophilus influenzae* type b; ^c Measles, mumps, and rubella; ^d Human papilloma virus; ^e m: months, y: years; ^f will be implemented in 2016.

Modified from the Public Health Agency of Sweden [15] and www.who.int [9].

3.3 CLINICAL TRIALS

Before any vaccine can be introduced, it must undergo rigorous safety and efficacy testing both in pre-clinical models and in humans. Human trials are referred to as clinical trials, and they are highly regulated to protect the participants of the study.

Before initiation, all clinical trials must be approved by the national Medical Products Agency and regional ethical review boards. Following approval, all clinical trials are required to register in an open international database (e.g., www.clinicaltrial.gov) [16] that provides public access to the study. Additionally, an Independent Data Monitoring Committee (IDMC) can be contracted to further ensure the safety and scientific integrity of the study. The IDMC consists of independent researchers with experience in the study field. The members advise on whether the study should be stopped prematurely due to risk of harm to the participants or because the study is not likely to reach its scientific goal. In the BPZE1 study (paper II), an IDMC was included to exclude any bias in the vaccine's safety evaluation. To ensure the safety of the participants, the IDMC was provided with data from one dose group before it approved continuation with the next, higher, dose group. The IDMC also ruled that the only case of a severe adverse event in the BPZE1 study was not vaccine related.

Ensuring participants' safety is the key issue in all clinical trials. Enrolment in a clinical study is voluntary, and all participants are free to discontinue participation at any time. Before enrolment, subjects must receive written and oral information about the risks and benefits of entering the trial, their rights, and their requirements as participants. Following the distribution of the oral and written information, they sign an informed consent form to indicate that they have received the information and are willing to participate in the study. No subject can be included in a clinical trial without signing the informed consent form.

Clinical trials are divided into different phases based on how far the new drug has come in its development. There are four phases of clinical trials [16], and these are summarized in Table 3. However, the criteria (stated in the table) can be adapted to suit the purpose of a specific trial and are, therefore, reasonably flexible. For instance, the BPZE1 study (papers II and III) included a placebo group, which usually is not a prerequisite for a phase 1 trial. The booster study (paper IV) was classified as a phase 4 study because the included vaccines were available on the market. However, the subjects were recruited from a specific cohort (previous participation in two specified clinical trials), and this does not strictly adhere to phase 4 criteria.

Clinical trials are also classified according to their masking and allocation. Trials can be open, single-blind, or double-blind in terms of masking and randomized or nonrandomized in terms of allocation [16]. In an open study, both the participant and the trial investigator know what type of vaccine or placebo the participant is given. In a single-blind study, either the participant or the trial investigator is blinded to what treatment is given, and in a double-blind study neither knows the intervention. The intervention code in double-blind trials is kept by a third party and can be broken prematurely following certain criteria that are set before the initiation of the trial. In randomized trials, the participants are allocated to the different study groups by chance [16]. Including masking and allocation reduces the risk of bias in the study and increases the reliability of the collected data.

From beginning to end, a clinical trial is a time-consuming process. Due to all of the safety precautions and regulations, taking a new vaccine from the laboratory into the market requires many years and costs enormous amounts of money. However, solid investigation of a new vaccine in an increasing number of subjects ensures a safe evaluation of its risks and benefits. Maintaining the quality of clinical trials is essential for obtaining new and safe treatments.

Table 3. General objectives and aims of clinical trials in different phases.

Phase	Objective	Number of subjects	Aims
1	Safety	<100	Identify adverse events. For novel vaccines, immunogenicity studies are often included.
2	Safety and efficacy	100–300	Compare efficacy (and safety) of new interventions with placebo and/or existing interventions.
3	Confirmation of safety and efficacy	>1000	Obtain more information on safety and efficacy in larger, more diverse populations.
4	Long-term safety and efficacy		Post-marketing studies. Additional information on safety and efficacy under less controlled settings. “Real-life” responses.

Modified from [17].

4 IMMUNOLOGY

The human body is constantly exposed to microbes; some of these are essential for our survival, but others are pathogenic. Our immune system is highly evolved to strike a balance between tolerating commensal microbes and fighting pathogens, and this homeostasis is carefully regulated. The immune system consists of several different cell populations, and each contributes alone and together with other cell types for efficient protection. Without this cooperation, we would quickly succumb to the numerous microbes found in our environment.

4.1 THE INNATE IMMUNE SYSTEM

Innate immunity is often described as a first line of defence against invading pathogens. The cells and the effector molecules of the innate response circulate in a functional state and are ready to rapidly respond to an invading pathogen. This local innate response can be sufficient to prevent infection, but adaptive immunity is usually required as well.

The innate immune system is activated by recognition of different pathogen-associated molecular patterns (PAMPs) expressed or secreted by pathogens as well as by recognition of self-molecules produced by damaged or dying cells (damage-associated molecular patterns [DAMPs]) [2]. These PAMPs and DAMPs are recognized by a large number of different pattern recognition receptors (PRRs) that exist as both cell membrane-bound receptors and soluble receptors in the cytoplasm. Toll-like receptors (TLRs) are a group of PRRs that have been extensively studied. Nine well-characterised members of the TLR family are expressed by humans, either at the cell surface (TLRs 1, 2, 4, 5, and 6) or in the endosomes (TLRs 3, 7, 8, and 9). The cell surface-associated TLRs recognise extracellular PAMPs and DAMPs, and the endosome-associated TLRs recognise PAMPs from phagocytosed pathogens. Upon binding, the TLRs activate different intracellular signalling cascades that ultimately lead to increased pro-inflammatory responses.

Cells that belong to the innate immune system contribute to the rapid clearance of invading pathogens, but these cells also work as part of the adaptive immune system. The macrophages and dendritic cells (DCs) of the innate system are so-called professional antigen-presenting cells (APCs). They scan the peripheral tissues in search of PAMPs, and upon encountering them they internalize the foreign antigen and present it in context with the major histocompatibility complex class II (MHCII) molecules. The APCs then travel to secondary lymphatic organs (SLOs), such as the spleen or lymph nodes, where they present the antigen and activate cells of the adaptive immune system.

4.2 THE ADAPTIVE IMMUNE SYSTEM

Whereas the cells of the innate immune system respond non-specifically to PAMPs, the adaptive immune system is a highly specialized system where each cell has unique antigen specificity. Initially, the adaptive response is slower than the innate response, but it provides a long-term pathogen-specific immunologic memory once established. The adaptive immune system normally consists of two responses: the cell-mediated immune response and the humoral immune response.

4.2.1 T cells

T cells play a central role in the cell-mediated immune response. They are divided into several different subgroups, each with different effector functions. Two major groups are $CD4^+$ T cells and $CD8^+$ T cells. The major function of $CD8^+$ T cells is to kill infected cells after recognising foreign antigen expressed in context with MHC class I molecules by nucleated cells.

The $CD4^+$ helper T cells (T_H) are the regulators of the adaptive immune system [2]. They are activated by the recognition of an antigen bound to MHCII molecules on APCs. Activated $CD4^+$ T cells orchestrate the adaptive immune response by up-regulating co-stimulatory molecules and secreting cytokines. $CD4^+$ T_H cells are further divided into subgroups, including T_{H1} and T_{H2} cells, regulatory T cells (T_{reg}), and T_{H17} cells. The different subgroups secrete different sets of cytokines, and each set will in turn facilitate the activation of a specific immune response. The main cytokine of the T_{H1} -type of response is interferon gamma ($IFN-\gamma$), which induces a response against intracellular pathogens. T_{H2} cells secrete IL-4 and IL-5, which initiate a response against extracellular pathogens. T_{reg} cells promote immune tolerance to inhibit excessive immune responses, and T_{H17} cells are pro-inflammatory and associated with autoimmune diseases [18].

A more recent discovery is the $CD4^+$ follicular helper T cells (T_{FH}). They are the mediators of the humoral immune response and have been found in both mice and humans (reviewed in [19]). They reside in SLOs in close contact with B cells. By secreting different cytokines and co-stimulatory molecules, they promote B-cell survival and proliferation as well as affinity enhancement of the humoral response.

4.2.2 B cells

B cells are the principal cells of the humoral response, and they exert their function through their effector molecules, antibodies. Antibodies, or immunoglobulins (Ig), are the secreted form of the B-cell receptor (BCR) and are unique to each B cell. The immense capacity of the B-cell population to produce antibodies with different antigen specificities is the key to the humoral response's ability to combat new invading pathogens throughout one's lifetime.

4.2.2.1 Antibodies

Antibodies are the effector molecules of B cells. There are five classes of antibodies: IgM, IgD, IgG, IgA, and IgE. IgG is the most abundant serotype, constituting approximately 65% of all the antibodies in sera, and has the longest half-life (9–23 days). IgA is the predominant antibody in mucosal secretions. It is secreted as a dimer and is essential for toxin and pathogen clearance at the mucosal surfaces. IgA can also be found as a monomer in the blood.

4.2.2.2 B-cell development – an introduction

To reach full maturation and function, the B cell undergoes multiple developmental stages. Each B cell expresses a unique BCR with a specific antigen-binding capacity, and the affinity of this binding determines the efficacy of the antibody response towards the pathogen. The B cells are derived from the bone marrow but migrate to the SLOs where they undergoes further development and maturation (schematically described in Figure 1).

4.2.3 Memory B cells and long-lived plasma cells

Memory B cells and long-lived plasma cells (LLPCs) both express BCRs with high affinity and represent the classical components of the humoral response. LLPCs are secreted as immature plasmablasts but mature and migrate to the bone marrow where they continuously secrete antibodies that maintain the serological protection against pathogens. Memory B cells home to SLOs but can also be detected in peripheral blood. The memory B-cell pool is the source of the humoral anamnestic response that is characterized by the rapid increase of antigen-specific antibodies detected after reinfection. Upon reactivation, they either differentiate into antibody-secreting cells (ASCs) or re-enter the germinal centre reaction, which increases the antigen-specific B-cell pool. With increasing age and with re-exposures, the memory B cells accumulate [20, 21], and detectable memory B cells are found many years after infection or vaccination [22-24].

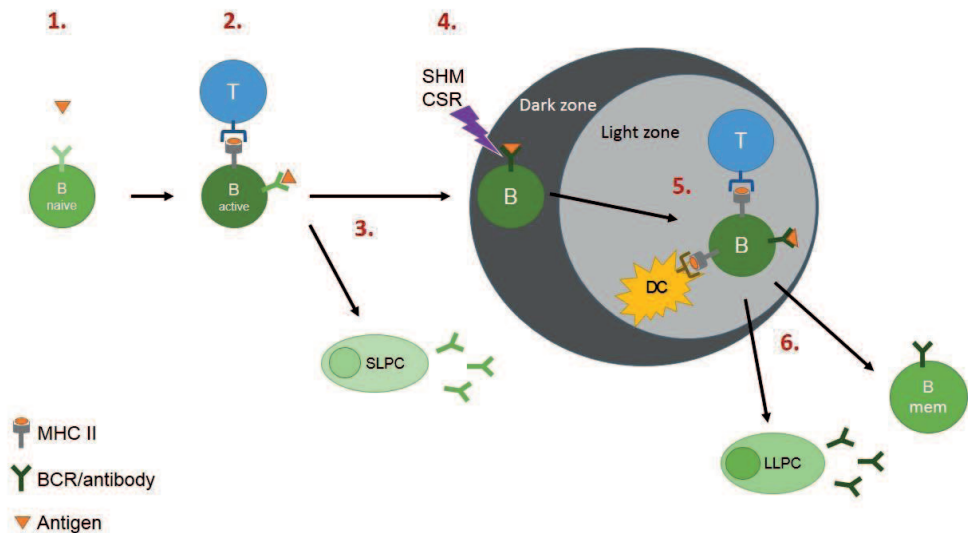


Figure 1. B-cell development and maturation.

1. In the secondary lymphoid organs (SLO), the naïve B cell is activated by recognition of its cognate antigen.
2. Activation leads to internalization and MHCII presentation of the antigen as well as upregulation of receptors that promote follicular helper T-cell (T_{FH}) interactions and further activation.
3. Following activation, the B cell can either enter into the extrafollicular pathway or the germinal centre (GC) reaction. The extrafollicular pathway is the origin of short-lived plasma cells (SLPC) that produce antibodies with low affinity.
4. In the dark zone of the GC reaction, two central processes in B-cell maturation take place: somatic hypermutation (SHM) for antibody affinity maturation and antibody class switch by class-switch recombination (CSR).
5. Following SHM and CSR, the B cell migrates into the light zone of the GC reaction where it further interacts with T_{FH} and follicular dendritic cells and undergoes additional antigen-dependent selection. High-affinity binding promotes survival, so poorly binding B cells are selected against.
6. B cells with high affinity antibodies leave the GC reaction as either a nonsecreting memory B cell going to SLOs or as an antibody-secreting long-lived plasma cell (LLPC) going to the bone marrow. Adapted from [2].

The humoral response has an extraordinary capacity for maintaining pathogen-specific protective antibody levels throughout a lifetime without any requirements for subsequent exposure to the pathogen or for booster vaccination [23, 25]. How this constant level is maintained is under debate. One theory suggests that the LLPCs are constantly being replenished in the bone marrow by an on-going cycle of activation and differentiation of memory B cells into LLPCs. The other theory proposes two separate paths for the memory B-cell and the LLPC responses where the two B-cell subsets act and contribute to immunity independently of each other.

In human studies that have measured the long-term presence of serum antibody and memory B-cell levels present in the blood, low or no correlation has been detected [23, 25, 26]. Other studies have also shown that memory B cells can be found in individuals without any detectable serum antibody levels with the same antigen specificity [27-29], and this supports the idea of two independently maintained populations of memory B cells and LLPCs. This theory is further strengthened by reports that antigen-specific serum antibody levels are maintained despite lack of antigen stimulation in both mice and humans [23, 30, 31] and that depletion of memory B cells has not been shown to affect the serum levels in mice [32-34]. However, one human study has found a correlation between long-term vaccine-induced memory B-cell and serum IgG responses, but the same study also detected subjects with antigen-specific memory B cells and no serum IgG [22]. This shows that the true relationship between the two B-cell responses has yet to be established.

4.2.4 Methods for detection of B-cell responses

The antigen-specific cellular B-cell responses and the serological antibody responses reported in this thesis were measured by ELISpot and ELISA, respectively. Put simply, both methods are based on a similar principle in which antibodies are identified by binding to their specific antigen and subsequent detection steps. Furthermore, this thesis also includes a flow cytometry analysis reported in paper III.

4.2.4.1 B-cell ELISpot

The B-cell ELISpot (enzyme-linked immunosorbent spot) method was first described by Czerkinsky et.al. in 1983 [35] and is now used for evaluation of an array of different antigen-specific ASCs. ELISpot can be applied to both plasma cells and memory B cells, but detection of memory B cells requires activation and differentiation of the memory B cells into ASCs. This is achieved by a prestimulation step with different B-cell activators. These activators are usually polyclonal, but specific antigens can also be added to the stimulation cocktail.

In the optimization and evaluation study of the novel B-cell ELISpot protocol reported in paper I, we ensured that the most optimal activation cocktail was used. Poor activation and differentiation of memory B cells into ASCs leads to under-reporting of the true number of antigen-specific ASCs present in the sample. Available B-cell activators target different receptors on the B cell, and several B-cell activation cocktails have been described [22, 36-38]. Pinna et al. [39] previously showed the potential of the combination of the TLR7/8 agonist R848 and IL-2 as a B-cell activator. In our study, we confirmed its superior induction of total IgG-producing cells compared to several other known B-cell activators (summarized in Table 4). We also found that the high activating potency of the cocktail enabled a decrease of the prestimulation time to 72 hours from the 6 days recommended by others [36, 37, 39]. This led us to conclude that the combination of R848 and IL-2 was the most optimal activator cocktail to use in the B-cell ELISpot protocol.

Table 4. Included B-cell activators in paper I and their effector molecule type.

B-cell activator	Type of effector molecule
R848	TLR7/8 agonist
CpG oligodeoxynucleotide (ODN)	TLR9 agonist
PWM ^a	Lectin/TLR agonist
SAC ^b	BCR cross-linking/TLR agonist
BAFF ^c	Cytokine
IL-2, IL-6, IL-21, IL-10	Cytokine
Anti-CD40	CD40 ligand

^a Pokeweed mitogen, ^b *Staphylococcus aureus* protein A, ^c B-cell activating factor

The basic principle of the established B-cell ELISpot protocol is shown in Figure 2A. The optimized B-cell ELISpot protocol uses an amplified detection system with monoclonal detection antibodies (Figure 2B). We found that the monoclonal detection system further increased detection sensitivity as reported in paper I.

We confirmed the overall performance of the novel protocol in a direct comparison with an established protocol [22]. A significant increase in the tetanus toxoid response in eight recently vaccinated subjects was detected with the novel protocol but not with the established protocol. Thus, the optimized protocol detects responses that otherwise would go undetected. The new protocol was easily adapted to several different antigens, and it readily detected both memory B cells and plasmablasts. We demonstrated its functionality and utility in papers III and IV.

In conclusion, the novel B-cell ELISpot protocol demonstrates a sensitive detection of antigen-specific ASCs, and the recent development with biotinylated antigens (paper I and [40]) shows that the assay can be further improved. In addition, the utility of the assay has been expanded for multiplex analysis through the development of the FluoroSpot protocol [41, 42].

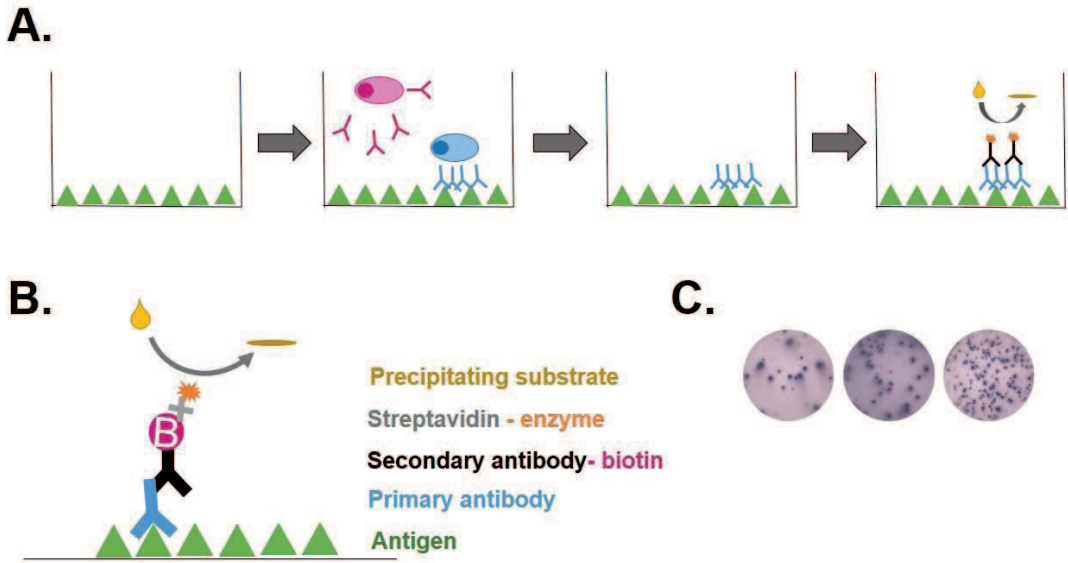


Figure 2. The B-cell ELISpot protocol.

A. The basic principle of the B-cell ELISpot. Multiscreen plates are coated with antigen. The antibody-secreting cells are added to the well and left overnight to secrete antibodies. Only the antibodies specific to the coated antigen will bind. Cells and nonspecific antibodies are then washed away to leave only antigen-specific primary antibodies bound to the membrane. Primary antibodies are detected with an enzyme-conjugated secondary antibody followed by the addition of substrate and subsequent precipitation on the well membrane. A spot forms on the well membrane for each single cell. Based on the number of spots, the number of antigen-specific B cells in the sample can be counted.

B. Amplified detection system. In paper I, an alternative detection system was used. This system utilizes two detection steps that include a secondary antibody conjugated to biotin followed by streptavidin-enzyme binding. This extra step amplifies the signal and enables more sensitive detection.

C. Examples of well images from the B-cell ELISpot amplified detection system.

4.2.4.2 ELISA

ELISA (enzyme-linked immunosorbent assay) is used to measure antibody levels in all types of fluids. A coating of either antigen or antibody captures the antibodies in the sample, and this is followed by detection with an enzyme-conjugated secondary antibody and colour conversion by substrate cleavage. The absorbance value of the sample is proportional to the amount of antibodies in the sample and is calculated by relating it to a reference with a known antibody concentration. The absorbance values are converted into either ELISA units/mL (EU/mL) or international units/mL (IU/mL) depending on the type of reference sera used.

There are three different types of ELISA: direct, indirect, and capture (also known as sandwich). They are all based on the same principle but vary in their detection system (see Figure 3). Generally, the capture ELISA is considered the most sensitive and specific followed by the indirect and finally the direct ELISA.

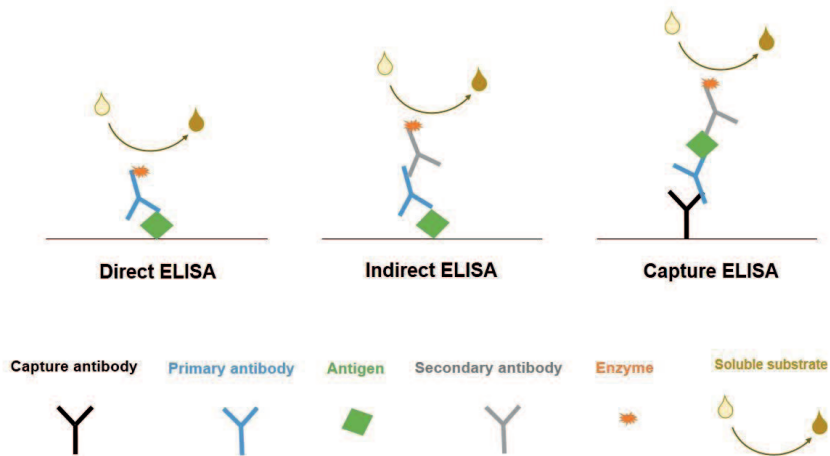


Figure 3. Different detection systems utilized by the ELISA method.

We used the indirect serum IgG ELISA in this thesis (papers II–IV). This method is accredited and has been used since 1992 when it was developed for laboratory use in collaboration with the Food and Drug Administration. Standardized methods and control sera enable comparison of different studies.

A more novel approach to the ELISA is the multiplex immunoassay (e.g., Luminex) [43, 44]. This method allows for the detection of antibodies of multiple antigen specificities in the same sample because antigen-coated beads are added to the sample instead of adding the sample to a well. This enables complex analyses on small sample volumes, for instance, when analysing blood samples from small children.

4.2.4.3 Flow cytometry

Flow cytometry is a multifaceted laser-based method that enables multiplex analysis of different cell populations within seconds. By labelling intracellular or surface components with fluorescent markers, the flow cytometer can detect and sort cell populations by their unique compositions of emitted light on a single-cell level.

In this thesis, flow cytometry was used to distinguish different B-cell subpopulations following BPZE1 vaccination in a few subjects (paper III). Changes in expression of surface molecules during cellular maturation allow for identification of different B-cell subpopulations. To get an in-depth view of the response induced by the BPZE1 vaccination, two flow cytometry panels were used (Table 5). This enabled detection of different memory B-cell subsets and class-switched B cells. B cells were first separated from the live peripheral blood mononuclear cell fraction by gating for CD19⁺, a universal marker for B cells. CD19 is expressed by B cells from early development but is lost in plasma cells. Further staining for CD27, CD21, and CD10 enabled identification of resting, activated, and tissue-like memory B-cell subsets (panel I). In panel II, CD27 and IgD staining was included to separate unswitched and switched memory B cells.

The analysis of the subjects in the BPZE1 study indicated that the vaccine induced the activated memory and the tissue-like memory types of B cells. This is encouraging because the tissue-like memory type would indicate the peripheral response sought by the vaccine. It was not clear if the detected response was, in fact, pertussis-specific because no antigen specificity was included in the flow cytometry analysis. Antigen-specific detection of B cells by flow cytometry is possible [45], and including this in the flow cytometry panels in future studies will offer more complex and comprehensive results from different immune responses.

Table 5. The two flow cytometry panels used to identify B-cell subpopulations in paper III.

Panel I		Panel II	
B cells	CD19 ⁺	B cells	CD19 ⁺
All memory B cells	CD19 ⁺ CD27 ⁺	All memory B cells	CD19 ⁺ CD27 ⁺
Transitional	CD19 ⁺ CD10 ⁺	Switched	CD19 ⁺ CD27 ⁺ IgD ⁻
Naïve	CD19 ⁺ CD10 ⁻ CD21 ⁺ CD27 ⁻	Unswitched	CD19 ⁺ CD27 ⁺ IgD ⁺
Activated memory	CD19 ⁺ CD10 ⁻ CD27 ⁺ CD21 ^{low}	Naïve	CD19 ⁺ CD27 ⁻ IgD ⁺
Resting memory	CD19 ⁺ CD10 ⁻ CD27 ⁺ CD21 ⁺	Double negatives	CD19 ⁺ CD27 ⁻ IgD ⁻
Tissue-like memory	CD19 ⁺ CD10 ⁻ CD27 ⁻ CD21 ⁻		

5 INDUCING IMMUNITY WITH VACCINES

In a recall antigen-specific response to a pathogen, the body utilizes two lines of immune defence (reviewed in [46]) as shown in Figure 4. For a vaccine to be efficient, both types of defences must be evoked. Moreover, the vaccine must induce the type of immune response that will give the best protection against the pathogen the vaccine is targeting, for example, a T_H1 -type of response for an intracellular organism. The immunological stimuli of the vaccine in combination with the local cytokine milieu will trigger different types of immune responses (reviewed in [46, 47]). The DCs will secrete different sets of cytokines depending on what PAMP/DAMP stimuli they are exposed to. Consequently, the vaccine content has a major influence on the type of immune response evoked. For whole-cell vaccines, the pathogen determines the response, but for acellular vaccines, the choice of adjuvants controls the type of immune response that will be evoked.

5.1 ADJUVANTS

Adjuvants are stimulatory substances that are included in vaccines to stimulate the uptake and presentation of the vaccine antigen. Originally, they were thought to function as passive depots for antigens that prolong antigen exposure to the immune system. Nowadays it is clear that adjuvants are important not only for inducing the immune responses but also for directing them. Adjuvants target the innate immune system [48] by functioning as PAMPs or DAMPs, and almost all PRRs are potential targets (reviewed in [49]). There are several on-going trials for new adjuvants [50], but there are only a few approved for human use: aluminium salts (alum), AS04, and oil-in-water emulsions (MF59, AS03).

Alum is the most well-known adjuvant and has been used for many years. Alum exerts its immunomodulatory effect by activation of the inflammasome and release of the pro-inflammatory cytokine IL-1 [51-53]. It has been shown to induce a T_H2 -skewed type of response [54]. AS04 is another adjuvant that contains alum in combination with the TLR4 agonist monophosphoryl lipid A. In contrast to alum, this adjuvant induces a T_H1 -type of response [55].

MF59 and AS03 are the oil-in-water emulsions that are approved for human use. They are based on squalene oil and induce a mixed T_H1/T_H2 -type of response [49]. The complete mechanisms of these adjuvants are not yet known, but studies have shown that MF59 broadens the antibody response and increases the magnitude and the functionality of the antibodies [56, 57].

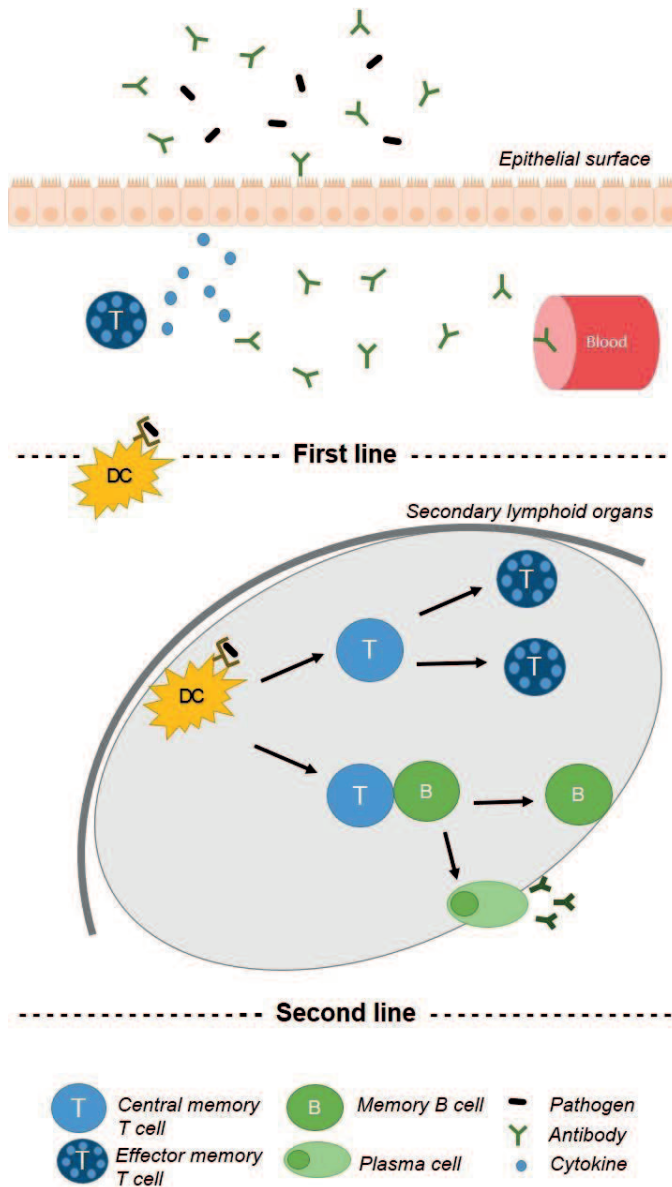


Figure 4. First and second lines of immune defence in an antigen-specific recall response. Following a primary infection, the immune system has two lines of defence against a re-invading pathogen. The first line functions to prevent attachment of the pathogen and initiation of the infection. It consists of ready-made antigen-specific antibodies present at the mucosal surface and in the circulation and peripheral antigen-specific effector T cells. However, if this line of defence fails, the second line consisting of central memory T and memory B cells is activated. The memory cells then rapidly proliferate and differentiate to prevent disease progression. *Modified from [4].*

5.2 PARENTERAL AND MUCOSAL ADMINISTRATION

Vaccines can be administered either parenterally (intramuscular, subcutaneous, or intradermal) or via the mucosa. The majority of vaccines are given parenterally, but oral polio and cholera vaccines as well as an intranasal influenza vaccine are available.

Homing of lymphocytes is primed at the site of activation [58], which suggests that the choice of administration route should reflect the desired local immune response (i.e., a mucosal administration for a mucosal response). Nevertheless, parenteral immunization has been shown to induce a strong mucosal response [46]. This could be explained by the finding that T cells activated in the spleen display promiscuous homing patterns and can home to the mucosa after activation [59]. Vaccine-induced neutralizing serum IgG has also been shown to transude over the mucosa to contribute to the vaccine-elicited protection [60]. However, the contribution of serum IgG has been shown to vary at different mucosal sites [61-63].

From the response evoked by BPZE1, it is evident that mucosal administration can induce a systemic IgG response with both serum antibody and memory B cells (paper II and III). The colonization of the attenuated vaccine strain offers a prolonged exposure of the pathogen to the immune system and the induction of a strong immune response. Mucosal administration has several advantages over parenteral-administered vaccines, such as needle-free administration, and the pursuit of mucosal vaccines should be prioritized.

6 BORDETELLA PERTUSSIS

Whooping cough, or pertussis, is caused by the small gram-negative coccobacillus *Bordetella pertussis* [64]. The disease is characterized by mucus-rich coughing attacks that end with a whooping inhalation. The first report of a pertussis epidemic is dated to 1578 (as stated in [65]), but the microbe was not identified until the early 20th century. It is named after its discoverer, Jules Bordet, and the Latin word for severe cough (*per* – severe, *tussis* – cough). There are eight identified species of *Bordetella*, and four of them have been found in humans [64]:

- *B. pertussis*, a strict human pathogen that infects the upper respiratory tract.
- *B. parapertussis*, which causes a milder form of pertussis disease in humans.
- *B. bronchiseptica*, which is most commonly found in animals such as dogs but has also been found to colonize humans.
- *B. holmesii*, a rare cause of sepsis.

6.1 PATHOGENESIS

B. pertussis is sensitive and cannot survive for long outside its host. It is strictly aerobic and is spread by infectious aerosol droplets from coughing. It is hard to isolate and cultivate and requires a special growth medium containing charcoal and blood. *B. pertussis* was first believed to be an extracellular bacterium but has been shown to persist intracellularly ([66] and reviewed in [67]). The bacterium expresses different adhesins that mediate bacterial attachment to epithelial cells in the respiratory tract, including filamentous hemagglutinin (FHA), pertactin (PRN), and fimbriae (Fim) (Figure 5). In addition, FHA and PRN also promote intracellular uptake and survival of the bacteria [64, 68].

B. pertussis also expresses different toxins that contribute to its pathogenesis [64]. Pertussis toxin (PT) is the only toxin specific to the pathogen, and it consists of five different subunits. The majority of the subunits enable binding of the protein, but the first (S1) exerts the toxicity. PT increases mucus production, which is a classical sign of pertussis. Adenylate cyclase toxin (ACT) has an effect similar to PT, but it also inhibits immune responses, which is an effect thought to be crucial for the establishment of the infection. Dermonecrotic toxin (DNT) causes vasoconstriction and is believed to contribute to the infection-inflicted tissue destruction. Tracheal cytotoxin (TCT) is an important contributor to the pertussis pathogenesis because it inhibits the cilia movement in the respiratory tract.

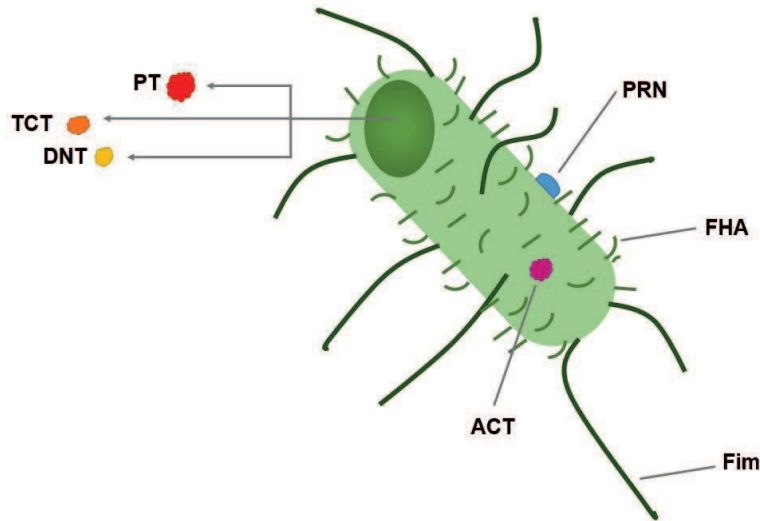


Figure 5. Virulence factors of *Bordetella pertussis*. Toxins: pertussis toxin (PT), tracheal cytotoxin (TCT), dermonecrotic toxin (DNT), and adenylate cyclase toxin (ACT). Adhesion proteins: pertactin (PRN), filamentous hemagglutinin (FHA), and fimbriae (Fim). Modified from Alison Weiss, ASM News, 1997.

For the BPZE1 vaccine used in papers II and III, the aim was to mimic a natural infection without causing disease. Therefore, in the attenuation of the novel BPZE1 vaccine strain it was crucial to reduce the pathogenicity without losing immunogenicity. To achieve this, three toxins were genetically altered or removed [69]. The DNT was deleted, and the toxic S1 subunit of PT was mutated by two point mutations into an inactive toxin. TCT secretion was depleted by exchanging the ineffective *B. pertussis* AmpG transporter protein with the functional *ampG* gene from *Escherichia coli*. This reduced the TCT production by more than 99%. These alterations eliminated the pathogenicity of the infection (as shown in mouse studies) without changing the morphology of the bacteria, its adherence capability, or its *in vitro* growth rate [69] thus fulfilling the aim of inducing a nonpathologic infection.

6.2 CLINICAL DISEASE

Pertussis is highly infectious and affects all people of all ages. It is estimated that 16 million cases occur globally each year with 95% of the cases found in developing countries [70]. This number is most likely an underestimation because studies have shown that pertussis is an underdiagnosed disease [71-73]. The severe and fatal cases are found in the infant population, and close to 200,000 children are estimated to die of pertussis each year [74]. This does not include children who succumb to fatal secondary infections.

A classical pertussis infection has three clinical stages, and it can take months for the affected individual to fully recover [64]. The incubation time between infection and the first signs of disease is quite long, 7–10 days. The catarrhal stage manifests with symptoms similar to a common cold, and the highest number of bacteria exists during this stage. This makes the individual highly contagious at the same time that the infection is usually mistaken for a cold, and this delays the precautions that should be taken to limit the spread of infection. The catarrhal stage is followed by the paroxysmal stage during which the classical pertussis symptoms manifest with coughing attacks followed by a whooping inhalation. At this stage, the bacterial toxins have inhibited cilia movement and increased the mucus production that lead to accumulation of mucus and restriction in the airways. The lack of cilia movement prevents airway clearance, and this initiates the coughing spasms. The spasms are often followed by the patient vomiting mucus. These attacks can be as frequent as 40–50 times a day leaving the patient exhausted. After 2–4 weeks, the paroxysmal stage recedes and the convalescent stage begins.

Treatment against whooping cough is mostly palliative because the disease is usually diagnosed at a late stage when the bacteria are already cleared. *B. pertussis* is, however, sensitive to macrolides such as erythromycin, and early treatment with antibiotics can lead to a milder disease progression if the infection is detected in time.

6.2.1 Pertussis case definitions

Pertussis can be classified according to several different criteria into the two major groups of clinical pertussis and laboratory-confirmed pertussis. As the names indicate, the former are confirmed by clinical manifestation and the latter are confirmed by laboratory findings. In general, a case is referred to as a probable case if only the clinical criteria are met; confirmed cases also require laboratory verification [75].

According to the WHO criteria for surveillance of pertussis, a clinical case is defined as more than 2 weeks of cough with one or more of the following symptoms: paroxysmal

cough, whoops, and/or post-coughing vomiting. Laboratory confirmation requires isolation of *B. pertussis*, a positive polymerase chain reaction (PCR) result, or positive pair serology [76]. The US Centers for Disease Control have a similar definition of the clinical disease, and for laboratory confirmed pertussis isolation of *B. pertussis* or positive PCR is accepted [75]. The differences between criteria (reviewed in [77]) hamper comparison between studies, and a harmonization of the criteria would be beneficial. Because pertussis disease is known to manifest differently with age, it has been suggested that the criteria should be age-specific [77].

6.3 VACCINATION AGAINST PERTUSSIS

Vaccines against pertussis have been available for more than 60 years and have led to a major decrease in pertussis incidence with a great reduction in severe and fatal cases [78]. Despite this, pertussis is still universally present and circulates in the population with cyclic peaks every 2–5 years [79].

Those with a previous pertussis infection and those who have been vaccinated generally experience a milder and subclinical infection rendering the majority of them unaware of their disease [80]. Therefore, it is thought that the pertussis reservoir is harboured by adults and adolescents, and studies have shown that parents and other close relatives are the main source of pertussis transmission to infants [81-83]. An Australian study showed that more than 60% of the cases in a pertussis epidemic occurred in those aged ≥ 10 years [84], which indicates that the major disease burden lies in the older age groups. Nevertheless, unvaccinated infants are the most vulnerable to pertussis, and the majority of the severe cases are found in this group [78, 85]. A Swedish study reported that as many as 72% of infected unvaccinated infants below three months of age had to be hospitalized due to pertussis-related complications [1].

The first pertussis vaccines introduced in the late 1940s consisted of killed whole-cell bacteria and greatly reduced the pertussis incidence and mortality. However, whole-cell pertussis (Pw) vaccines have been associated with higher reactogenicity, and this led to the development of acellular pertussis (Pa) vaccines in the 1980s [86]. Acellular vaccines are available with up to five antigens of *B. pertussis*: detoxified PT (PTd), FHA, PRN, and Fim serotypes 2 and 3 (Fim2/3). Both vaccine types are recommended by the WHO [87]. Pa and Pw are given in combination with diphtheria and tetanus as part of the DTaP and DTwP vaccinations, respectively. Vaccination schemes vary between nations, but in general 3 or 4 doses are given during the first year of life. This scheme is often complimented with one or two booster doses that contain a low-dose diphtheria and pertussis vaccine (Tdap) [88].

6.3.1 Booster vaccinations

Several studies reported a short-term efficacy of the priming doses after the introduction of Pa vaccines in the 1990s, and this led to a recommendation for a preschool booster [89-93]. There has also been a shift in pertussis incidence towards older age groups, and an increase has been reported in adolescents [93-95]. Therefore, the adolescent Tdap booster has been suggested in order to reduce the disease burden in that population. An adolescent booster is safe [96-103], induces long-lasting immunity [97, 104, 105], and successfully lowers the incidence of pertussis in that age group.

The introduction of an adolescent booster was also thought to have an impact on the incidence of infant pertussis. However, one study investigating infant pertussis found a much larger transmission from other age groups than adolescents [106], and another group reported that the yearly peak for adolescent pertussis incidence does not coincide with the infant peak [83]. Moreover, a British contact study found very little interaction between infants and adolescents [107]. This raises questions about the effect that an adolescent booster will have on the infant pertussis incidence. Two studies investigating this have reported contradicting results. One study concluded that the introduction of the adolescent booster was “partially effective in preventing pertussis hospitalizations among infants” [108]. However, in contrast, another study concluded that the adolescent booster did not influence the infant pertussis incidence [109]. Although the adolescent booster has a positive effect on reducing pertussis incidence in that age group, the benefits for infant pertussis are not clear.

Adolescents generally experience mild and asymptomatic pertussis infections, and a natural infection at that age is most likely well tolerated and would induce a longer lasting immunity compared to vaccination. The risk with the short efficacy of an adolescent Pa-booster is that it could postpone the susceptibility to pertussis into childbearing years and thus increase the risk of transmission to infants (discussed in [91, 110]). In the future, a more controlled and safer approach to the natural infection could be to introduce the BPZE1 vaccine as an adolescent booster. With the close resemblance to a natural infection, the BPZE1 vaccine could offer immunity similar to a natural infection without the disadvantages of a wild-type infection.

6.3.2 Pertussis vaccination in Sweden

Sweden introduced Pw vaccines into the national childhood vaccination scheme in the early 1950s. However, Pw vaccination was discontinued in 1979 due to increasing reports of low vaccine efficacy and severe side effects, and this led to a rapid resurgence of pertussis in the population [111, 112]. With the development of the Pa vaccine, the safety profile of pertussis vaccination improved and vaccine efficacy trials were

performed in Sweden during the vaccine-free period [6, 113-115]. These studies confirmed the efficacy and safety of the Pa vaccines, and pertussis vaccination was reintroduced in 1996. The introduction led to a significant decrease in pertussis in the country [1] as shown in Figure 6. However, the 17-year hiatus led to a unique, natural immunity in the Swedish unvaccinated population when compared to other countries with uninterrupted pertussis vaccination programs. For the BPZE1 study, only subjects born during the non-vaccinating time period were included (paper II).

Laboratory reported pertussis cases in Sweden 1989-2012.

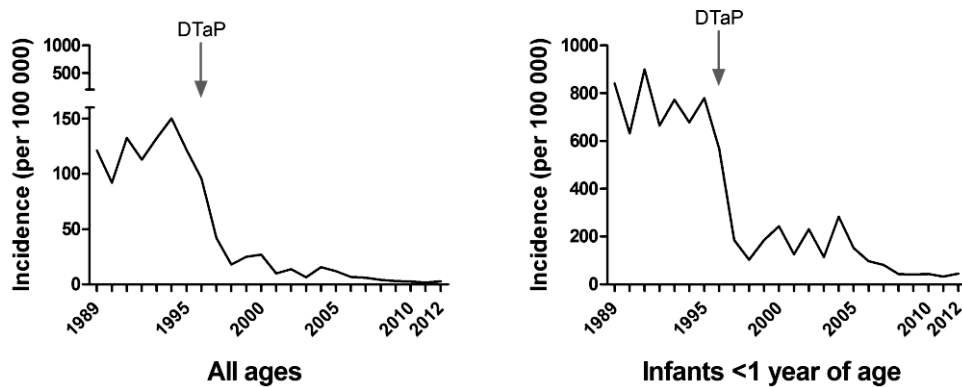


Figure 6. Incidence of laboratory-reported pertussis cases in Sweden between 1989 and 2012 in all age groups (left) and in infants (right). The arrow indicates the introduction of the acellular pertussis vaccine in combination with diphtheria and tetanus (DTaP). Modified from [1].

Currently, Sweden employs a pertussis vaccination scheme that consists of three doses of either the two- or three-component vaccine at 3, 5, and 12 months. This is followed by a preschool booster dose at 5–6 years (introduced in 2007 after a safety and efficacy study [5]). In 2016 a school-leaving booster at 14–16 years of age will be introduced. Preceding the introduction of the adolescent booster, a safety and efficacy study was performed in Swedish adolescents. Specific safety data from Swedish adolescents are important because the effect of the hiatus in pertussis immunization on the population's immunity was not clear. Some of the data obtained in this study are reported in paper IV.

Sweden consists of 21 different counties that all implement the national immunization program. The vaccine brand that is used, however, is independently decided. Thus different pertussis vaccines are used in the country, and the brand varies over time within the same county. The available pertussis vaccines in Sweden are listed in Table 6. All vaccines are given as combination vaccines, but only the pertussis antigens are listed for simplicity. In paper IV, a booster vaccine named diTekiBooster was used (for the contents of the vaccine, see Table 7 in §7.1). This vaccine is not available on the Swedish market but was approved for use in the trial.

Table 6. Available pertussis vaccines in Sweden.

Manufacturer	Brand name	PTd ^a	FHA ^a	PRN ^a	Fim2/3 ^a
S.P. ^b	Tetravac®/Pentavac®/Hexyon	25	25	-	-
	Triaxis® ^d	2.5	5	3	5
GSK ^c	Infanrix®	25	25	8	-
	Boostrix® ^d	8	8	2.5	-

^a Concentration of antigen (µg/0.5-mL dose), ^b Sanofi Pasteur, ^c GlaxoSmithKline, ^d Low-dose vaccine

6.4 RESURGENCE OF PERTUSSIS

In the last few years, there have been major pertussis outbreaks with several infant mortalities in countries with high vaccination coverage [116-118]. Waning immunity, pathogen adaptation, and better detection of the infection due to more sensitive diagnostics have been suggested to contribute to the increase in confirmed cases ([119, 120] and reviewed in [121]). The global change to Pa vaccines from Pw vaccines is thought to be the major cause of resurgence (as discussed in [122]). A study using a novel baboon pertussis model [123] showed that even though Pa vaccines protected against disease, vaccinated animals were still colonized by the bacteria to the same extent as naïve animals [124]. Importantly, the Pa-vaccinated animals were also shown to transmit pertussis to naïve animals thus identifying a weakness of Pa vaccines. Convalescent animals were fully protected against colonization, but the Pw-vaccinated baboons were also colonized. They, however, cleared the infection more quickly compared to Pa-vaccinated animals. Furthermore, a study of *B. bronchiseptica* in its natural host (mice) showed that even though the Pw vaccination does not prevent colonization, it blocks bacterial shedding and transmission of the disease [125].

In humans, Pw-primed children are less susceptible to pertussis than Pa-primed children [126]. Children receiving a mixed Pw/Pa vaccination schedule were also better protected if the first dose was Pw vaccine instead of Pa vaccine [127, 128]. The poor protective efficacy of Pa vaccines is, therefore, most likely to contribute to the present resurgence of pertussis. The pertussis vaccines available today must be improved to stop the resurgence of pertussis and the resulting severe and fatal cases [116, 126, 127].

7 PROTECTION AGAINST PERTUSSIS

A natural infection does not offer life-long protection against pertussis but instead induces a longer lasting protection compared to immunization. A Swedish seroprevalence study performed 10 years after the introduction of Pa vaccines, suggested that natural immunity lasts for approximately 15 years, but the protection gained after three doses of Pa vaccine lasts only for approximately 5 years [91, 129].

7.1 VACCINE EFFICACY

Pw vaccines are generally considered more effective than Pa vaccines [130-132]. The efficacy of Pa vaccines is determined by the number of components they contain. Vaccines including three or more components (PT, FHA, and PRN, with or without Fim2/3) have higher efficacy compared to 1- or 2-component vaccines ([133, 134] and reviewed in [135, 136]).

There are many Pa vaccines on the international market, but there is no consensus concerning antigen concentration or adjuvants (see [137] for examples). In the booster study (paper IV), two different vaccines were included with varying numbers of antigens and antigen concentrations (see Table 7). As expected, the number of components influenced the response, and the adolescents responded only against the antigens included in the booster dose. The antigen concentration also influenced the magnitude of the immune responses induced by the vaccine.

The great impact that pertussis vaccines have on disease incidence is widely known. However, determination of the optimal type of vaccine is hampered by the variety in multiple factors such as the vaccine content, schedule, follow-up, and case criteria used in the different efficacy studies. Environmental factors such as natural immunity and under-reporting of pertussis introduce bias into the evaluations. In summary, Pa vaccines are efficient at reducing pertussis incidence, and a single dose of Pa vaccine has been shown to reduce the risk of severe disease in infants [138]. They also have an excellent safety profile with fewer side effects than Pw vaccines. However, the Pw vaccines are considered more efficacious (reviewed in [70, 136]), but their usage is impeded by their reactogenicity. This indicates that with improvement of the Pw vaccine safety profile this type of vaccine could be more efficient at inducing a strong immune response against pertussis.

Table 7. Vaccines included in the booster study.

Antigen (per 0.5 mL)	Tdap5 ^b (TRIAXIS®)	Tdap1 ^c (diTekiBooster)
Tetanus toxoid	5 Lf ^a	6.25 Lf
Diphtheria toxoid	2 Lf	6.25 Lf
Pertussis toxoid	2.5 µg	20 µg
Filamentous hemagglutinin	5 µg	-
Pertactin	3 µg	-
Fimbriae 2 and 3	5 µg	-
Adjuvant	Aluminium phosphate (0.33 mg Al)	Aluminium hydroxide hydrate (0.5 mg Al)

^a Lf: Limit of Flocculation, ^b 5-component pertussis vaccine, ^c 1-component pertussis vaccine

7.2 IMMUNE RESPONSES

Cells belonging to the innate immune system help by controlling the pertussis infection (reviewed in [139]) and DCs and macrophages are detected early in the infection. Macrophages contribute to the protective immunity against pertussis and engulf and destroy the bacteria. Depending on the stimuli, DCs have been shown to induce T_H1-, T_H17-, and T_{reg}-cell responses in pertussis infections and thus play an important role in modulating the immune response ([140, 141] and reviewed in [139]).

Natural pertussis infections and Pw vaccines induce similar T_H1-type responses [142-145], but Pa vaccines induce a more mixed T_H2/T_H1-type response [146-150] (Figure 7). T_H1-type responses mediated by IFN-γ are important for resolving pertussis infections, and mice lacking IFN-γ are highly susceptible to lethal pertussis infections [151]. The T_H2-type of response seems to play a minor role in protection against natural infection. Children infected with pertussis do not induce a T_H2-type response [145], and in mice antibodies offer no major impact on bacterial clearance [152]. However, antibodies are thought to contribute to immunity by neutralization, opsonisation, and inhibition of pathogen binding to the host [153].

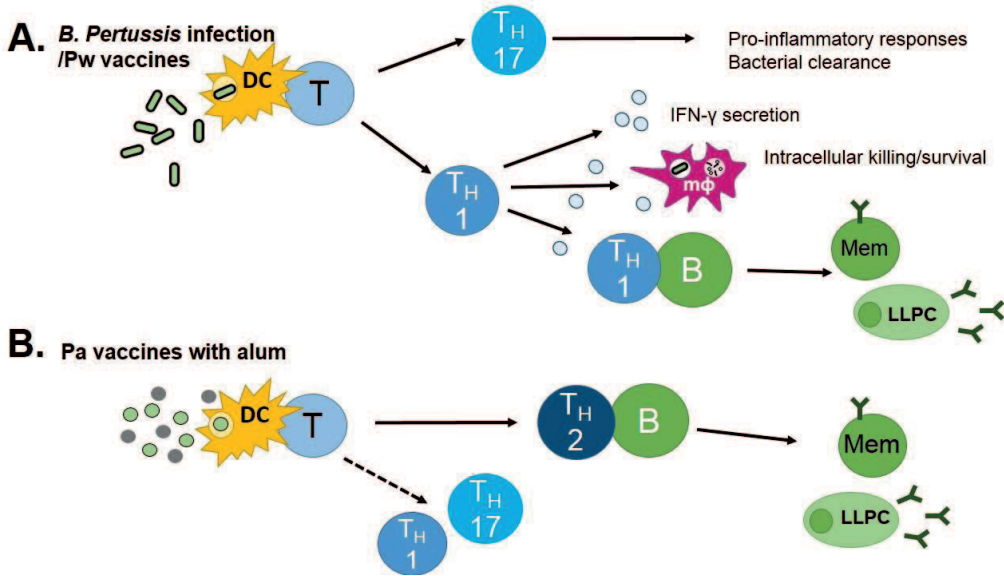


Figure 7. Immune responses induced by pertussis infection or vaccination. The dendritic cells (DCs) take up bacteria or antigen and present it to naïve T cells. A. Natural pertussis infection and whole-cell pertussis (Pw) vaccines lead to a T_H1 -type response where T_H1 cells secrete interferon gamma (IFN- γ) and activate naïve B cells into memory B cells and long-lived plasma cells (LLPC). The macrophages (m ϕ) are also activated and engulf bacteria, which leads to intracellular killing, but intracellular survival of *B. pertussis* is also seen. T_H17 cells are also activated and contribute to bacterial clearance and other pro-inflammatory responses. B. Acellular pertussis (Pa) vaccines with alum as the adjuvant induce a more mixed T_H2/T_H1 -type response with strong activation of B cells and antibody production. Blue: T cells, Green: B cells. Adapted from [3].

7.2.1 B-cell responses

The traditional way to evaluate B-cell responses after pertussis vaccination is to measure antibody levels in serum, but not much focus has been on cellular B-cell responses. In studies including cell-mediated immunity, the focus has been mainly on T-cell responses [100, 154-156]. However, more data are emerging about the cellular B-cell responses after infection and vaccination against various pathogens, including pertussis [22-25, 27].

There is increasing evidence that B cells are important for efficient protection against pertussis. Mouse studies have shown a protective role for B cells without the influence of antibodies [152, 157], and mice with a depleted B-cell pool were less efficient at clearing a pertussis infection compared to immunocompetent mice [144]. In a *B. bronchiseptica* model, the shedding of bacteria was negatively correlated with

increasing levels of B cells in the nasal cavities of mice. This decrease was, however, also influenced by T cells and antibodies [125]. Apart from being effector cells, B cells also function as APCs. Mice with deficient B-cell compartments or lacking MHCII-expression by B cells have been shown to have impaired CD4⁺ T-cell proliferation and differentiation following viral infections [158, 159], and this highlights the contribution of the B-cell APC function. This demonstrates that the B cells most likely play multiple roles in the protection against pertussis and other pathogens. Limiting the evaluation of B-cell responses to only serology could be disadvantageous because immune responses would go undetected.

Whereas other pathogens induce strong, stable B-cell responses after infection or vaccination [20, 23], pertussis does not seem to evoke high numbers of memory B cells after vaccination [22, 160] and is generally considered to be a poor immune inducer [89, 161]. In a comprehensive study by Stenger et al., discrepancies in the B-cell response were identified in mice vaccinated and boosted with either the Pa or Pw vaccine [162]. Following the vaccination and booster, pertussis-specific serum IgG levels, plasma cells in bone marrow, and splenic memory B cells increased. Interestingly, following the peak levels, the pertussis-specific serum IgG decreased but the plasma cell levels increased, and this indicates an imbalance in the number of plasma cells and antibody secretion. The splenic memory B cells dropped to background levels, but their numbers could be rapidly increased with revaccination or antigen challenge. Curiously, this induction did not result in increased serum IgG levels. This led the authors to conclude that a plausible reason for the poor immunological response induced by pertussis might be due to a combination of poor production of antibody by plasma cells in the bone marrow and malfunction of the anamnestic memory B-cell response.

Evaluations of long-term B-cell responses in humans are generally limited to peripheral blood with a few exceptions [28]. The spleen is the major reservoir for memory B cells, and memory B-cell frequencies are very low in peripheral blood [28, 163]. In the two clinical studies included in this thesis (papers II–IV), we measured the presence of memory B cells and antibody levels in blood both pre- and post-vaccination. The studies were not designed for long-term evaluation of the B-cell response following pertussis vaccination. The BPZE1 study did, however, have a follow-up of 6 months that enabled a slightly longer evaluation compared to the booster study with only a 4–6 week follow-up (papers III and IV, respectively). The maintained or increased levels of serum IgG at 5–6 months post BPZE1 vaccination in the colonized (culture-positive) subjects is indicative of an active pool of LLPCs (see Figure 8). The decrease in memory B cells after peak levels at day 28 is in agreement with other studies [20, 105, 164] and was expected because memory B cells home to the SLO and do not circulate extensively.

Encouragingly, although the memory B-cell levels had declined at 6 months, some subjects still had elevated levels compared to pre-vaccination, which indicates an increased memory B-cell pool.

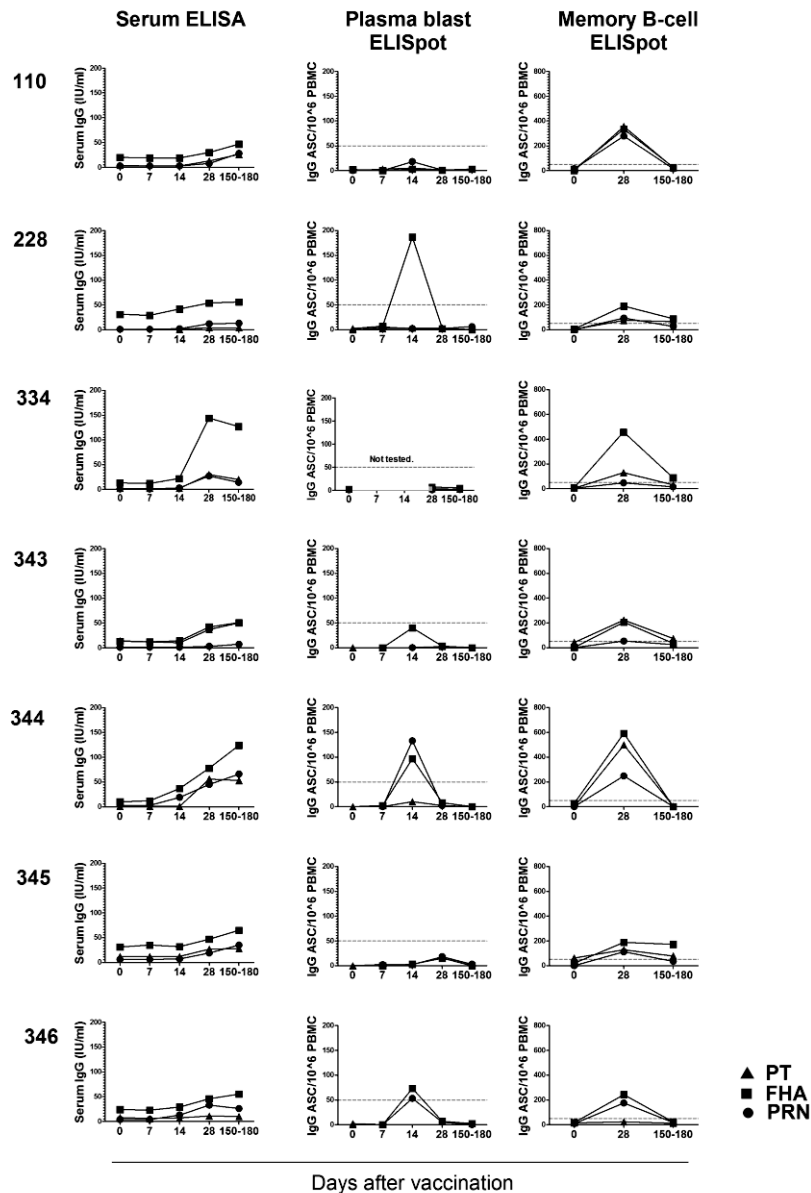


Figure 8. The B-cell responses detected in the seven culture-positive subjects in the BPZE1 study (from paper III).

In the short follow-up of the booster study, the subjects were found to respond similarly with antigen-specific memory B cells and serum IgG, and significant correlation could be detected for the 1-component group (Figure 9). However, this response was detected during the time when the acute response is still detectable in human blood [165] and, therefore, does not truly reflect the long-term presence and relationship between memory B cells and serum IgG.

The complexity and contributions of the immune responses induced by pertussis remain to be elucidated. The fact that even a natural infection induces a relatively short-lived protection indicates that *B. pertussis* itself is highly evolved to escape strong immune recognition. Circumventing these obstacles is a challenge for optimizing the protection against pertussis.

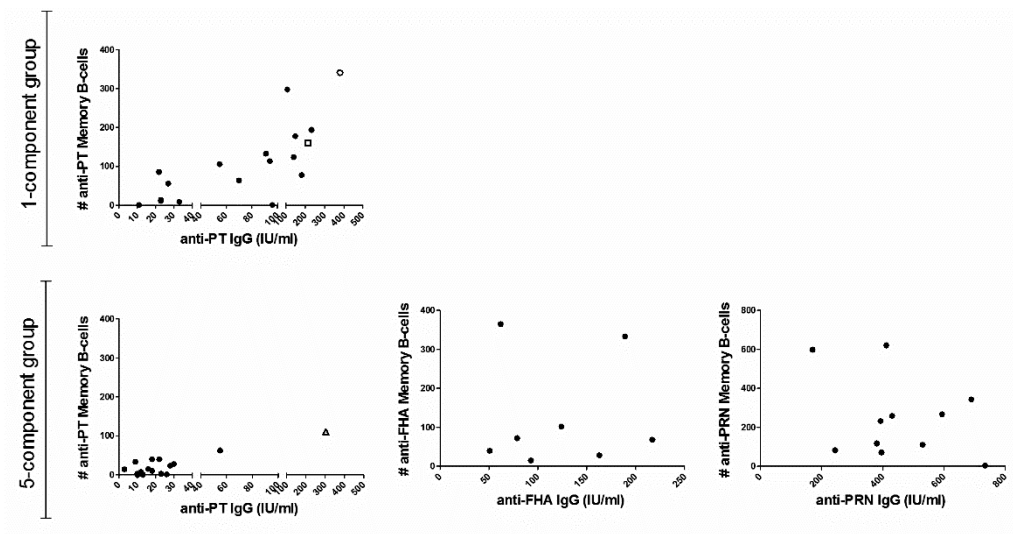


Figure 9. Correlation between the memory B-cell response and the serological response detected in the booster study (from paper IV). A significant correlation (Spearman $r=0.7314$) could be detected only in the PT-specific response for the 1-component vaccine group.

7.2.2 Comparison between a live, attenuated whole-cell vaccine and Pa vaccines

This thesis included the evaluations of a live, attenuated vaccine (BPZE1, papers II and III) and two Pa vaccines (1- and 5-component, paper IV). In both studies, vaccine-induced responses were evaluated pre-vaccination and 28–42 days post-vaccination with respect to pertussis-specific serum IgG levels (detected by ELISA) and IgG-producing memory B cells (detected by ELISpot). It could be rightfully argued that the large differences between the two studies and study populations do not allow for a comparison between the two types of vaccines. Because clinical trials on live, attenuated pertussis vaccines are rare, however, this is a good opportunity to reflect upon the similarities and differences between the two types of vaccines. Listed below are the major discrepancies between the studies:

1. *Previous vaccination scheme.* The BPZE1 individuals (aged 19–34 years) had not received any previous pertussis vaccination but were not considered naïve, meaning that all had pertussis specific antibodies pre-vaccination. The adolescents (aged 14–15 years) that received the Pa vaccines had a primary vaccination consisting of three DTPa doses at 3, 5, and 12 months and an additional Tdap booster at 5½ years.
2. *Administration route.* BPZE1 was given intranasally, but Pa vaccines were given parenterally.
3. *Adjuvants.* The BPZE1 vaccine did not contain any adjuvant, but the Pa vaccines given to the adolescents contained alum.
4. *Number of subjects.* See Table 8 for subjects included in the comparison. In the BPZE1 study, only seven subjects were colonized by the bacteria and exhibited a pertussis-specific response and were thus eligible for comparison. The variation in the number of included adolescents for memory B-cell responses was due to limited availability of cells.
5. *Antigen concentration and content.* Because BPZE1 is a whole-cell vaccine, the exact antigen concentration is not determinable, but all antigens included in the comparison are expressed by the bacteria. The antigen content and concentration varied between the Pa vaccines (see Table 7 in §7.1).

Table 8. Number of subjects included for the comparison between BPZE1 and acellular pertussis (Pa) vaccines (1- or 5-component).

		BPZE	Pa1	Pa5
Memory B-cell ELISpot	PT	7	18	16
	FHA	7	8	8
	PRN	7	11	11
Serum IgG ELISA	PT	7	18	16
	FHA	7	18	16
	PRN	7	18	16

The intranasal vaccination with the BPZE1 vaccine induced a strong memory B-cell response against all included antigens (Figure 10). The adolescents responded against the antigen included in their booster dose. However, the 5-component group did not mount a strong PT-specific response. The PT-specific serum response was similar between the BPZE1 and the 5-component vaccine but lower compared to the 1-component vaccine. The 5-component vaccine induced higher antibody levels against FHA and PRN compared to the intranasal whole-cell vaccine. The inclusion of the T_H2 -skewing adjuvant alum in the Pa vaccine and the differences in the previous vaccination history could explain this. Although the BPZE1 vaccine is not yet optimized, it appears to induce an immune response more similar to that seen after a natural infection compared to the Pa vaccine.

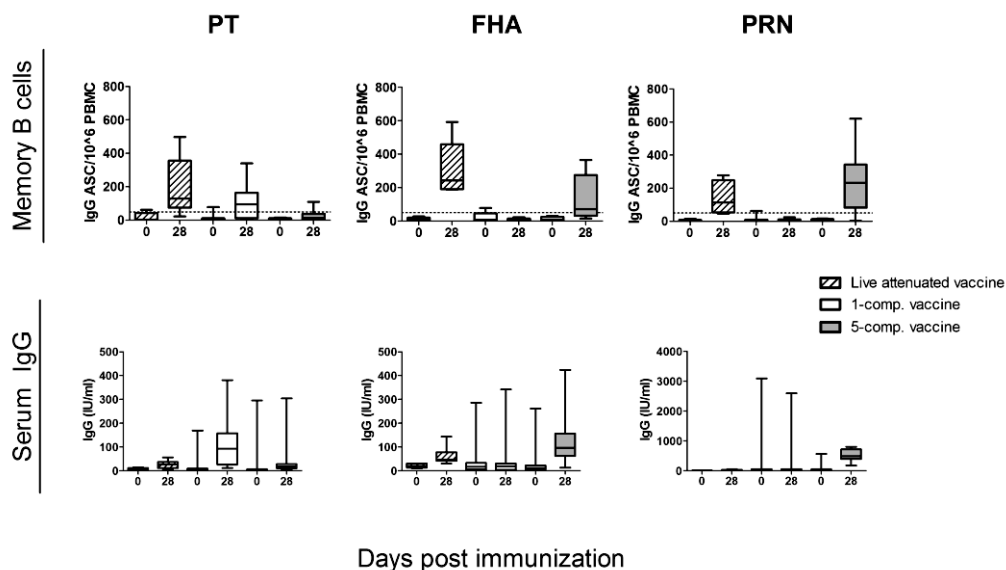


Figure 10. A comparison of the responses detected after vaccination with a live, attenuated whole-cell (Pw) vaccine (BPZE1) and two acellular pertussis (Pa) vaccines (1- and 5-component). The results were obtained in two very different studies abrogating any true conclusions, but the Pw vaccine seems to induce a stronger cellular response compared to serological response, whereas the Pa vaccine induces a more mixed cellular and serological response.

7.3 CORRELATES OF PROTECTION

Several attempts have been made to estimate a correlate of protection against pertussis, but no consensus has been reached. Fim antibodies have been shown to impair *B. pertussis* attachment to respiratory epithelial cells [68] and were shown to offer protection at a group level in an early Pw vaccine study [166]. Later, a more complex correlation was shown where PRN, Fim, and PT antibodies correlate either singly or synergistically with protection at a group level [80, 167-170]. Cellular immunity has also been shown to be essential in pertussis protection, which suggests that several factors contribute to the protection against pertussis and impedes the conclusion regarding a single correlate of protection.

In the BPZE1 study, an interesting finding regarding the antibody specificity and protection against colonization was discovered (paper II). An anti-PT level above 20 IU/mL was set as an exclusion criterion to account for any recent pertussis infection, and no other antibody specificity was regarded. However, when analysing the pre-vaccination samples in the high-dose group, the culture-negative subjects were found to have significantly higher antibody levels compared to the culture-positive subjects (see Figure 11), and this clearly indicates a protective role of antibodies with specificities other than PT against BPZE1 colonization.

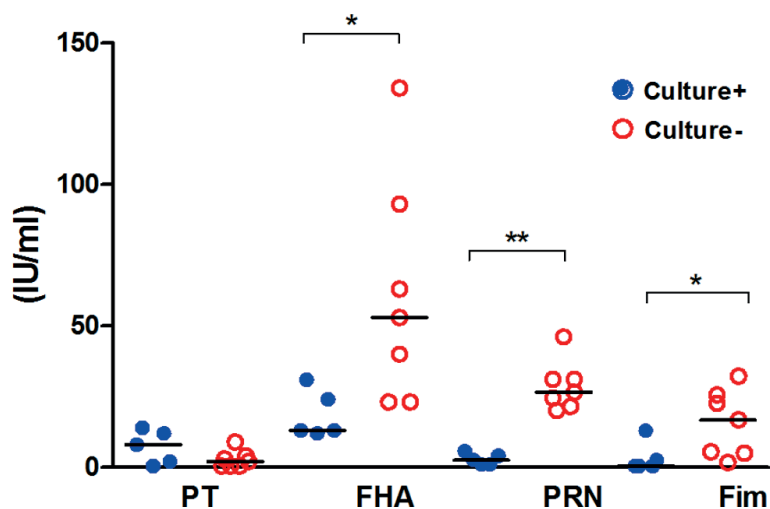


Figure 11. The pre-vaccination levels of pertussis-specific antibodies in sera in the culture-positive (n = 5) and the culture-negative subjects (n = 7) in the high-dose group in the BPZE1 study (from paper II). The culture-negative subjects had significantly higher levels compared to the culture-positive subjects against all antigens except PT. This indicates that PRN-, FHA-, and Fim-specific antibodies might offer protection against *B. pertussis* colonization.

7.4 NEW APPROACHES FOR PROTECTION AGAINST PERTUSSIS

At present, there is no effective strategy to prevent pertussis in the most vulnerable, unvaccinated infants in their first months of life. Optimizing the current vaccination scheme for efficient protection in this age group is, therefore, of high priority.

7.4.1 Cocoon, maternal, and neonatal vaccination strategies

The cocoon strategy involves vaccination of the infant's closest contacts right after birth. Because parents and other close household contacts are known sources of infant pertussis, this strategy is thought to limit transmission to the infant during the first critical months. Although this strategy is likely to have an impact on infant pertussis, it does not prevent transmission from casual contact that infants are also exposed to [107, 171].

Inducing protective antibody levels in the infants via maternal vaccination is an alternative for infant protection. In a baboon model, maternal Pa vaccination was shown to protect against disease in infants; however, this protection did not prevent colonization following pertussis challenge [172]. In humans, maternal vaccination against pertussis has been found safe [173, 174] and is recommended in several countries (summarized in [175]). The low adherence to the program is a concern, however (reviewed in [176]).

A more direct strategy to evoke the infant immune system is neonatal vaccination. Pw and Pa vaccination in infant mice induces protective immunity towards pertussis challenge [177], and in young infants, a natural pertussis infection or Pw vaccination promotes a T_H1 -type response [143]. Even premature infants mount pertussis-specific responses following vaccination [178], which demonstrates the maturity of the infant immune system. Pa vaccination of neonates induces a strong T_H2 -type response [179] but is safe and immunogenic [180-182]. Studies in neonatal baboons also showed that Pa vaccination induced protection against pertussis disease but not against colonization [172].

7.4.2 Novel vaccines

An alternative approach to achieving better vaccine-induced protection for infants is optimization of the currently available vaccines. Increasing vaccine-efficacy could lead to a reduction of pertussis incidence in the general population and thus reduce transmission to infants. Suggestions for improvement have been to develop Pw vaccines with less reactogenicity, to add more antigens to the Pa vaccine, and/or to include other adjuvants to better boost and direct the immune responses.

Pa vaccine efficacy is believed to improve by inclusion of adjuvants that induce a more T_H1 -skewed immune response. Addition of the TLR9 agonist CpG to an alum-adjuvant Pa vaccine has given encouraging results for a more balanced induction of the T_H1/T_H2 -type response in mice [183, 184]. Therefore, adding additional adjuvants to the licenced vaccines could provide protection that is more efficient. In parallel, the inclusion of more antigens is also believed to increase efficacy because it would induce a broader response. This is supported by the findings that vaccine efficacy increases with the number of antigens included [133, 135, 136]. A possible candidate for inclusion in the Pa vaccines is ACT. Adding ACT to the Pa vaccine enhances immune responses in mice [185], and ACT on its own induces protective immunity [186]. All together, the efficacy of Pa vaccines is likely to be improved with novel formulations. New Pw vaccines are being investigated due to their high efficacy, but the key issue is to reduce side effects.

7.4.3 BPZE1

BPZE1 is designed to mimic a natural infection and is intended to be offered as a birth dose to complement the current vaccination program. Several pre-clinical studies have investigated the safety and immunogenicity of BPZE1. It is genetically stable with no reversion despite numerous *in vitro* and *in vivo* passages [187]. BPZE1 colonization in adult and infant mice is similar to virulent strains but without any pathological changes [69]. One dose of BPZE1 offers better protection compared to two doses of Pa vaccine [69], and this protection is maintained up to one year after vaccination [188, 189]. *In vivo* studies in mice and an *in vitro* study with human DCs demonstrate the induction of strong T_H1 - and T_H17 -type responses similar to natural infection responses. Specific B cells and antibodies are also induced by BPZE1 and contribute to protection [190].

Mucosal vaccination with a live, attenuated pertussis vaccine has not been performed before in a clinical setting. Killed Pw vaccines have, however, been shown to be immunogenic when administered intranasally with a spray [191, 192]. In addition, intranasal vaccination with a cholera subunit vaccine induced strong, long-lasting antibody responses both in serum and in nasal secretions [193].

Similar to natural infection, BPZE1 was found in nasopharynx samples between day 4 and day 28 after vaccination in the culture-positive subjects (paper II) [194], and this demonstrates full functionality of the modified bacteria in humans. However, because only 7 of the 36 subjects that received the active vaccine were colonized by the bacteria, the vaccine response seemed to be suboptimal. Five of the culture-positive subjects were in the high-dose group, which indicates a dose-dependent vaccine response. Similar results were also found in the pre-clinical studies where vaccine efficacy and immune responses increased with BPZE1 dosage [195]. An explanation for the low vaccine

response in humans could be the low cfu/dose. Because BPZE1 had not been used in humans before, the dosages were estimated based on results obtained in mouse studies [195]. A dose of 10^6 cfu was well tolerated in mice, but the maximum dose in the clinical trial was only 10^7 cfu (paper II). In a baboon animal model, a dosage of 10^9 – 10^{10} cfu of virulent *B. pertussis* is used to infect animals [123], and an intranasal vaccine against kennel cough includes $\geq 10^8$ cfu/dose. This indicates that higher dosages could be tested in humans to increase the vaccine response.

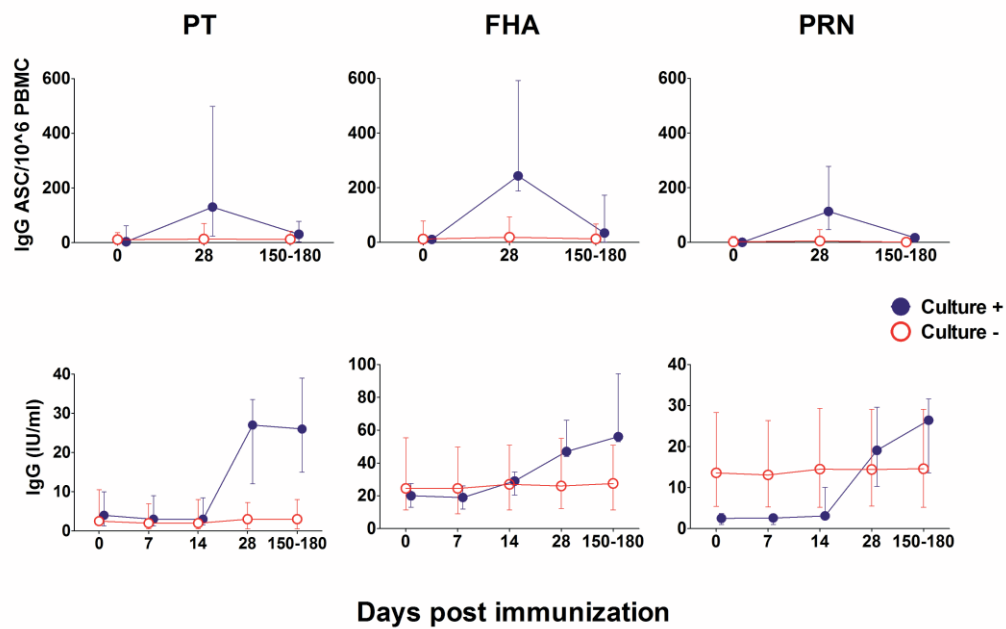


Figure 12. B-cell responses from the BPZE1 trial. Culture-positive subjects (n = 7) had increased pertussis-specific serum IgG levels and memory B-cell responses following BPZE1 vaccination. Memory B-cell response peaked at day 28 but declined 150–180 days post-vaccination. Serum IgG antibody levels increased after 7–14 days and were maintained or increased 150–180 days post-immunization. No response was seen in the culture-negative group (n = 29). Median values with max and min values are shown.

From the results obtained in the BPZE1 study (reported in papers II and III), we concluded that the BPZE1 vaccination was safe for human use and that colonized subjects induced strong and long-term pertussis-specific serological and B-cell responses (as measured up to 6 months post-vaccination; see Figure 12). Included in the

study design but not reported in the papers were the T-cell responses evoked by BPZE1. The presence of pertussis-specific IFN- γ and IL-2 secreting cells were evaluated by T-cell ELISpot on days 0, 14, and 28 and 5–6 months post-vaccination. Due to a methodological error, only two culture-positive subjects (no. 110 from the low-dose group and no. 228 from the medium-dose group) had available samples from all time points. According to established criteria (see Figure 13), subject no. 110 responded with IL-2-secreting T cells against all antigens tested for, but only the PT-specific IFN- γ responses met the response criteria. Subject no. 228 produced both IFN- γ and IL-2 against FHA and PT but did not meet the response criteria for PRN (see Figure 13). Parenteral administration of pertussis vaccines has been demonstrated to induce a strong IFN- γ response one month after immunization in humans [150, 196, 197], and mouse studies of BPZE1 have shown that vaccination induces protective levels of splenic CD4⁺ T cells [190]. More subjects would have enabled a stronger conclusion, but these limited results do indicate that BPZE1 vaccination induces pertussis-specific T-cell responses in humans.

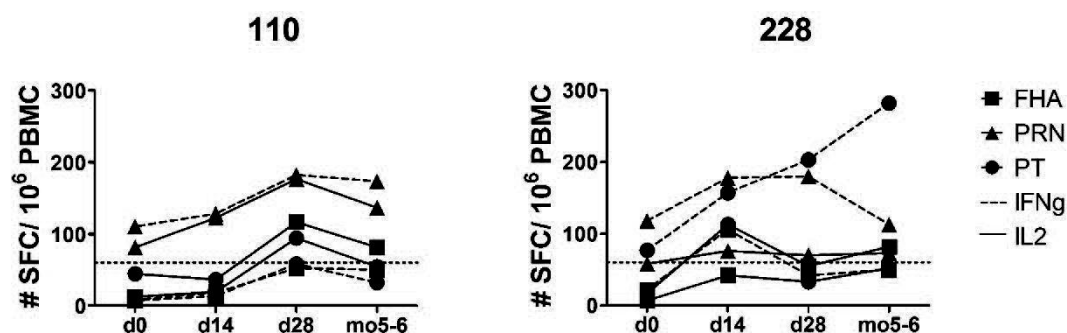


Figure 13. IFN- γ - and IL-2-secreting T cells at different time points following BPZE1 vaccination in two culture-positive subjects. Subject no. 110 responded with IL-2-specific T cells against all antigens but with IFN- γ -specific T cells only against PT. Subject no. 228 responded with both IL-2- and IFN- γ -specific T cells against FHA and PT but did not meet the response criteria for PRN due to high pre-vaccination levels. Response criteria were set to be more than double the number of spots post-vaccination compared to pre-vaccination and above 55 spots/10⁶ PBMC for IFN- γ . For IL-2, no cut-off value was established.

Pertussis-specific serum IgA levels were also determined (see Figure 14). The culture-positive subjects responded with IgA against all included antigens except PRN. The culture-negative group was non-responsive throughout the trial except for some fluctuations in PRN. Even though increases were seen for the culture-positive group, the

differences were not statistically significant. This result is most likely influenced by the high antibody levels in the culture-negative group.

In conclusion, the results from the first human study of the novel BPZE1 vaccine strain are promising, and the vaccine strain was found to be safe and immunogenic. However, the vaccine is not yet optimized, and the dosages and vaccine formulation need to be improved. With these changes, the true potential of the novel vaccine strain can be established in future studies.

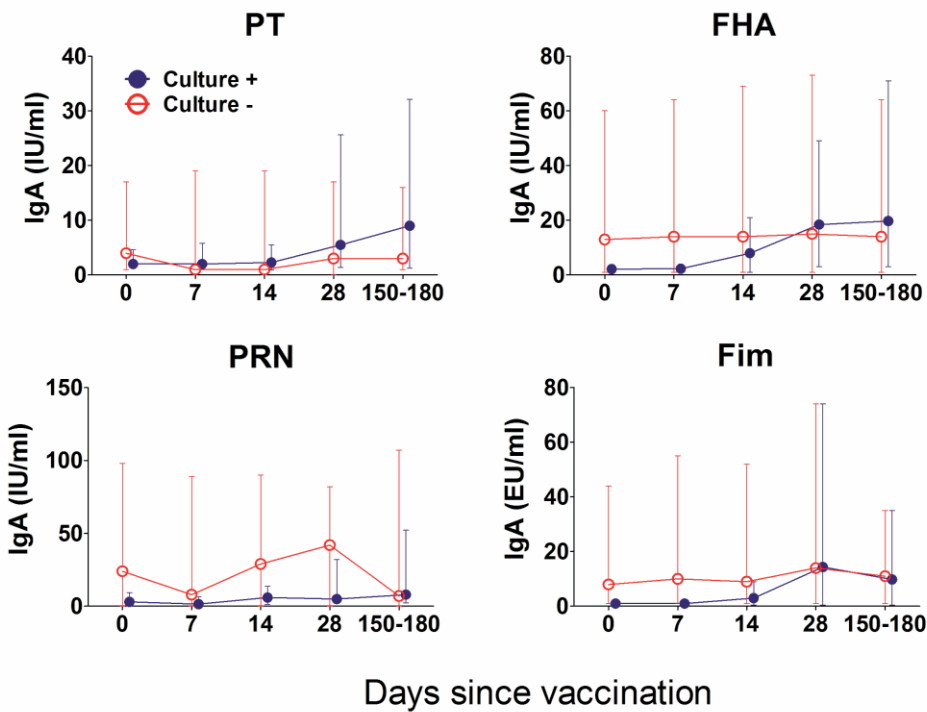


Figure 14. Pertussis-specific serum IgA levels in culture-positive (n = 7) and culture-negative (n = 29) subjects. Increases in the PT-, FHA-, and Fim-specific antibody levels were detected at day 28 post-immunization in the culture-positive group, but the increases were not significantly higher compared to culture-negative subjects. Median values with min and max values are shown.

8 CONCLUDING REMARKS

Despite years of vaccination, *B. pertussis* is still a concern to the general population and a serious threat to unvaccinated infants. Numerous studies have evaluated the efficacy of pertussis vaccines, but comparison between studies is often hampered by the differences in the study designs. The optimization of the B-cell ELISpot protocol described in this thesis clarified the impact that the inclusion of the optimal activators and detection system has on the detection sensitivity of a positive result. A strong consensus on standardised protocols and controls would greatly facilitate the analysis of a broader array of data obtained in different studies.

Traditionally, evaluation of pertussis has centred on serological antibody responses, but there is no established single serological correlate of protection. The protection against a natural pertussis infection is multifaceted, and there is still much to learn about the pertussis-specific immune response. In the two clinical trials included in this thesis, we detected strong and broad memory B-cell and serological antibody responses following both mucosal and parenteral pertussis immunization. This shows that without a broad evaluation, vaccine-induced responses would go undetected. Therefore, it seems beneficial to investigate several different arms of the immune responses to better comprehend the pertussis-induced immunity.

Within the scope of this thesis, we have reported on an optimized B-cell ELISpot and the immunogenicity after two types of pertussis vaccination (acellular and live, attenuated) in two different Swedish populations (previously vaccinated and unvaccinated). Although the vaccines were found safe and immunogenic, the analyses did have limitations. In general, few samples from subjects were available for each analysis due to low vaccine response in the BPZE1 study and low cell availability for the boosted adolescents. More subjects would have strengthened the statistical evaluation of the responses.

Most of the results reported in this thesis were obtained within the scope of clinical trials, which, to some extent, was also a limiting factor. Clinical trials are highly regulated to protect the participants and are meticulously planned. No deviation from the clinical study protocol or the informed consent document is allowed, which results in an inflexible analysis plan after approval of the study. Moreover, the clinical trials included analyses of several immune parameters but only a limited sample size, which impeded an extensive evaluation of a specific response. The methodological part of this thesis is, therefore, limited because generally only ELISpot and ELISA were implemented.

Working with clinical trials does, however, require specific expertise. All of the work performed for this thesis, from the planning to the archiving of results, was done according to Good Clinical and Laboratory Practice (GCLP). This is a detailed and time-consuming process but vital for quality-assured results. These regulations should be applied to all types of clinical research, and experience with GCLP is a highly valuable asset. The GCLP knowledge gained within the scope of this thesis is believed to compensate for the lack of methodological variation.

Increasing the efficacy of pertussis vaccines is an urgent matter. Improving the reactogenicity of Pw vaccines and switching back to whole-cell vaccines should lead to better efficacy, as would including better adjuvants or more antigens in the available Pa vaccines. Increasing efficacy of available vaccines would lead to an increased protection in the population and reduced pertussis transmission to infants. However, protecting the infant by adjusting its environment is complicated, and a more appealing approach is to improve the infant's own protection. Neonatal vaccination is, therefore, a hopeful approach, especially in combination with more efficient vaccines.

Eradication of pertussis is an unlikely solution today. Therefore, the focus lies on optimizing protection for infants and small children. With more knowledge about the immune responses required for protection, we can also learn how to optimize the vaccines that target pertussis. With patience, open-mindedness, and collaboration, the riddle of pertussis can be solved.

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10 REFERENCES

1. Nilsson, L., et al., *Pertussis surveillande in Sweden - Fifteen year report*. 2013, Swedish Institute for Communicable Disease Control.
2. Abbas AK, L.A., Pillai S, *Cellular and molecular immunology*. 7 ed. 2012, Philadelphia: Elsevier Saunders.
3. 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.
4. Bevan, M.J., *Understand memory, design better vaccines*. Nat Immunol, 2011. **12**(6): p. 463-5.
5. Gustafsson, L., et al., *Antibody responses and clinical reaction to booster doses of TdP+IPV, TdP-IPV at 5½ years of age after primary immunization with DTcP (DTPa5) at 3, 5 and 12 months of age in Pertussis Vaccine Trial II [Technical Report]*. Smittskyddsinstitutet publikationer, 2000.
6. Olin, P., et al., *Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine*. Ad Hoc Group for the Study of Pertussis Vaccines. Lancet, 1997. **350**(9091): p. 1569-77.
7. Riedel, S., *Edward Jenner and the history of smallpox and vaccination*. Proc (Bayl Univ Med Cent), 2005. **18**(1): p. 21-5.
8. Ehreth, J., *The global value of vaccination*. Vaccine, 2003. **21**(7-8): p. 596-600.
9. WHO. *WHO recommendations for routine immunization - summary tables*. 2013 [cited 2014 8th of January]; Available from: http://www.who.int/immunization/policy/immunization_tables/en/index.html.
10. Uhnoo, I., A. Tegnell, and R. Thorstensson, *Vaccinuppföljning*. 2013: Smittskyddsinstitutet.
11. WHO. *WHO Immunization coverage*. 2013 [cited 2014 7th of January]; Available from: <http://www.who.int/mediacentre/factsheets/fs378/en/>.
12. Wakefield, A.J., et al., *Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children*. Lancet, 1998. **351**(9103): p. 637-41.
13. *Retraction--Ileal-lymphoid-nodular hyperplasia, non-specific colitis, and pervasive developmental disorder in children*. Lancet, 2010. **375**(9713): p. 445.
14. McBrien, J., et al., *Measles outbreak in Dublin, 2000*. Pediatr Infect Dis J, 2003. **22**(7): p. 580-4.
15. Folkhälsomyndigheten. *The Public Health Agency of Sweden*. 2013 [cited 2014 January 9th]; Available from: <http://folkhalsomyndigheten.se/amnesomraden/smittskydd-och-sjukdomar/vaccinationer/barnvaccinationer/>.
16. NIH. *U.S National Institutes of Health*. 2012 [cited 2014 17th of February]; Available from: <http://clinicaltrials.gov>.
17. NIAID. *Types of Vaccines*. 2012 [cited 2014 27th of February]; Available from: <http://www.niaid.nih.gov/topics/vaccines/understanding/pages/typesvaccines.aspx>.
18. Eisenstein, E.M. and C.B. Williams, *The T(reg)/Th17 cell balance: a new paradigm for autoimmunity*. Pediatr Res, 2009. **65**(5 Pt 2): p. 26R-31R.
19. Chen, M., et al., *The development and function of follicular helper T cells in immune responses*. Cell Mol Immunol, 2012. **9**(5): p. 375-9.
20. Nanan, R., et al., *Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes*. Vaccine, 2001. **20**(3-4): p. 498-504.
21. van Twillert, I., et al., *Age related differences in dynamics of specific memory B cell populations after clinical pertussis infection*. PLoS One, 2014. **9**(1): p. e85227.
22. Buisman, A.M., et al., *Long-term presence of memory B-cells specific for different vaccine components*. Vaccine, 2009. **28**(1): p. 179-86.
23. Crotty, S., et al., *Cutting edge: long-term B cell memory in humans after smallpox vaccination*. J Immunol, 2003. **171**(10): p. 4969-73.
24. Kakoulidou, M., et al., *Kinetics of antibody and memory B cell responses after MMR immunization in children and young adults*. Vaccine, 2013. **31**(4): p. 711-7.
25. Amanna, I.J., N.E. Carlson, and M.K. Slifka, *Duration of humoral immunity to common viral and vaccine antigens*. N Engl J Med, 2007. **357**(19): p. 1903-15.

26. Leyendeckers, H., et al., *Correlation analysis between frequencies of circulating antigen-specific IgG-bearing memory B cells and serum titers of antigen-specific IgG*. Eur J Immunol, 1999. **29**(4): p. 1406-17.
27. Bauer, T. and W. Jilg, *Hepatitis B surface antigen-specific T and B cell memory in individuals who had lost protective antibodies after hepatitis B vaccination*. Vaccine, 2006. **24**(5): p. 572-7.
28. Mamani-Matsuda, M., et al., *The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells*. Blood, 2008. **111**(9): p. 4653-9.
29. Rosado, M.M., et al., *Switched memory B cells maintain specific memory independently of serum antibodies: the hepatitis B example*. Eur J Immunol, 2011. **41**(6): p. 1800-8.
30. Hammarlund, E., et al., *Duration of antiviral immunity after smallpox vaccination*. Nat Med, 2003. **9**(9): p. 1131-7.
31. Manz, R.A., et al., *Survival of long-lived plasma cells is independent of antigen*. Int Immunol, 1998. **10**(11): p. 1703-11.
32. Ahuja, A., et al., *Maintenance of the plasma cell pool is independent of memory B cells*. Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4802-7.
33. DiLillo, D.J., et al., *Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice*. J Immunol, 2008. **180**(1): p. 361-71.
34. Vallerskog, T., et al., *Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE*. Clin Immunol, 2007. **122**(1): p. 62-74.
35. Czerkinsky, C.C., et al., *A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells*. J Immunol Methods, 1983. **65**(1-2): p. 109-21.
36. Cao, Y., et al., *An optimized assay for the enumeration of antigen-specific memory B cells in different compartments of the human body*. J Immunol Methods, 2010. **358**(1-2): p. 56-65.
37. Crotty, S., et al., *Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system*. J Immunol Methods, 2004. **286**(1-2): p. 111-22.
38. Weiss, G.E., et al., *High efficiency human memory B cell assay and its application to studying Plasmodium falciparum-specific memory B cells in natural infections*. J Immunol Methods, 2012. **375**(1-2): p. 68-74.
39. Pinna, D., et al., *Clonal dissection of the human memory B-cell repertoire following infection and vaccination*. Eur J Immunol, 2009. **39**(5): p. 1260-70.
40. Dosenovic, P., et al., *Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization*. J Immunol, 2009. **183**(5): p. 3373-82.
41. Ahlborg, N. and B. Axelsson, *Dual- and triple-color fluorospot*. Methods Mol Biol, 2012. **792**: p. 77-85.
42. Czerkinsky, C., et al., *A novel two colour ELISPOT assay. I. Simultaneous detection of distinct types of antibody-secreting cells*. J Immunol Methods, 1988. **115**(1): p. 31-7.
43. Pickering, J.W., et al., *A multiplexed fluorescent microsphere immunoassay for antibodies to pneumococcal capsular polysaccharides*. Am J Clin Pathol, 2002. **117**(4): p. 589-96.
44. van Gageldonk, P.G., et al., *Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus*. J Immunol Methods, 2008. **335**(1-2): p. 79-89.
45. Amanna, I.J. and M.K. Slifka, *Quantitation of rare memory B cell populations by two independent and complementary approaches*. J Immunol Methods, 2006. **317**(1-2): p. 175-85.
46. Sallusto, F., et al., *From vaccines to memory and back*. Immunity, 2010. **33**(4): p. 451-63.
47. Pulendran, B. and R. Ahmed, *Immunological mechanisms of vaccination*. Nat Immunol, 2011. **12**(6): p. 509-17.
48. McKee, A.S., M.W. Munks, and P. Marrack, *How do adjuvants work? Important considerations for new generation adjuvants*. Immunity, 2007. **27**(5): p. 687-90.
49. Coffman, R.L., A. Sher, and R.A. Seder, *Vaccine adjuvants: putting innate immunity to work*. Immunity, 2010. **33**(4): p. 492-503.
50. O'Hagan, D.T. and E. De Gregorio, *The path to a successful vaccine adjuvant--'the long and winding road'*. Drug Discov Today, 2009. **14**(11-12): p. 541-51.

51. 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.
52. Kool, M., et al., *Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome*. *J Immunol*, 2008. **181**(6): p. 3755-9.
53. Li, H., S. Nookala, and F. Re, *Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release*. *J Immunol*, 2007. **178**(8): p. 5271-6.
54. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants*. *Curr Opin Immunol*, 2009. **21**(1): p. 23-9.
55. Didierlaurent, A.M., et al., *AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity*. *J Immunol*, 2009. **183**(10): p. 6186-97.
56. Galli, G., et al., *Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted pre-pandemic vaccine*. *Proc Natl Acad Sci U S A*, 2009. **106**(19): p. 7962-7.
57. Khurana, S., et al., *MF59 adjuvant enhances diversity and affinity of antibody-mediated immune response to pandemic influenza vaccines*. *Sci Transl Med*, 2011. **3**(85): p. 85ra48.
58. Sigmundsdottir, H. and E.C. Butcher, *Environmental cues, dendritic cells and the programming of tissue-selective lymphocyte trafficking*. *Nat Immunol*, 2008. **9**(9): p. 981-7.
59. Masopust, D., et al., *Dynamic T cell migration program provides resident memory within intestinal epithelium*. *J Exp Med*, 2010. **207**(3): p. 553-64.
60. Schwarz, T.F. and O. Leo, *Immune response to human papillomavirus after prophylactic vaccination with AS04-adjuvanted HPV-16/18 vaccine: improving upon nature*. *Gynecol Oncol*, 2008. **110**(3 Suppl 1): p. S1-10.
61. Kaur, R., et al., *Antibody in middle ear fluid of children originates predominantly from sera and nasopharyngeal secretions*. *Clin Vaccine Immunol*, 2012. **19**(10): p. 1593-6.
62. Renegar, K.B., et al., *Role of IgA versus IgG in the control of influenza viral infection in the murine respiratory tract*. *J Immunol*, 2004. **173**(3): p. 1978-86.
63. Sorensen, C.H., U.B. Sorensen, and J. Henriksen, *Local production of IgG to pneumococcal C-polysaccharide in upper airway secretions from children with recurrent acute otitis media*. *Microb Pathog*, 1989. **6**(3): p. 183-91.
64. Murray, P., K. Rosenthal, and M. Pfaller, *Medical Microbiology*. 6th ed. 2009, Canada: Mosby Elsevier.
65. Guiso, N., *Bordetella pertussis and pertussis vaccines*. *Clin Infect Dis*, 2009. **49**(10): p. 1565-9.
66. Friedman, R.L., et al., *Uptake and intracellular survival of Bordetella pertussis in human macrophages*. *Infect Immun*, 1992. **60**(11): p. 4578-85.
67. Mills, K.H., *Immunity to Bordetella pertussis*. *Microbes Infect*, 2001. **3**(8): p. 655-77.
68. Rodriguez, M.E., et al., *Bordetella pertussis attachment to respiratory epithelial cells can be impaired by fimbriae-specific antibodies*. *FEMS Immunol Med Microbiol*, 2006. **46**(1): p. 39-47.
69. Mielcarek, N., et al., *Live attenuated B. pertussis as a single-dose nasal vaccine against whooping cough*. *PLoS Pathog*, 2006. **2**(7): p. e65.
70. WHO, *Pertussis vaccines: WHO position paper*. *Wkly Epidemiol Rec*, 2010. **85**(40): p. 385-400.
71. Fernandez-Cano, M.I., et al., *Incidence of whooping cough in Spain (1997-2010): an underreported disease*. *Eur J Pediatr*, 2013.
72. Hodder, S.L., et al., *Antibody responses to Bordetella pertussis antigens and clinical correlations in elderly community residents*. *Clin Infect Dis*, 2000. **31**(1): p. 7-14.
73. Stefanoff, P., et al., *Incidence of pertussis in patients of general practitioners in Poland*. *Epidemiol Infect*, 2013: p. 1-10.
74. 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.
75. Faulkner, A., et al. *Manual for the surveillance of vaccine-preventable diseases*. *Vaccines & Immunizations 2011* [cited 2014 28th of January]; Available from: <http://www.cdc.gov/vaccines/pubs/surv-manual/chpt10-pertussis.pdf>.
76. WHO, *WHO-recommended standards for surveillance of selected vaccine-preventable diseases*, V.A.a.M. team, Editor. 2003.

77. Cherry, J.D., et al., *Clinical definitions of pertussis: Summary of a Global Pertussis Initiative roundtable meeting, February 2011*. Clin Infect Dis, 2012. **54**(12): p. 1756-64.
78. Clark, T.A., N.E. Messonnier, and S.C. Hadler, *Pertussis control: time for something new?* Trends Microbiol, 2012. **20**(5): p. 211-3.
79. Forsyth, K., *Pertussis, Still a Formidable Foe*. Clinical Infectious Diseases, 2007. **45**(11): p. 1487-91.
80. 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.
81. Baron, S., et al., *Epidemiology of pertussis in French hospitals in 1993 and 1994: thirty years after a routine use of vaccination*. Pediatr Infect Dis J, 1998. **17**(5): p. 412-8.
82. Crowcroft, N.S., et al., *Severe and unrecognised: pertussis in UK infants*. Arch Dis Child, 2003. **88**(9): p. 802-6.
83. Lavine, J., et al., *Imperfect vaccine-induced immunity and whooping cough transmission to infants*. Vaccine, 2010. **29**(1): p. 11-6.
84. Burgess, M.A., P.B. McIntyre, and T.C. Heath, *Pertussis re-emerging: who is responsible?* Aust N Z J Public Health, 1998. **22**(1): p. 9-10.
85. Heininger, U., D. Weibel, and J.L. Richard, *Prospective nationwide surveillance of hospitalizations due to pertussis in children, 2006-2010*. Pediatr Infect Dis J, 2014. **33**(2): p. 147-51.
86. Sato, Y., M. Kimura, and H. Fukumi, *Development of a pertussis component vaccine in Japan*. Lancet, 1984. **1**(8369): p. 122-6.
87. WHO, *Pertussis vaccines--WHO position paper*. Wkly Epidemiol Rec, 2005. **80**(4): p. 31-9.
88. ECDC. *Scientific panel on childhood immunisation schedule: Diphtheria-tetanus-pertussis (DTP) vaccination*. ECDC GUIDANCE 2009 [cited 2014 January 29]; Available from: www.ecdc.europa.eu.
89. Gustafsson, L., et al., *Long-term follow-up of Swedish children vaccinated with acellular pertussis vaccines at 3, 5, and 12 months of age indicates the need for a booster dose at 5 to 7 years of age*. Pediatrics, 2006. **118**(3): p. 978-84.
90. Forsyth, K.D., et al., *New pertussis vaccination strategies beyond infancy: recommendations by the global pertussis initiative*. Clin Infect Dis, 2004. **39**(12): p. 1802-9.
91. Hallander, H.O., L. Nilsson, and L. Gustafsson, *Is adolescent pertussis vaccination preferable to natural booster infections?* Expert Rev Clin Pharmacol, 2011. **4**(6): p. 705-11.
92. 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.
93. Lavine, J.S., et al., *Short-lived immunity against pertussis, age-specific routes of transmission, and the utility of a teenage booster vaccine*. Vaccine, 2012. **30**(3): p. 544-51.
94. Cherry, J.D., *Epidemiology of pertussis*. Pediatr Infect Dis J, 2006. **25**(4): p. 361-2.
95. von Konig, C.H., et al., *Pertussis of adults and infants*. Lancet Infect Dis, 2002. **2**(12): p. 744-50.
96. Chang, S., et al., *U.S. Postlicensure safety surveillance for adolescent and adult tetanus, diphtheria and acellular pertussis vaccines: 2005-2007*. Vaccine, 2013. **31**(10): p. 1447-52.
97. Edelman, K., et al., *Immunity to pertussis 5 years after booster immunization during adolescence*. Clin Infect Dis, 2007. **44**(10): p. 1271-7.
98. Hara, M., et al., *Immunogenicity and safety after booster vaccination of diphtheria, tetanus, and acellular pertussis in young adults: an open randomized controlled trial in Japan*. Clin Vaccine Immunol, 2013. **20**(12): p. 1799-804.
99. Prelog, M., et al., *Differences of IgG antibody avidity after an acellular pertussis (aP) booster in adolescents after a whole cell (wvP) or aP primary vaccination*. Vaccine, 2013. **31**(2): p. 387-93.
100. Rieber, N., et al., *Differences of humoral and cellular immune response to an acellular pertussis booster in adolescents with a whole cell or acellular primary vaccination*. Vaccine, 2008. **26**(52): p. 6929-35.
101. Tran Minh, N.N., et al., *Antibody and cell-mediated immune responses to booster immunization with a new acellular pertussis vaccine in school children*. Vaccine, 1998. **16**(17): p. 1604-10.

102. Tran Minh, N.N., et al., *Acellular vaccines containing reduced quantities of pertussis antigens as a booster in adolescents*. Pediatrics, 1999. **104**(6): p. e70.
103. Ward, J.I., et al., *Bordetella Pertussis infections in vaccinated and unvaccinated adolescents and adults, as assessed in a national prospective randomized Acellular Pertussis Vaccine Trial (APERT)*. Clin Infect Dis, 2006. **43**(2): p. 151-7.
104. Bailleux, F., et al., *Predicted long-term persistence of pertussis antibodies in adolescents after an adolescent and adult formulation combined tetanus, diphtheria, and 5-component acellular pertussis vaccine, based on mathematical modeling and 5-year observed data*. Vaccine, 2008. **26**(31): p. 3903-8.
105. Hendriks, 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.
106. Bisgard, K.M., et al., *Infant pertussis: who was the source?* Pediatr Infect Dis J, 2004. **23**(11): p. 985-9.
107. van Hoek, A.J., et al., *The social life of infants in the context of infectious disease transmission; social contacts and mixing patterns of the very young*. PLoS One, 2013. **8**(10): p. e76180.
108. Auger, K.A., S.W. Patrick, and M.M. Davis, *Infant Hospitalizations for Pertussis Before and After Tdap Recommendations for Adolescents*. Pediatrics, 2013.
109. Skoff, T.H., et al., *Early Impact of the US Tdap vaccination program on pertussis trends*. Arch Pediatr Adolesc Med, 2012. **166**(4): p. 344-9.
110. Locht, C. and N. Mielcarek, *New pertussis vaccination approaches: en route to protect newborns?* FEMS Immunol Med Microbiol, 2012. **66**(2): p. 121-33.
111. Isacson, J., et al., *How common is whooping cough in a nonvaccinating country?* Pediatr Infect Dis J, 1993. **12**(4): p. 284-8.
112. Romanus, V., R. Jonsell, and S.O. Bergquist, *Pertussis in Sweden after the cessation of general immunization in 1979*. Pediatr Infect Dis J, 1987. **6**(4): p. 364-71.
113. *Placebo-controlled trial of two acellular pertussis vaccines in Sweden--protective efficacy and adverse events*. Ad Hoc Group for the Study of Pertussis Vaccines. Lancet, 1988. **1**(8592): p. 955-60.
114. Gustafsson, L., et al., *A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine*. N Engl J Med, 1996. **334**(6): p. 349-55.
115. Trollfors, B., et al., *A placebo-controlled trial of a pertussis-toxoid vaccine*. N Engl J Med, 1995. **333**(16): p. 1045-50.
116. CDC, *Pertussis epidemic--Washington, 2012*. MMWR Morb Mortal Wkly Rep, 2012. **61**(28): p. 517-22.
117. Boulton, J., *The UK pertussis epidemic: implications for immunisation*. Br J Nurs, 2013. **22**(18): p. 1046-50.
118. Winter, K., et al., *California pertussis epidemic, 2010*. J Pediatr, 2012. **161**(6): p. 1091-6.
119. 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.
120. Mooi, F.R., V.D.M. NA, and H.E. De Melker, *Pertussis resurgence: waning immunity and pathogen adaptation - two sides of the same coin*. Epidemiol Infect, 2013: p. 1-10.
121. Cherry, J.D., *Pertussis: challenges today and for the future*. PLoS Pathog, 2013. **9**(7): p. e1003418.
122. Cherry, J.D., *Why do pertussis vaccines fail?* Pediatrics, 2012. **129**(5): p. 968-70.
123. Warfel, J.M., et al., *Nonhuman primate model of pertussis*. Infect Immun, 2012. **80**(4): p. 1530-6.
124. 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, 2013.
125. Smallridge, W.E., et al., *Different effects of whole cell and acellular vaccines on Bordetella transmission*. J Infect Dis, 2014.
126. 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.

127. 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.
128. Witt, M.A., et al., *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*. Clin Infect Dis, 2013. **56**(9): p. 1248-54.
129. Hallander, H.O., et al., *Seroprevalence of pertussis antitoxin (anti-PT) in Sweden before and 10 years after the introduction of a universal childhood pertussis vaccination program*. APMIS, 2009. **117**(12): p. 912-22.
130. Canthaboo, C., et al., *Investigation of cellular and humoral immune responses to whole cell and acellular pertussis vaccines*. Vaccine, 2000. **19**(6): p. 637-43.
131. Smits, K., et al., *Different T cell memory in preadolescents after whole-cell or acellular pertussis vaccination*. Vaccine, 2013. **32**(1): p. 111-8.
132. 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.
133. Cherry, J.D., et al., *Antibody response patterns to Bordetella pertussis antigens in vaccinated (primed) and unvaccinated (unprimed) young children with pertussis*. Clin Vaccine Immunol, 2010. **17**(5): p. 741-7.
134. Hallander, H.O. and L. Gustafsson, *Efficacy and effectiveness of acellular pertussis vaccines: a 20-year Swedish experience*. Expert Rev Vaccines, 2009. **8**(10): p. 1303-7.
135. Jefferson, T., M. Rudin, and C. DiPietrantonj, *Systematic review of the effects of pertussis vaccines in children*. Vaccine, 2003. **21**(17-18): p. 2003-14.
136. Zhang, L., et al., *Acellular vaccines for preventing whooping cough in children*. Cochrane Database Syst Rev, 2012. **3**: p. CD001478.
137. Edwards, K.M., et al., *Comparison of 13 acellular pertussis vaccines: overview and serologic response*. Pediatrics, 1995. **96**(3 Pt 2): p. 548-57.
138. Nilsson, L., et al., *Pertussis vaccination in infancy lowers the incidence of pertussis disease and the rate of hospitalisation after one and two doses: analyses of 10 years of pertussis surveillance*. Vaccine, 2012. **30**(21): p. 3239-47.
139. Higgs, R., et al., *Immunity to the respiratory pathogen Bordetella pertussis*. Mucosal Immunol, 2012. **5**(5): p. 485-500.
140. 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.
141. 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.
142. Barbic, J., et al., *Role of gamma interferon in natural clearance of Bordetella pertussis infection*. Infect Immun, 1997. **65**(12): p. 4904-8.
143. Mascart, F., et al., *Bordetella pertussis infection in 2-month-old infants promotes type 1 T cell responses*. J Immunol, 2003. **170**(3): p. 1504-9.
144. 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.
145. Ryan, M., et al., *Bordetella pertussis respiratory infection in children is associated with preferential activation of type 1 T helper cells*. J Infect Dis, 1997. **175**(5): p. 1246-50.
146. 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.
147. Dirix, V., et al., *Cytokine and antibody profiles in 1-year-old children vaccinated with either acellular or whole-cell pertussis vaccine during infancy*. Vaccine, 2009. **27**(43): p. 6042-7.
148. Esposito, S., et al., *Long-term pertussis-specific immunity after primary vaccination with a combined diphtheria, tetanus, tricomponent acellular pertussis, and hepatitis B vaccine in comparison with that after natural infection*. Infect Immun, 2001. **69**(7): p. 4516-20.
149. Rowe, J., et al., *Antigen-specific responses to diphtheria-tetanus-acellular pertussis vaccine in human infants are initially Th2 polarized*. Infect Immun, 2000. **68**(7): p. 3873-7.

150. 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.
151. 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.
152. 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.
153. Rodriguez, M.E., et al., *Fc receptor-mediated immunity against Bordetella pertussis*. J Immunol, 2001. **167**(11): p. 6545-51.
154. Guiso, N., et al., *Long-term humoral and cell-mediated immunity after acellular pertussis vaccination compares favourably with whole-cell vaccines 6 years after booster vaccination in the second year of life*. Vaccine, 2007. **25**(8): p. 1390-7.
155. Tran Minh, N.N., et al., *Cell-mediated immune responses to antigens of Bordetella pertussis and protection against pertussis in school children*. Pediatr Infect Dis J, 1999. **18**(4): p. 366-70.
156. Zepp, F., et al., *Immunogenicity of reduced antigen content tetanus-diphtheria-acellular pertussis vaccine in adolescents as a sixth consecutive dose of acellular pertussis-containing vaccine*. Vaccine, 2007. **25**(29): p. 5248-52.
157. 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.
158. Crawford, A., et al., *Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells*. J Immunol, 2006. **176**(6): p. 3498-506.
159. Whitmire, J.K., et al., *Requirement of B cells for generating CD4+ T cell memory*. J Immunol, 2009. **182**(4): p. 1868-76.
160. 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.
161. 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.
162. 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.
163. Perez-Andres, M., et al., *Human peripheral blood B-cell compartments: a crossroad in B-cell traffic*. Cytometry B Clin Cytom, 2010. **78 Suppl 1**: p. S47-60.
164. Nduati, E.W., et al., *Distinct kinetics of memory B-cell and plasma-cell responses in peripheral blood following a blood-stage Plasmodium chabaudi infection in mice*. PLoS One, 2010. **5**(11): p. e15007.
165. Blanchard-Rohner, G., et al., *Appearance of peripheral blood plasma cells and memory B cells in a primary and secondary immune response in humans*. Blood, 2009. **114**(24): p. 4998-5002.
166. *VACCINATION against whooping-cough; relation between protection in children and results of laboratory tests; a report to the Whooping-cough Immunization Committee of the Medical Research Council and to the medical officers of health for Cardiff, Leeds, Leyton, Manchester, Middlesex, Oxford, Poole, Tottenham, Walthamstow, and Wembley*. Br Med J, 1956. **2**(4990): p. 454-62.
167. Cherry, J.D., et al., *A search for serologic correlates of immunity to Bordetella pertussis cough illnesses*. Vaccine, 1998. **16**(20): p. 1901-6.
168. Granstrom, M. and G. Granstrom, *Serological correlates in whooping cough*. Vaccine, 1993. **11**(4): p. 445-8.
169. 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.
170. Versteegh, F.G., et al., *Age-specific long-term course of IgG antibodies to pertussis toxin after symptomatic infection with Bordetella pertussis*. Epidemiol Infect, 2005. **133**(4): p. 737-48.
171. Wendelboe, A.M., et al., *Estimating the role of casual contact from the community in transmission of Bordetella pertussis to young infants*. Emerg Themes Epidemiol, 2007. **4**: p. 15.

172. Warfel, J.M., et al., *Maternal and Neonatal Vaccination Protects Newborn Baboons From Pertussis Infection*. J Infect Dis, 2014.
173. Shakib, J.H., et al., *Tetanus, diphtheria, acellular pertussis vaccine during pregnancy: pregnancy and infant health outcomes*. J Pediatr, 2013. **163**(5): p. 1422-6 e1-4.
174. *Updated recommendations for use of tetanus toxoid, reduced diphtheria toxoid and acellular pertussis vaccine (Tdap) in pregnant women and persons who have or anticipate having close contact with an infant aged <12 months --- Advisory Committee on Immunization Practices (ACIP), 2011*. MMWR Morb Mortal Wkly Rep, 2011. **60**(41): p. 1424-6.
175. Chiappini, E., et al., *Pertussis re-emergence in the post-vaccination era*. BMC Infect Dis, 2013. **13**: p. 151.
176. Lindsey, B., B. Kampmann, and C. Jones, *Maternal immunization as a strategy to decrease susceptibility to infection in newborn infants*. Curr Opin Infect Dis, 2013. **26**(3): p. 248-53.
177. Roduit, C., et al., *Immunogenicity and protective efficacy of neonatal vaccination against Bordetella pertussis in a murine model: evidence for early control of pertussis*. Infect Immun, 2002. **70**(7): p. 3521-8.
178. 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.
179. White, O.J., et al., *Th2-polarisation of cellular immune memory to neonatal pertussis vaccination*. Vaccine, 2010. **28**(14): p. 2648-52.
180. Halasa, N.B., et al., *Poor immune responses to a birth dose of diphtheria, tetanus, and acellular pertussis vaccine*. J Pediatr, 2008. **153**(3): p. 327-32.
181. Knuf, M., et al., *Neonatal vaccination with an acellular pertussis vaccine accelerates the acquisition of pertussis antibodies in infants*. J Pediatr, 2008. **152**(5): p. 655-60, 660 e1.
182. Wood, N., et al., *Acellular pertussis vaccine at birth and one month induces antibody responses by two months of age*. Pediatr Infect Dis J, 2010. **29**(3): p. 209-15.
183. Sugai, T., et al., *A CpG-containing oligodeoxynucleotide as an efficient adjuvant counterbalancing the Th1/Th2 immune response in diphtheria-tetanus-pertussis vaccine*. Vaccine, 2005. **23**(46-47): p. 5450-6.
184. Gracia, A., et al., *Antibody responses in adult and neonatal BALB/c mice to immunization with novel Bordetella pertussis vaccine formulations*. Vaccine, 2011. **29**(8): p. 1595-604.
185. Cheung, G.Y., et al., *Effect of different forms of adenylate cyclase toxin of Bordetella pertussis on protection afforded by an acellular pertussis vaccine in a murine model*. Infect Immun, 2006. **74**(12): p. 6797-805.
186. Guiso, N., M. Szatanik, and M. Rocancourt, *Protective activity of Bordetella adenylate cyclase-hemolysin against bacterial colonization*. Microb Pathog, 1991. **11**(6): p. 423-31.
187. Feunou, P.F., et al., *Genetic stability of the live attenuated Bordetella pertussis vaccine candidate BPZE1*. Vaccine, 2008. **26**(45): p. 5722-7.
188. 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.
189. Skerry, C.M. and B.P. Mahon, *A live, attenuated Bordetella pertussis vaccine provides long-term protection against virulent challenge in a murine model*. Clin Vaccine Immunol, 2011. **18**(2): p. 187-93.
190. Feunou, P.F., J. Bertout, and C. Loch, *T- and B-cell-mediated protection induced by novel, live attenuated pertussis vaccine in mice. Cross protection against parapertussis*. PLoS One, 2010. **5**(4): p. e10178.
191. Berstad, A.K., et al., *A nasal whole-cell pertussis vaccine induces specific systemic and cross-reactive mucosal antibody responses in human volunteers*. J Med Microbiol, 2000. **49**(2): p. 157-63.
192. Berstad, A.K., et al., *Induction of antigen-specific T cell responses in human volunteers after intranasal immunization with a whole-cell pertussis vaccine*. Vaccine, 2000. **18**(22): p. 2323-30.
193. Bergquist, C., et al., *Intranasal vaccination of humans with recombinant cholera toxin B subunit induces systemic and local antibody responses in the upper respiratory tract and the vagina*. Infect Immun, 1997. **65**(7): p. 2676-84.

194. Mattoo, S. and J.D. Cherry, *Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to Bordetella pertussis and other Bordetella subspecies*. Clin Microbiol Rev, 2005. **18**(2): p. 326-82.
195. Mielcarek, N., et al., *Dose response of attenuated Bordetella pertussis BPZE1-induced protection in mice*. Clin Vaccine Immunol, 2010. **17**(3): p. 317-24.
196. Meyer, C.U., et al., *Cellular immunity in adolescents and adults following acellular pertussis vaccine administration*. Clin Vaccine Immunol, 2007. **14**(3): p. 288-92.
197. Schure, R.M., et al., *Differential T- and B-Cell Responses to Pertussis in Acellular Vaccine-Primed versus Whole-Cell Vaccine-Primed Children 2 Years after Preschool Acellular Booster Vaccination*. Clin Vaccine Immunol, 2013. **20**(9): p. 1388-95.