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PERTUSSIS: INFECTION, VACCINATION AND GENE POLYMORPHISMS

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To my family

ABSTRACT

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Pertussis: infection, vaccination and gene polymorphisms

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Pertussis or whooping cough is a human respiratory tract infection and a vaccine-preventable disease that is caused by *Bordetella pertussis* bacteria. Pertussis vaccination has been part of the Finnish national vaccine program since 1952. Despite extensive vaccinations, the incidence of pertussis has increased in many countries during the last decades. Large epidemics have been observed also in countries with high vaccine coverage. Inter-individual variation in immune responses is always encountered after vaccination. Low vaccine responses may cause vulnerability to pertussis even straight after vaccination. Reasons for low responses are not fully understood. The innate immune system is responsible for the initial recognition of pathogens and vaccine antigens. The role of innate immunity on pertussis immunity has not been thoroughly investigated. Mannose-binding lectin (MBL) and toll-like receptor 4 (TLR4) are important molecules of the innate immune system and in the recognition of pathogens. Cytokines form a signaling network that have a notable role in immune responses after infections as well as after vaccinations. Single nucleotide polymorphism (SNP) is common in genes encoding these molecules and the polymorphisms have been reported to affect vaccine response after viral and bacterial vaccines.

This study investigated the gene polymorphisms of *MBL2, TLR4* and interleukin (*IL)-10* promoter and their association with vaccine responses after acellular pertussis (aP) vaccination in Finnish adolescents and infants. Cell-mediated immune responses were investigated ten years after the previous pertussis vaccinations in young adults. In addition, the role of MBL deficiency in pertussis infection susceptibility was evaluated.

The results of this study show that subjects with *TLR4* polymorphism had lower antibody production and persistence after aP vaccination compared with normal allele. A specific SNP in the *TLR4* gene was associated with decreased antibody responses and persistence in adolescents after aP booster vaccination. Cell-mediated immune responses were partly detected ten years after the previous vaccination; booster vaccine clearly enhanced the responses. In addition, subjects with IL-10 polymorphism had altered cell-mediated immune responses. MBL deficiency was found to be more frequent in pertussis patients than healthy controls but the polymorphism of *MBL2* was not associated with antibody responses after acellular pertussis vaccination.

The novel finding of this study was that genetic variation in the innate immune system seems to play a role in altered pertussis vaccine responses as well as in pertussis infection. These new findings enlighten the mechanisms behind the low responses after pertussis vaccination and help to predict risk factors related to this phenomenon.

Key words: *Bordetella pertussis*, pertussis, vaccination, vaccine response, gene polymorphism, TLR, MBL, IL-10

TIIVISTELMÄ

KIRSI GRÖNDAHL-YLI-HANNUKSELA

Hinkuyskä: infektio, rokottaminen ja geenien vaihtelu

Turun yliopisto, Lääketieteellinen tiedekunta, Lastentautioppi; Turun yliopiston kliininen tohtoriohjelma (TKT); Turun yliopistollinen keskussairaala; Infektiotaudit osasto, Bakteeri-infektiot yksikkö, Terveyden ja hyvinvoinnin laitos, Turku

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Hinkuyskä on *Bordetella pertussis* –bakteerin aiheuttama hengitystieinfektio, jota vastaan on Suomessa rokotettu jo vuodesta 1952. Hinkuyskän esiintyvyys on noussut useissa maissa siitä huolimatta, että näissä maissa on saavutettu korkea rokotekattavuus. Laajoja epidemioita on havaittu myös näissä maissa, joissa rokotekattavuus on korkea. Kuten yleensä rokotuksilla, myös hinkuyskärokotuksen yhteydessä havaitaan rokotevasteissa yksilöiden välistä vaihtelua. Syytä alhaiseen rokotevasteeseen ei täysin tunneta. Luontainen immuniteetti muodostaa ensivasteen patogeenejä vastaan. Lisäksi se osallistuu rokoteantigeenien tunnistamiseen. Luontaisen immunteetin osuutta elimistön puolustuksessa hinkuyskää vastaan on tutkittu vain vähän. Mannosia sitova lektiini (MBL) ja tollin kaltainen reseptori 4 (TLR4) ovat tärkeitä luontaisen immuniteetin molekyylejä, jotka osallistuvat patogeenien tunnistamiseen. Interleukiinit (IL) ovat sytokiineihin kuuluvia signalointimolekyylejä, joiden muodostamilla signaalinvälitysketjuilla on merkittävä rooli immuunipuolustuksessa. Monimuotoisuus eli polymorfia on yleistä näitä molekyylejä koodaavissa geeneissä ja osan näistä polymorfioista on huomattu vaikuttavan rokotevasteisiin virus- ja bakteerirokotteiden yhteydessä.

Tässä väitöskirjassa tutkittiin *MBL2*-, *TLR4*- ja *IL-10* geenipolymorfian yhteyttä rokotevasteisiin hinkuyskärokotteen jälkeen suomalaisilla nuorilla aikuisilla sekä lapsilla. Hinkuyskäspesifisen soluvälitteisen immuunivasteen kehittymistä seurattiin kymmenen vuoden aikajänteellä. Lisäksi selvitettiin lisääkö MBL-proteiinin puutos alttiutta hinkuyskäinfektiolle.

Tulokset osoittavat, että soluttoman hinkuyskärokotteen jälkeen tutkimukseen osallistuneista henkilöistä niillä, jotka kantavat *TLR4* geenipolymorfiaa, oli alentunut hinkuyskäspesifinen vasta-aineiden tuotanto ja pysyvyys, verrattuna normaaliin geenimuodon kantajiin. Soluvälitteinen immuniteetti säilyi osittain kymmenen vuotta edellisestä rokotteesta ja tehosterokote nosti soluvälitteistä immuunivastetta merkittävästi. Lisäksi havaittiin ero soluvälitteisen immuniteetin vasteissa *IL-10* geenipolymorfiaa kantavien ja normaalin geenimuodon kantajien välillä. Hinkuyskäpotilailla puolestaan löydettiin useammin MBL-proteiinin puutos kontrolliryhmään verrattuna, mutta *MBL2* geenipolymorfia ei vaikuttanut hinkuyskärokotevasteisiin.

Väitöskirjan tulokset viittaavat siihen, että geneettinen vaihtelu luontaisen immuniteetin molekyyleissä vaikuttaa sekä rokotevasteisiin soluttoman hinkuyskärokotteen jälkeen että hinkuyskäinfektioalttiuteen. Tutkimuksen tulokset auttavat ymmärtämään mekanismeja ja ennakoimaan riskitekijöitä, jotka vaikuttavat poikkeavaan rokotevasteeseen ja infektioalttiuteen.

Avainsanat: Bordetella pertussis, hinkuyskä, rokottaminen, rokotevaste, geenipolymorfia, TLR, MBL, IL-10

CONTENTS

ABBREVIATIONS

aP	acellular pertussis vaccine
brk	*Bordetella* resistance to killing
bp	base pair
cAMP	cyclic adenosine monophosphate
cpm	counts per minute
CRD	carbohydrate-recognition domain
DC	dendritic cells
DTaP	diphtheria-tetanus-acellular pertussis vaccine (for primary vaccination)
dTap	diphtheria-tetanus-acellular pertussis vaccine (for adolescent booster vaccination)
DTwP	diphtheria-tetanus-whole cell pertussis vaccine
ECDC	European Centre for Disease Prevention and Control
ELISA	enzyme-linked immunosorbent assay
Fim	fimbriae
FHA	filamentous hemagglutinin
GMC	geometric mean concentration
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IRF	interferon regulatory factors
IS	insertion sequence
KTL	Kansanterveyslaitos
kDa	kilodalton
LPS	lipopolysaccharide
LOS	lipooligosaccharide
MALDI-TOF	matrix-assisted laser desorption/ionization time-of-flight mass spectrometer

MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MyD88	myeloid differentiation primary response gene
NaCl	natrium chloride
NK	natural killer
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
dNTP	deoxynucleoside triphosphate, in the text commonly written as "nucleotides"
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PRN	pertactin
PT	pertussis toxin
ptl	pertussis toxin liberation
PWM	pokeweed mitogen
RGD	arginyl (R) – glycyl (G) - aspartic acid (D) motif
RNA	ribonucleic acid
RSV	respiratory syncytial virus
SNP	single nucleotide polymorphism
SI	stimulation index
tcf	tracheal colonization factor
THL	Terveyden ja hyvinvoinnin laitos
TLR	toll-like receptor
TNF	tumor necrosis factor
TIRAP	TIR adaptor protein
TIR	Toll-interleukin-1 receptor
TRAM	TRIF-related adaptor molecule
TRAF	TNF receptor-associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
vag	*vir*-locus activated genes
WHO	World Health Organization
wP	whole cell pertussis vaccine

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles which are referred to in the text by Roman numerals (I – V). Unpublished data are also included.

I. Gröndahl-Yli-Hannuksela Kirsi, Vuononvirta Juho, Barkoff Alex-Mikael, Viander Markku, Van Der Meeren Olivier, Mertsola Jussi and He Qiushui. Gene polymorphism of toll-like receptor 4: effect on antibody production and persistence after acellular pertussis vaccination in adolescence. J Infect Dis. 2012 Apr 15; 205(8):1214-9.

II. Gröndahl-Yli-Hannuksela Kirsi, Vuononvirta Juho, Peltola Ville, Mertsola Jussi and He Qiushui. Lack of association between mannose binding lectin and antibody responses after acellular pertussis vaccinations. PLoS One. 2014 Feb 18;9(2):e88919.

III. Gröndahl-Yli-Hannuksela Kirsi, Kauko Leni, Van Der Meeren Olivier, Mertsola Jussi and He Qiushui. Pertussis specific cell-mediated immune responses ten years after acellular pertussis booster vaccination in young adults. *Manuscript submitted.*

IV. Gröndahl-Yli-Hannuksela Kirsi, Vahlberg Tero, Ilonen Jorma, Mertsola Jussi and Qiushui He. Polymorphism of IL-10 gene promoter region: association with cell mediated immunity after acellular pertussis vaccination in adults. *Manuscript submitted.*

V. Gröndahl-Yli-Hannuksela Kirsi, Viander Markku, Mertsola Jussi and He Qiushui. Increased risk of pertussis in adult patients with mannose-binding lectin deficiency. APMIS. 2013 Apr;121(4):311-5.

1. INTRODUCTION

Pertussis is a vaccine-preventable disease of the upper respiratory tract, which is caused by the gram-negative bacterium *Bordetella pertussis*. In the prevaccine era, pertussis was one of the leading causes of infant mortality (Cherry 1999). Vaccinations against pertussis were started in the 1930's with the experimental whole cell pertussis vaccines (wP). After the vaccine efficacy had been approved, between the late 1940's and the early 1950's, many countries started the nationwide pertussis vaccinations with diphtheria-tetanus-whole cell pertussis vaccine (DTwP). In Finland, pertussis vaccinations were started in 1952. Vaccinations reduced dramatically the pertussis incidence. However, due to the reactogenicity of the DTwP vaccine, it was widely replaced in industrialized countries with more tolerated acellular pertussis vaccine (DTaP) in the middle of 1990's. Around the same period, the incidence of pertussis has started to increase again and several large epidemics have been reported (Mooi *et al.* 2014). In Finland, DTaP vaccine has been the sole pertussis vaccine since 2005. It is known that antibodies decay fast after DTaP vaccination, but some protection can be provided also after that by cell-mediated immunity (CMI) (Ausiello *et al.* 1999, Hendrikx *et al.* 2011b, Mahon *et al.* 2000). However, it is not known how long the protection lasts. When large scale vaccinations are performed, there is always inter-individual variation observed. Some individuals do not respond properly to the vaccine by producing antibodies or inducing CMI (Poland *et al.* 2008, Kimman *et al.* 2007). This may cause increased risk to pertussis in these low- or non-responders. Several factors can affect this phenomenon such as age, nutrition and previous vaccinations. However, it is acknowledged that genetic variation in humans influences immune responses as the genes of the immune network are highly polymorphic (Kimman *et al.* 2007, Poland *et al.* 2011).

Pattern recognition molecules are important proteins of the innate immune system. They are involved in the initial recognition of extracellular pathogens. Toll-like receptor 4 (TLR4) and mannose-binding lectin (MBL) is a membrane-bound and a soluble pattern recognition molecule of the innate immune system, respectively. Cytokines are soluble signaling molecules of the immune system. All these molecules are known to be highly polymorphic and polymorphisms have been shown to be associated with altered antibody responses and infection susceptibility (Garred 2008, Schroder &Schumann 2005, Smith &Humphries 2009, Heitzeneder *et al.* 2012, Kaslow *et al.* 2008). In addition, MBL deficiency, which is caused by the gene polymorphism, has been linked to the susceptibility to infectious diseases (Heitzeneder *et al.* 2012). However, it has not been studied whether polymorphism in innate immune system affect vaccine response after acellular pertussis vaccine.

The primary aim of this study was to examine whether genetic variation observed in innate immune system is related to low immune response after dTap vaccination. In addition, connection between polymorphism in innate immune system and pertussis

infection frequency was evaluated. Cell-mediated immunity accounts for the protection in some extents after the waning of pertussis specific antibodies. One of the objectives of this study was to evaluate the CMI responses after dTap vaccination and the association of genetic variation with observed low CMI responses.

2. REVIEW OF THE LITERATURE

2.1. Pertussis

Pertussis, or whooping cough, is an acute, upper respiratory tract infection, caused mainly by a strictly human pathogen, gram-negative bacterium *Bordetella pertussis*. *B. parapertussis* and *B. holmesii* are other, less common, causative agent of pertussis with milder symptoms and shorter duration of the disease. The first reported pertussis epidemic in young children was described already in 1578 in Paris, France (Cone 1970). The clinical symptoms of pertussis have remained similar to those of the prevaccine era, nonetheless the severe complications were more frequently reported before the mass vaccinations (Cherry 1999).

2.1.1. Clinical pertussis

B. pertussis is transmitted from human to human with droplets from cough. After exposure to *B. pertussis*, bacteria adhere to the respiratory epithelium of the host (Paddock *et al.* 2008, Melvin *et al.* 2014). This incubation period lasts 5 – 21 day, most commonly it is seven to ten days (Crowcroft &Pebody 2006). Pertussis infection is characteristically divided into three stages: catarrhal, paroxysmal and convalescent phase. The catarrhal phase, which lasts from 7 to 14 days, resembles the common cold with low fever and mild cough. At this stage, pertussis is highly contagious but seldom diagnosed, as the symptoms resemble many other respiratory tract infections (Crowcroft &Pebody 2006, Wood &McIntyre 2008). Catarrhal phase is developed to paroxysmal phase after a few weeks. Paroxysmal phase is the representative stage of pertussis. During paroxysm, forceful coughing repeats five to ten times within a single expiration. The coughing has a typically whooping sound during the inspiration phase due to the massive inspiratory effort (Mattoo &Cherry 2005). During paroxysms, cyanosis and posttussive vomiting are common and the symptoms often are present without fever. Between the paroxysm episodes, patient can be asymptomatic (Hewlett &Edwards 2005). Paroxysmal phase lasts for 4 – 6 weeks, after which the frequency of paroxysms decreases and slow improving of the symptoms is seen. This convalescent phase normally lasts a week or two, but infrequently it can even take months, like the Chinese term for pertussis “cough of 100 days” well describes.

Complications are more often described in unvaccinated infants. Complications such as pneumonia, hyperleucocytosis, apnea, seizures and encephalopathy can be recognized with classical pertussis. The highest mortality rate to pertussis is in infants less than six months of age and especially in young infants less than two months of age. Infants account for almost 90 % of the fatal cases from pertussis in the United States (Hewlett &Edwards 2005).

Patients who have been previously immunized, especially adolescents and adults, often have an atypical pertussis infection, where prolonged coughing may be the only

evidence of pertussis (Hewlett &Edwards 2005). However, the longitude of the disease in adolescents and adults can be high with mean duration of 36 – 48 days (von Konig *et al.* 2002). Like in infants, also in adults the coughing can be paroxysmal, which disturbs the sleep but it may lack the classical whooping sound (von Konig *et al.* 2002, Cherry *et al.* 2005). The frequency of other symptoms such as vomiting, sweating attacks and syncope varies a lot (Hewlett &Edwards 2005). In addition, adults seek to the clinicians more slowly, usually after several weeks of coughing. This together with clinicians' unawareness of adult pertussis may lead to misdiagnosis as asthma, chronic sinusitis, mycoplasma infection or other lung infection (Cherry *et al.* 2005, Yaari *et al.* 1999). The prolonged diagnosis can lead to several secondary cases in more vulnerable age groups.

It has been estimated that one case can infect 17 susceptible secondary cases (Heininger 2012), which is as high as the transimission rate caused by measles (Winter *et al.* 2012). Household contacts are the major source of *B. pertussis* infection in infants, accounting for 73 – 82 % of the cases (de Greeff *et al.* 2010). Especially the parents are critical in transmitting pertussis to infants, as they are the source of 55 % of the primary cases (de Greeff *et al.* 2010, Wendelboe *et al.* 2007).

2.1.2. Diagnostics

Diagnosis of pertussis is a combination of clinical suspicion combined with proper laboratory tests. The World Health Organization (WHO) and European Center for Diseases Prevention and Control (ECDC) have the case definition for suspicion of pertussis (EU Comission decision 2008, WHO 2003). According to ECDC's and WHO's clinical case definition, a suspicion of pertussis should be considered when a patient has a cough of two weeks or longer and at least one of the following symptoms: 1) paroxysms of cough 2) inspiratory whooping or 3) post-tussive vomiting. A laboratory confirmed case should have at least one of the following: isolated *B. pertussis* from a clinical specimen, detection of *B. pertussis* nucleic acid from a clinical specimen or *B. pertussis* specific antibodies. Diagnostic laboratory test should be selected based on the age of the patient and the duration of the symptoms. Commonly used diagnostic methods are those listed in the WHO's case definition; culture, polymerase chain reaction (PCR) and serology.

Culture is thought to be the golden standard for the diagnosis. The specificity of the culture is known to be high. However, the sensitivity of the culture is affected by several factors. The positivity rate of the culture decreases drastically if the sample is taken more than two weeks after the beginning of the symptoms or if the patient has received antimicrobial treatment. The sensitivity remains 1 – 3 %, if the specimen is collected after three weeks from the onset of cough (Kretsinger *et al.* 2006). The sensitivity is reduced also if the specimen is improper, meaning that it is a per nasal or throat swab or a sweep from the anterior nares (Crowcroft &Pebody 2006). The correct place for the specimen is in the posterior nasopharynx as *B. pertussis* mainly attaches to the ciliated epithelium of the nasopharynx (Paddock *et al.* 2008). In addition to swabs, nasopharyngeal aspirates are often taken, especially from infants. The specific medium for *B. pertussis* culture is

Reagan-Lowe agar and Bordet-Gengou agar. Proper culture condition is ambient ≥+35°C, additional CO_2 is not needed (Kerr &Matthews 2000). Colonies of light grey, highly convex, pearly and mercury-like appearance are visible in average 72 hours but longer incubation increases the sensitivity (Katzko *et al.* 1996). The clinical laboratories answers the positive result commonly after three days but the confirmation of the negative result is given after seven days of incubation (UTUlab 2008).

In addition of the diagnostic purpose, collecting the *B. pertussis* strains from pertussis patients is important for the molecular typing of the circulating bacteria and antibiotic susceptibility testing. Molecular typing helps to understand the bacterial adaptation to vaccine induced immunity and the emergence of new variants of *B. pertussis*. Antibiotic susceptibility testing is important for monitoring of antimicrobial resistance strains. Recently, erythromycin resistant *B. pertussis* has emerged in China and Iran (Wang *et al.* 2014, Shahcheraghi *et al.* 2014).

Amplification of the nucleic acid using PCR has sustained its position as a valuable diagnostic method since the early 1990's, when it was described as a sensitive method for detecting *B. pertussis* from the nasopharyngeal specimens (Meade &Bollen 1994, He *et al.* 1994). The benefit of PCR is its speed, sensitivity and specificity. In the PCR reaction, nucleic acid is amplified, which allows the method to detect even very low numbers of bacteria or non-viable bacteria. The sensitivity of PCR compared with culture can be four-fold higher and it can be used for samples taken from patients with longer period after onset of the symptoms (He *et al.* 1996). This is a clear advantage for the pertussis diagnostics, as a positive result can be detected even after the culture becomes negative (Edelman *et al.* 1996). *B. pertussis* genome contains several elements which have been used as a target in the diagnostic PCR, such as the commonly used pertussis toxin (PT) promoter (Houard *et al.* 1989) and insertion sequence (IS) element 481 (Glare *et al.* 1990). The most widely used target is a transposable element IS481, which is present in more than 200 copies in the *B. pertussis* genome (Parkhill *et al.* 2003). However, IS481 sequence is also found in the genome of *B. holmesii*, but only 8 – 10 copies (Reischl *et al.* 2001). Due to this cross reactivity of IS481 between two species, secondary targets such as RecA have been proposed to differentiate these two species (Loeffelholz 2012, Antila *et al.* 2006). This may though decrease the analytical sensitivity of the PCR (Kosters *et al.* 2002) since only one copy of the gene in the *B. pertussis* genome. The high copy number of IS481 increases the sensitivity of the PCR which may increase the risk for false positive results mainly due to contaminations at different stages of the process (Cherry *et al.* 2005).

After infection, increase is noticed in the serum concentration of immunoglobulins (Ig) G, IgA and IgM against specific antigens of *B. pertussis*, such as pertussis toxin (PT), pertactin (PRN) and filamentous hemagglutinin (FHA). The basis of serology is the measurement of antibody concentrations against *B. pertussis* specific antigens from serum samples using enzyme-linked immunosorbent assay (ELISA). Purified PT and FHA are commonly used as a capture molecule, PRN and fimbriae (fim) less extensively (Guiso *et al.* 2011). From these, only PT is solely specific for *B. pertussis* and it is

thus recommended to be used in serological diagnosis (Guiso *et al.* 2011, ECDC 2012). The first serological pertussis ELISA described for the diagnostics had sonicated whole bacteria as a coating molecule and this method is still used in pertussis diagnostics in Finland (Viljanen *et al.* 1982). Increase in the IgG concentration against PT, FHA and PRN is seen after infection. PT is *B. pertussis* specific whereas anti-FHA and anti-PRN IgG may be caused by cross-reactivity with other *Bordetellas* or other species such as *Haemophilus* species and *Mycoplasma pneumoniae* (Guiso *et al.* 2011). IgA against PT, FHA and PRN is produced less extensively, only in 20 – 50 % of the subjects (Muller *et al.* 1997). Even PT is specific for *B. pertussis*, serological tests do not distinguish the antibodies created from infection and vaccination, as it is present both in the bacteria and in the vaccines. This may interfer the interpretation of the results and that is why the vaccination status and age of the patient should be taken into accout when evaluating the results. Increase in the antibody concentration is seen rather late, only after one or two week from the onset of the symptoms. Serology can be performed from paired sera, the first taken from the acute phase and the second from the convalescent phase. However, in the clinical practice a single serum is used more commonly. In neonates and infants younger than two years of age, PCR or/and culture should be used as a diagnostic method, due to the possible interference of vaccination doses given. In addition, the antibody production after infection may be slow in this age group (Guiso *et al.* 2011, Muller *et al.* 1997). In older children, adolescents and adults serology is the most commonly used method in the diagnostics. Table 1 summarizes the three commonly used pertussis diagnostic methods.

Table 1. Summary of culture, PCR and serology in pertussis diagnostics.

Method	Duration of symptoms	Suitable age	Advantages	Disadvantages
Culture	< 3 weeks	All patients	Cheap Collection of circulating strains for surveillance High sensitivity in infants	Decreased sensitivity with time Time-consuming
PCR	up to 4 – 6 weeks	All patients	Sensitive Specific Speed Longer usability	Contamination risk Specificity depends on the target
Serology	2 – 12 weeks	>2 years of age >1 year from the last pertussis vaccine	Longer usability Easy sampling	No differentiation between vaccine and infection induced response

Finland has a long tradition and a pioneering role in the initiation of pertussis diagnostics with serology in the 1980's (Viljanen *et al.* 1982) and PCR in the 1990's (He *et al.* 1994). Currently in Finland, serology is the main diagnostic method for pertussis accounting for 82 % of the laboratory-confirmed pertussis cases and the rest (18 %) is diagnosed by PCR or culture (National Infectious Disease Register, THL).

2.1.3. Pertussis epidemiology

In the pre-vaccine era, pertussis was mainly reported as a childhood disease. Most of the children had pertussis by the age of five to seven years. The immunity in the population, both in the children and adults, was regularly boosted by children's infections. Between 1920 and 1940, the estimate of pertussis attack rates in some parts of the USA has been as high as 872/100 000 population (Cherry 1999). The start of the nationwide vaccinations in the 1950's had a positive impact on the incidence in many countries and the control of the disease was clear. The incidence of pertussis started to peak again in many developed countries after the 1990's. According to WHO's estimate, in 2008 there were 16 million pertussis cases worldwide and on average 195 000 pertussis related deaths. Most of the cases, 95 %, are recorded in the developing countries (WHO 2010). These statistics keeps pertussis as one of the leading causes of vaccine-preventable deaths worldwide.

The epidemic cycle of pertussis has remained static 2 – 5 years since the prevaccine era, as the bacteria continue to circulate globally despite the mass vaccinations (Mills *et al.* 2014). Epidemics and high incidence has been noticed in many countries such as The Netherlands (van der Maas *et al.* 2013), Spain (Sizaire *et al.* 2014) and Australia (Spokes *et al.* 2010). California, in the USA, witnessed the largest epidemic in more than 60 years in 2010, when more than 10 000 pertussis cases and 10 deaths among infants were reported (Winter *et al.* 2012). Another epidemic peaked in 2014, with almost 11 200 reported cases (record in March 2015) (CDPH 2015). A large outbreak with similar figures was witnessed in the United Kingdom between 2012 and 2013. In this epidemic, 17 deaths were reported, all in unvaccinated infants aged 2 – 9 weeks at the onset of the symptoms (Amirthalingam *et al.* 2014).

In addition to the highest incidence observed in infants, the burden of pertussis has shifted more to older children, adolescents and adults. These age groups serve as a big reservoir for *B. pertussis* and they cause a significant risk of pertussis for infants too young to be vaccinated. Adults who have been vaccinated only as a child and have no natural boosting of the immunity from the community, then transmit the bacteria to young infants (Hewlett &Edwards 2005). The increased incidence may also be related to increased awareness and better diagnostics and surveillance. In Europe, the incidence of adolescents older than 14 years of age was increased 115 % from the year 1998 to 2000 (Celentano *et al.* 2005). Between 2003 and 2007, the total incidence in 20 European countries was 4.1/100 000 (EUVAC.net 2011). The proportion of patient >15 years of age increased yearly (Zepp *et al.* 2011). Nevertheless, the age specific incidence of pertussis in young infants, less than 6 months of age, is much higher compared with other age groups. For example in the USA in 2013, the incidence of infants younger than 6 months was 138/100 000, incidence of infants 6 to 11 months of age was 39.4/100 000. In Europe, the average incidence for infants younger than 1 year of age was 35.5/100 000 in 2003 – 2007, even though big variation is noticed between the countries. (Celentano *et al.* 2005, EUVAC.net 2011, CDC 2013) However, the incidence of these young infants has remained stable in Europe whereas in the USA even increase is seen (Wood &McIntyre 2008, Celentano *et al.* 2005, Zepp *et al.* 2011). The comparison of incidences

between countries is rather difficult and should be made with caution. This is due to the different case definitions, diagnostic methods, surveillance and reporting systems as well as possible under recognition of adult patients with unusual or lack of symptoms.

2.1.4. Pertussis in Finland

Pertussis has been recognized in Finland already in the 19th century. In the middle of 19th century, the mortality due to pertussis was estimated to be 7.5 % of the total mortality rate of the population (36 ‰). After that, the mortality due to pertussis has gradually decreased to 0.02 ‰ from the whole population in the 1920's and 0/100 000 inhabitants in the 1970's (Huovila 1981, Forsius 2005). However, even though the mortality decreased, the incidence remained high until the 1950's. Between 1920 and 1950, before the vaccination law came into effect in 1952, the incidence of pertussis varied from 160.8 to 424.8/100 000 inhabitants. Vaccinations decreased the incidence to 2.8/100 000 inhabitants in the 1970's (Huovila 1981). Since 1995, only laboratory confirmed cases have been reported to National Infectious Disease Register collected by National Institute for Health and Welfare (THL, former Kansanterveyslaitos KTL). Similarly to other western countries, also in Finland, the beginning of pertussis re-emergence was seen in the 1980's (Huovila 1981, Elomaa *et al.* 2005). The number of annually reported cases has been rather stable since the last epidemic in 2003 – 2004 when 1263 and 1631 laboratory confirmed cases were reported, respectively (Figure 1.). The increase in the proportion of pertussis cases in older age groups can also be seen in Finland. In 1995, 37.1 % of the reported cases were in the age group of older than 15 years, whereas in 2013 the same age group accounted 57.3 % of the cases (Figure 2.).

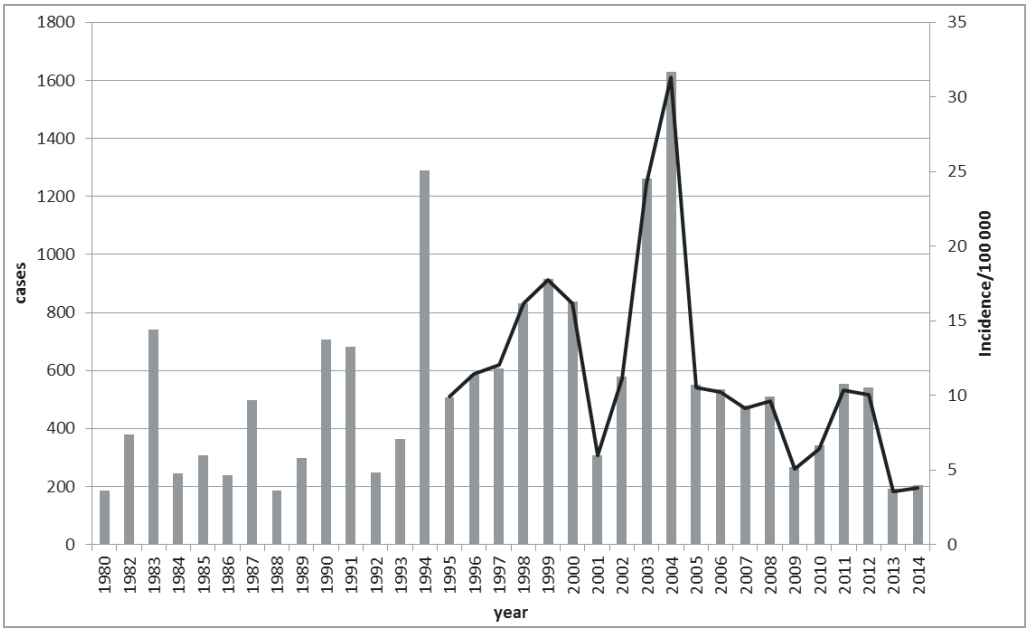

Figure 1. Laboratory confirmed pertussis cases (grey bars) and incidence per 100 000 (black line) in Finland between 1980 and 2014. Based on (THL 2014, WHO 2014)

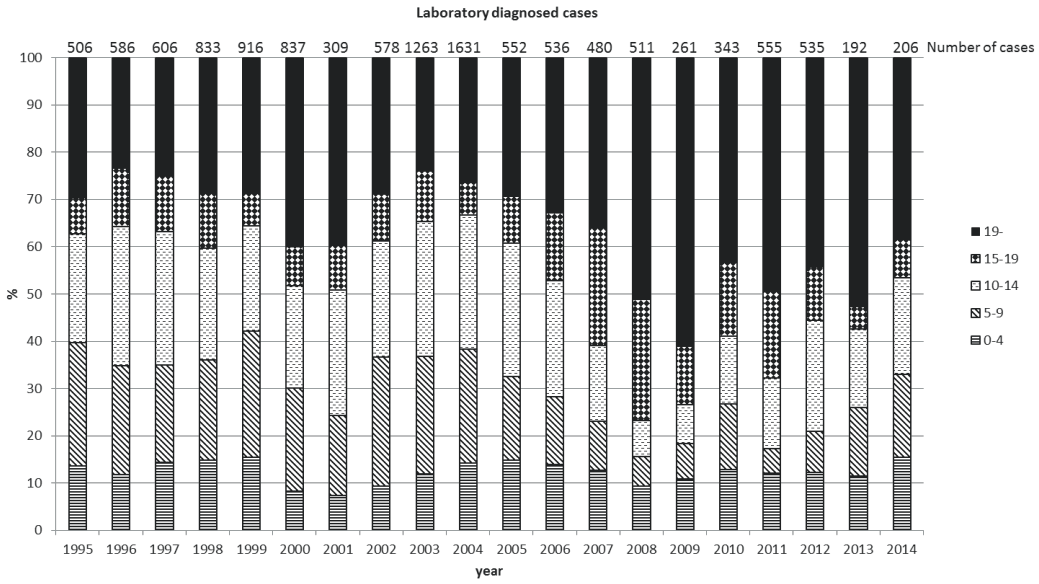

Figure 2. Laboratory diagnosed, reported cases and the proportion of different age groups. Based on National Infectious Disease Register, THL

2.2. *Bordetella pertussis*

In 1900, Jules Bordet observed the causative agent of pertussis microscopically from a sputum sample of a pertussis patient. It was six years later J. Bordet reported with Octave Gengou that they had successfully isolated *B. pertussis*, after discovering the optimal growth medium, Bordet Gengou medium, which is still used for the culture (Guiso 2014). *B. pertussis* was first called as *Haemophilus pertussis* as it needs blood on the agar. Later in the 1950's the name was changed to *Bordetella pertussis* as an honor for Jules Bordet (Gerlach *et al.* 2001). *B. pertussis* is a strictly aerobic, gram-negative coccobacillus that is classified to the genus Bordetellae.

2.2.1. Bordetellae genus

The Bordetella genus includes nine species: *B. ansorpii, B. avium, B. bronchiseptica*, *B. hinzii, B. holmesii*, *B. parapertussis, B. pertussis*, *B. petrii* and *B. trematum* (Gross *et al.* 2010). From these *B. pertussis*, *B. parapertussis*, *B. holmesii,* and in immunocompromised patients also *B. bronchiseptica,* are human pathogens. *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are the most widely studied species of the genus. Compared with other pathogenic bacteria which have notable genotypic diversity, such as *Eschericia coli*, *Haemophilus influenzae* and *Streptococcus pyogenes*, the genetic diversity of *Bordetellas* is constricted, due to very late divergence from the evolutionary progenitor (van der Zee *et al.* 1997). In 2003, the whole-genome sequencing project verified the previous findings (van der Zee *et al.* 1997), that *B. pertussis* and *B. parapertussis* are

both independently evolved from *B. bronchiseptica,* and from these two, *B. pertussis* has diverged more from the common progenitor (Parkhill *et al.* 2003, Bart *et al.* 2014). The genome of *B. pertussis* is notably smaller compared with *B. bronchiseptica* and *B. parapertussis* (4.1 x10^6 base pairs (bp) vs 5.3x10^6 and 4.7x10^6 bp, respectively). *B. pertussis* and *B. parapertussis* have rather similar deletions and rearrangements in their genomes, but in *B. pertussis* these events are even more extreme. Reduction in the genome has mainly been caused by transposable elements called insertion sequence (IS) elements. *B. pertussis* has more than 200 copies of IS481 which has caused the rearrangements, deletions and pseudogene formation during the late evolution (Parkhill *et al.* 2003).

2.2.2. Virulence factors

During the colonization to the epithelium of the respiratory tract, *B. pertussis* is forced to compete for the space and nutrient in the epithelium with normal flora as well as evade the host's immune mechanisms. For the survival, *B. pertussis* produces a wide range of toxins and adhesins that are also critical for the pathogenesis of the bacteria. These virulence factors are also the antigens which has been selected in various combinations for the currently used pertussis vaccines.

2.2.2.1. Adhesins

The adhesins that are needed for the adherence and colonization are also the main virulence factors of *B. pertussis*; filamentous hemagglutinin (FHA), pertactin (PRN) and fimbria (Fim). In addition to these, *B. pertussis* produces also other adhesins; BrkA named based on its location in *Bordetella* resistance to killing –locus (Fernandez &Weiss 1994), tracheal colonization factor (Tcf) (Finn &Stevens 1995) and Vag8, named for its gene belonging to a group of *vir*-locus activated genes (Finn &Amsbaugh 1998). Hereafter the focus is only on the adhesins FHA, PRN and Fim, since these are included in many of the acellular pertussis vaccines.

Filamentous hemagglutinin

FHA is a dominant adhesion molecule of *B. pertussis* against eukaryotic cells (Relman *et al.* 1989, van den Berg *et al.* 1999). It is also highly immunogenic and that is why it is included as an antigen in many of the currently used acellular vaccines. FHA is a prototypic protein of the two-partner secretion (TPS) (Coutte *et al.* 2001). TPS includes two proteins, one cargo protein, here FHA, which is transported through the outer membrane of the gram-negative bacterium by transporter protein, FhaC (Noinaj &Buchanan 2014, Jacob-Dubuisson *et al.* 1999, Guedin *et al.* 2000). FHA is first synthetized as a large ~370 kilodaltons (kDa) precursor protein which is cleaved to ~220 kDa trans-membrane protein (Locht *et al.* 1993). FhaC is a 16-stranded, transmembrane β-barrel, which forms a pore across the membrane. This pore is used for the secretion of FHA through the membrane.(Clantin *et al.* 2007) FHA has at least four different binding

domains which are used for the attachment to the epithelium (Mattoo &Cherry 2005, de Gouw *et al.* 2011); the classical arginyl-glycyl-aspartic acid (RGD)-motif, heparin binding motif, carbohydrate recognition domain and integrin binding domain (Ishibashi *et al.* 2001, Prasad *et al.* 1993, Menozzi *et al.* 1994). The FHA's ability to bind heparin is associated with the erythrocyte agglutination during *B. pertussis* infection (Menozzi *et al.* 1994). Typically adhesins remain attached at the bacterial surface. However, a significant amount of FHA is also released from the surface into the extracellular space (Locht *et al.* 1993). Secreted FHA is needed for the bacteria to persist and multiply in the lungs of a mouse model, as this free FHA helps the bacteria disperse from microcolonies and detach from the epithelium (Coutte *et al.* 2003). This type of loose connections between bacterial adhesion molecules and the host facilitate the bacterial spread in the respiratory track (de Gouw *et al.* 2011). In addition to the adhesion, FHA functions as an immunomodulator that interferes with the host immune responses against *B. pertussis*. In macrophages and dendritic cells, *in vitro*, FHA suppresses the pro-inflammatory interleukin (IL)-12 production by increasing the expression of IL-10 (McGuirk &Mills 2000, McGuirk *et al.* 2002). FHA also mediates proapoptotic response in monocytic and bronchial epithelial cells *in vitro* (Abramson *et al.* 2001). Despite of the critical role of FHA as an adhesive and immunomodulator, very recently, *B. pertussis* isolate not expressing FHA has been observed in France (Hegerle *et al.* 2012).

Pertactin

Pertactin (PRN) is a common component of the acellular vaccines due to its distinguished role as an inducer of antibody production which has been proven in clinical trials (Storsaeter *et al.* 1998, Cherry *et al.* 1998, Gustafsson *et al.* 1996). It is a 69 kDa sized autotransporter protein located in the outer membrane of *B. pertussis* (Charles *et al.* 1989). Autotransporters are typically proteins that are able to channel their own secretion through the outer membrane (Henderson &Nataro 2001). PRN functions as an adhesin by using the RGD-motif of the protein (Leininger *et al.* 1991). The role of PRN as an adhesion protein has been contradicting, as in human bronchial and laryngeal epithelial cells, PRN deficient *B. pertussis* retains its ability to attach the cells (van den Berg *et al.* 1999, Roberts *et al.* 1991). The ligands of PRN in the host have not been verified either (de Gouw *et al.* 2011). The rise of *B. pertussis* not expressing PRN has been observed in many countries such as Japan, USA, Australia, France, Finland, the Netherlands, Sweden, Norway and Israel. (Otsuka *et al.* 2012, Bouchez *et al.* 2009, Barkoff *et al.* 2012, Pawloski *et al.* 2014, Zeddeman *et al.* 2014, Bamberger *et al.* 2015). These evidences support the dispensable role of PRN for *B. pertussis* and the adaptation of *B. pertussis* to vaccinated populations in countries that use aP vaccine.

Fimbriae

Similar to FHA, fimbriae (Fim) are also filamentous adhesion molecules bound at the outer membrane of *B. pertussis*. However, FHA and Fim belong to different protein families. Fim consists of two subunits. The major subunit is either Fim2 or Fim3, that

are 22,5 kDa and 22 kDa, respectively. Fim2 and Fim3 also correspond to the serotypes of the Fim protein (Robinson *et al.* 1989, Blom *et al.* 1983). The minor subunit is FimD, with molecular size of 40 kDa (Geuijen *et al.* 1997, Willems *et al.* 1993). Three serotypes of Fim are reported with the expression of Fim2, Fim3 or both Fim2,3 (Robinson *et al.* 1989). The difference between the serotypes is caused by a variation in the promoter region of the *fim* gene operon. Insertion or deletion of cysteines in a cysteine rich region leads to a phase-variation in the expression of the *fim* genes (Willems *et al.* 1990). The major domain of Fim recognizes sulfated sugar-containing molecules on the respiratory epithelia (Mooi *et al.* 1992), whereas FimD is important for the binding to integrins on monocytic cells (Hazenbos *et al.* 1995). Fim is considered as the major adhesin together with FHA (Funnell &Robinson 1993). However, in a mouse model, it has been shown that FHA is more crucial for the adherence than Fim. *B. pertussis* defective in Fim retained their ability to colonize mice, whereas with the FHA deficient strains, the adherence was severly disrupted (Mooi *et al.* 1992). This may be due to the differing role of these molecules during the infection. FHA is needed for the initial adherence, whereas Fim is responsible for the persistence of adherence and spreading of the bacteria to other locations in the respiratory tract (Mooi *et al.* 1992). Fim is also immunogenic and antibodies against Fim have been reported to correlate with the protection (Storsaeter *et al.* 1998, Cherry *et al.* 1998).

2.2.2.2. Toxins

After entering the nasopharynx, *B. pertussis* adheres to the epithelium and produces a wide array of virulence factors including both adhesins and toxins. The main toxin produced exclusively by *B. pertussis* is pertussis toxin (PT). *B. parapertussis* and *B. bronchiseptica* have the *ptx* gene as well, but they do not express PT due to mutations in the promoter region of the gene (Arico &Rappuoli 1987). However, PT is not the only toxin; also adenylate cyclase toxin (ACT), lipopolysaccharide (LPS), tracheal cytotoxin (TCT) and dermonecrotic toxin (DNT) are produced. Here, only the main toxins PT, ACT and LPS are described.

Pertussis toxin

PT is a protein composed of hexameric AB_5 structure and it is 105 kDa in size. AB toxins, like PT, cholera toxin and diphtheria toxin are ADP-ribosylating toxins (Katada &Ui 1982). PT hexamer consists of a catalytic subunit referred as A or S1 subunit and B_5 subunit consisting of S2, S3, two S4 and S5. Subunits S1 – S5, in order of decreasing size, are produced by the genes *ptxA* to *ptxE* (Locht &Keith 1986, Nicosia *et al.* 1986). S2 – S5 form a pentamer, including two S4 subunits. This pentamer is used for the transportation of the catalytic S1 into the host cell. Before exporting PT, it is assembled in the periplasm of *B. pertussis* (Pizza *et al.* 1990). After this, PT is transported with pertussis toxin liberation (Ptl) type IV secretion system through the inner and outer membrane to the extracellular space (Weiss *et al.* 1993). PT is able to bind to almost all cell types and ligands are typically glycoproteins with sialic acid but glycosylation

is not a prerequisite for binding (Locht *et al.* 2011, Saukkonen *et al.* 1992). PT enters the cytosol of the host cells and in the cytoplasmic membrane the S1 subunit ADP-ribosylates the guanine nucleotide protein (G protein), inactivating it (Locht *et al.* 2011). This G protein inactivation causes the accumulation of cyclic adenosine monophosphate (cAMP), an important messenger in the cell signaling, leading to dysregulation of immune responses. This causes a variety of biological actions, depending on the targeted cells, such as histamine sensitization, leukocytosis, lymphocytosis and delayed recruitment of neutrophils to the respiratory tract (Munoz *et al.* 1981, Verschueren *et al.* 1991, Morse &Morse 1976). The role of PT for the bacterial adhesion is currently considered as a facilitating factor but the main function is the modulation of host immune responses (de Gouw *et al.* 2011, Saukkonen *et al.* 1992, Carbonetti *et al.* 2003). Antibodies against PT are important for the protection against pertussis and that is why PT is included, alone or in combination with other antigens, in all currently used acellular vaccines (Locht *et al.* 2011). Nevertheless, similar to the strains not expressing FHA and PRN, also *B. pertussis* strains not expressing PT have been reported (Bouchez *et al.* 2009).

Adenylate cyclase toxin

ACT is a 200 kDa protein with adenylate cyclase and hemolytic activity. In addition to *B. pertussis,* also *B. parapertussis* and *B. bronchiseptica* produce ACT. It is produced as an inactive protein, which is transformed to the active form posttranslationally.(Glaser *et al.* 1988) The active protein is transported to the extracellular space, in which it resides in the proximity of the outer membrane of the bacteria (Hewlett *et al.* 1976). Interaction between FHA and the membrane-bound ACT seems to help the retention of ACT in the proximity of the bacteria and facilitate its adhesion function (Zaretzky *et al.* 2002). Only part of the produced ACT enters the host cell, where the calmodulin binding domain causes the calmodulin derived activation of the adenylate cyclase, which leads to increased production of cAMP. The hemolysin activity of ACT is based on its ability to form cation-selective channels in membranes (Ladant &Ullmann 1999). In addition, inhibition of chemotaxis and phagocytosis as well as apoptotic function has also been reported with ACT (Boyd *et al.* 2005). ACT is not included in any of the currently used acellular vaccines.

Lipopolysaccharide

LPS is a characteristic and dominant element of the outer membrane of gram-negative bacteria, likewise *B. pertussis*. It is also an endotoxin. The basic structure of LPS consists of a lipid A molecule and a two-part polysaccharide; the core oligosaccharide and a polysaccharide protruding outward the bacterial membrane called O-antigen (Fedele *et al.* 2013). In *B. parapertussis* and *B. bronchiseptica,* LPS includes the O-antigen whereas in *B. pertussis* LPS is lacking the O-antigen structure (Caroff *et al.* 2001). The LPS absent of O-antigen is commonly called LOS. Because of the LPS' wide prevalence in gram-negative bacteria, host immune defense senses it easily by molecules such as surfactants and toll-like receptor 4 (TLR4) (Augusto *et al.* 2002, Poltorak *et al.* 1998).

However, *B. pertussis* is able to evade this recognition via surfactant due to the lacking of O-antigen. Whereas for TLR4 recognition, the lipid A moiety of LPS is the main domain identified (Saitoh *et al.* 2004).

2.2.3. Strain variation

Since the introduction of pertussis vaccinations, the circulating *B. pertussis* populations have been observed to change genetically (Kallonen &He 2009). The variation has been most extensively studied in the genes associated with the virulence, especially the genes coding for pertactin (*prn*), the A subunit of PT (*ptxA*) and fimbriae (*fim*) as well as in pertussis toxin promoter (*ptxP*) (van Gent *et al.* 2012). The variation is commonly caused by a single nucleotide polymorphism (SNP), but for *prn* the difference is insertions, deletions or repeats of nucleotides at the variable region. There are several alleles identified from each of the virulence genes; *ptxP* (17 alleles), *ptxA* (5), *prn* (13), *fim2* (2) and *fim3* (3) (Mooi *et al.* 2014). In addition to SNPs, gene loss has been observed with genome-wide hybridization analysis (Caro *et al.* 2006, Heikkinen *et al.* 2007, Kallonen *et al.* 2011, King *et al.* 2010). Temporal changes in the *B. pertussis* have been observed in several countries and commonly predominant strains are distributed widely (Mooi 2010). Probably *B. pertussis* uses genetic variation as an advantage to adapt in an environment with high selection pressure from the vaccinated population. It has been questionned whether this affects the vaccine effectiveness (Mooi *et al.* 2001).

2.3. Pertussis vaccination

After the first isolation of the *B. pertussis* in the early 1900's, and due to the high mortality of the pertussis disease, the vaccine development against this serious disease was started. The first experimental vaccine against pertussis was designed in 1933. Slight protection was reported with this vaccine composed of suspended bacteria. By that time, the same preparation was used as a cure for pertussis infection and to immunize against pertussis. (Cherry 1996) Between the 1940's and the 1950's several industrialized countries started the large-scale vaccinations against pertussis. Until this milestone, pertussis had been the main cause of infant mortality and more than every other child had suffered from it before school entry (Halperin 2007). The introduction of the whole cell pertussis (wP) vaccine decreased drastically the incidence and mortality from pertussis.

2.3.1. Vaccine types

Two types of pertussis vaccines are current in use; whole cell pertussis (DTwP) and acellular pertussis (DTaP) vaccine, both in combination with diphtheria (D) and tetanus (T). For the vaccine nomenclature; the size of the letter describes the amount of antigen included in the vaccine. Capital letter indicates higher amount of antigen whereas small letter is a sign of reduced antigen composition, which is common for booster vaccines.

At the beginning of vaccinations, wP was administrated as a monocomponent, but soon a combination of DTwP were available and recommended (Mattoo &Cherry 2005). The isolation of pertussis toxin and its use as a vaccine component was not similarly possible as it was with diphtheria and tetanus toxoids, because of the difficulties in the growing of *B. pertussis*.

Whole-cell vaccine is produced from the heat-killed bacteria. The bacterial strains selected for the synthesis of the vaccine were those circulating during the development phase of DTwP (Guiso 2014). However, the reproducibility of the DTwP vaccine is not simple because of the difficulty of *B. pertussis* growth. The effect of DTwP vaccination on pertussis incidence was significant. In fifteen years, the incidence of pertussis in the USA decreased from 209 cases/100 000 to 51 cases/100 000 (Shapiro-Shapin 2010). Even DTwP was effective against pertussis; the drawback of this vaccine was its reactogenicity. This aspect is extensively described in a review by Mattoo and Cherry (Mattoo &Cherry 2005). Local reactions at the injection site such as redness and swelling were commonly recorded, as well as fever (≥38^{o}C). The most adverse reactions were related to neurological disorders and sudden deaths, although they occurred rarely. The fear of the adverse vaccine reactions even led to the discontinuation of pertussis vaccination in Sweden from 1979 to 1996 (Romanus *et al.* 1987). In Japan, two fatalities led to a temporary, two months, suspension of vaccination. A broad refusal of the DTwP vaccine was followed, causing the vaccine coverage to decline to 10 % (Sato &Sato 1999). Nevertheless, the DTwP vaccines are still administrated in Poland and Serbia in Europe and in most of the developing countries as the cost of the DTwP vaccine compared with DTaP is more than 10-times lower, which makes the DTaP not affordable for the low-income countries (Zawadka *et al.* 2014, Dakic *et al.* 2010, Sheridan *et al.* 2014).

The development of a replacement vaccine for DTwP started after the 1970's, when it was technically possible to extract and purify the antigen components of the *B. pertussis* (Mattoo &Cherry 2005). Acellular vaccine is composed of the purified antigens of the *B. pertussis*. Japan was the first country to report the replacement of DTwP with DTaP in 1981 (Sato &Sato 1999). After that, most of the developed countries have followed. In the 1990's series of randomized, double-blinded efficacy trials were conducted to evaluate the immunogenicity, efficacy and safety of acellular vaccine. These studies were performed in countries such as Italy, Sweden and Germany, who all had previously used DTwP in their vaccine program (Gustafsson *et al.* 1996, Greco *et al.* 1996, Schmitt *et al.* 1996). The supportive data obtained on DTaP vaccines safety and efficacy led to the licensure of the vaccine in many developed countries. Currently available DTaP vaccines contain one to five antigens: PT, PRN, FHA, Fim2 and Fim3 in combination of PT-FHA, PT-PRN-FHA, PT alone or all five antigens together. To strengthen the immune response, antigens are combined with alum adjuvant. DTaP is significantly less reactogenic and more tolerated compared with DTwP and due to this; DTaP can also be administrated to adolescents and adults (Zepp *et al.* 2011, Jacquet *et al.* 2006).

The recent large pertussis epidemics in parts of the USA and in Australia have demonstrated a new pattern of infection. High incidence is noticed in pre-adolescents and young adolescents who have received their primary pertussis vaccines during the transition from DTwP to DTaP. These age-groups are the first cohorts that have received purely acellular pertussis vaccines as a primary vaccine. Several retrospective studies have been conducted to estimate whether the transition of vaccine has affected the incidences. Several recent publications has shown evidences that widely used DTaP vaccines seem to provide a shorter period of protective immunity compared with DTwP (Sheridan *et al.* 2014, Liko *et al.* 2013, Klein *et al.* 2012, Witt *et al.* 2012).

2.3.2. Vaccination in Finland

Vaccinations were started in 1952 using a vaccines produced by the Finnish pharmaceutical company, Orion. The vaccines used were diphtheria-pertussis vaccine *Per-Dif-Vaccin*, and monocomponent pertussis vaccines *Per-Vaccin forte* and *Per-Vaccin mite,* which all included several virulent strains of *B. pertussis.* The difference between the vaccines was in the amount of bacteria; *Per-Dif* and *forte* included at least 15 $x10^9$ cells per milliliter whereas *mite* only $2x10^9$ cells per milliliter.(Lääketehdas Orion Oy 1952) Between 1962 and 1976 the Finnish DTwP vaccine included only one virulent strain 18530 and it was produced by the National Public Health Institute, Helsinki, Finland. After 1976, the strain 1772 was added to the vaccine and this composition was used until 2005 when DTwP vaccine was ceased (Elomaa *et al.* 2005). The vaccination schedule was stable from the 1970's to 2003 with primary immunization given with DTwP at the age of 3, 4, 5 and 24 months. The first acellular pertussis vaccine taken into the national vaccination program in 2003 was a tricomponent (PT, FHA, PRN) acellular pertussis booster vaccine (dtap, Boostrix®, GlaxoSmithKline Biologicals, Belgium) administrated for 6 year old children to reduce the disease in children of school age (He &Mertsola 2008). Two years later in 2005, DTwP was replaced from the primary vaccination with DTaP-inactivated poliovirus vaccine (IPV) (Tetravac™, Sanofi Pasteur, France), which includes two components (PT and FHA). The vaccine schedule was also changed to 3, 5 and 12 months. A dtap booster vaccine was administrated at the age of 4 years (Boostrix®, GlaxoSmithKline Biologicals). In 2009, the primary vaccine was changed from two-component for pertussis to three-component (PT, FHA and PRN) DTaP-IPV-*Haemophilus influenza* type B (DTaP-IPV-Hib; Infanrix Polio Hib, GlaxoSmithKline). Current booster doses are given at the age of 4 years (DTaP-IPV, Tetravac™, Sanofi Pasteur), 14 – 15 years of age (dtap, Boostrix®, GlaxoSmithKline) and 18 – 20 year old persons attending the military service.

2.4. Innate Immunity

In general, innate immunity functions to discriminate between self and non-self. It is the essential, first line defense against pathogens. The initial recognition of conserved metabolic products of the microbes is one of the main functions of innate immunity. This host defense mechanism activates immediately after recognizing microbial

invasion and holds the infection until the adaptive immune system is effective (Akira *et al.* 2006, Kumar *et al.* 2011, Janeway 1992). Many of the structures recognized from the microorganisms are unique and not produced by the host. Molecules such as LPS, lipoproteins and double-stranded RNA are produced by bacteria or viruses but not eukaryotic cells. Microorganisms are not able to alter these structures without changing the core functions of them. This would happen only with several separate mutations, which could eventually change the pathogenicity of the microbe. The evolutionary time has probably selected these conserved regions as the targets for the initial recognition (Janeway 1992, Medzhitov 2001). These molecules of microorganism that are recognized by the innate immunity are commonly called pathogen-associated molecular patterns (PAMP). The cells that function in the innate immunity include dendritic cells (DC), mast cells, neutrophils, eosinophils and natural killer (NK) cells (Kumar *et al.* 2011). Hereafter, the focus is on two pattern recognition molecules of the innate immune system, one soluble and one membrane bound receptor, mannose-binding lectin (MBL) and Toll-like receptor 4 (TLR), respectively.

2.4.1. Mannose-binding lectin

Mannose-binding lectin (MBL) was first described in the late 1940's, as a factor able to inhibit the hemagglutination induced by influenza virus. In the 1990's it was discovered that the factor is MBL (Anders *et al.* 1990). MBL is a soluble serum protein that is principally synthetized in hepatocytes of humans. It belongs to collectins, a family of proteins able to recognize sugar structures on the surface of various microorganisms. Other major collectins are surfactant protein A and D functioning in the lungs.(Holmskov *et al.* 2003) MBL is produced as a 25 kDa monomeric protein, which consists of calcium-dependent carbohydrate-recognition domain (CRD), α-helical neck region and a collagenous rod-shaped region (Ezekowitz *et al.* 1988, Larsen *et al.* 2004). The structural subunit of MBL contains three identical monomeric polypeptides that form a triple helix in the collagenous region (Sheriff *et al.* 1994). In the blood, MBL circulates as higher oligomers which are formed from dimers to hexamers. These oligomers are oriented like a bouquet, the form, which is essential for the binding ability of MBL (Figure 3). Only tetramers or higher oligomers are active (Yokota *et al.* 1995). MBL forms a complex with a MBL-associated serine protease (MASP), MASP-1, MASP-2, MASP-3 and nonprotease sMAP, a small fragment of MASP-2 (Holmskov *et al.* 2003). MBL is a major pattern-recognition molecule and it mainly recognizes sugar molecules of the microorganisms. A bond is formed via CRD on sugars including mannose, N-acetyl-d-glucosamine, N-acetyl-mannosamine, fucose and glucose, but not galactose or sialic acid. The specificity depends on the orientation of the 3' and 4' hydroxyl group of the sugar. This may help MBL to separate self from non-self, as many human carbohydrate molecules terminate with galactose or sialic acid (Ip *et al.* 2009, Dommett *et al.* 2006). In addition to sugar molecules, it has been reported that MBL is able to bind to nucleic acids, phospholipids and non-glycosylated proteins (Ip *et al.* 2009, Kilpatrick 1998, Palaniyar *et al.* 2004).

2.4.1.1. The function of MBL

MBL recognizes a wide variety of microorganisms including gram-negative and gram-positive bacteria, viruses, protozoa and fungi (Table 2) (Dommett *et al.* 2006). There are no studies on whether MBL is able to recognize *B. pertussis*. However, the structurally highly similar *Haemophilus influenzae* is a common target for MBL (Neth *et al.* 2000). The binding of CRD on the surface of microorganism causes conformational change in the MBL which leads to autoactivation of the MASP-2 attached to the MBL (Dong *et al.* 2007). The main function of MBL is to activate the lectin pathway of complement (Takahashi &Ezekowitz 2005). There are three different complement pathways; the classical, lectin and alternative. From these, only the lectin pathway is discussed here. Binding to microorganism activates the MBL-MASP-2 complex, which causes the cleavage of C4 and C2 to form C4bC2a, the C3 convertase. C3 cleavage results in the lytic pathway, which is common to all three complement pathways. Eventually the cascade results to the formation of a membrane attack complex and lysis of the pathogen. In addition to MBL, ficolins can activate the lectin pathway (Liu *et al.* 2005). MBL functions also as a direct opsonin, facilitating the phagocytosis of the pathogens as well as apoptotic cells (Steinberger *et al.* 2002, Ogden *et al.* 2001).

Table 2. Examples of microorganisms' recognized by MBL

Bacteria	**Reference**
Haemophilus influenzae	(Neth *et al.* 2000, van Emmerik *et al.* 1994)
Streptococcus pneumoniae	(Neth *et al.* 2000)
Staphylococcus aureus	(Neth *et al.* 2000)
Chlamydia pneumonia	(Swanson *et al.* 1998)
Virus	
Influenza A	(Hartshorn *et al.* 1993)
Herpes simplex virus 2	(Fischer *et al.* 1994)
HIV	(Saifuddin *et al.* 2000)
Fungi	
Candida albicans	(Neth *et al.* 2000)
Aspergillus fumigatus	(Neth *et al.* 2000)

2.4.1.2. Gene polymorphism of MBL2

MBL is encoded by *MBL2* gene, which is located on the chromosome 10 (Sastry *et al.* 1989). Another MBL gene, *MBL1*, is a pseudogene that is positioned close to *MBL2,* but in humans, MBL is produced only from *MBL2* (Mogues *et al.* 1996). The *MBL2* gene consists of four exons and three introns (Taylor *et al.* 1989). In the exon1 there are three common single nucleotide polymorphism (SNP) sites (Figure 3.). All three point mutations cause an amino acid change during the translation of the messenger RNA: in codon 52 arginine is replaced with cysteine as a result of nucleotide substitution of cytosine to thymine (C>T) in mRNA position +154 (in nucleotide triplet CGT to TGT), the reference code for this SNP is rs5030737; in codon 54 glycine is replaced with aspartic acid as a result of nucleotide

substitution of guanine to adenine (G>A), in mRNA position +161 (in nucleotide triplet GGC to GAC), the reference code is rs1800450; and in codon 57 glycine is changed to glutamic acid as a result of nucleotide substitution of G>A in mRNA position +170 (in nucleotide triplet GGA to GAA) and the reference code is rs1800451 (summarized in Table 3) (Sumiya *et al.* 1991, Lipscombe *et al.* 1992, Madsen *et al.* 1994). These exon1 mutant alleles are commonly referred as variant D, B and C, respectively, whereas the normal allele is called A. The variant alleles D, B and C are in the literature combined as a variant O. In a heterozygote variant, two different alleles of the gene are present; one normal and one mutated and this is referred as A/O (A/B, A/C and A/D), whereas in a homozygote variant both alleles are mutated and it is marked as O/O (B/B, D/D and C/C). In the literature, also compound heterozygotes B/C, B/D and C/D are referred as variant O/O.

All these three variant alleles, B, C and D have a significant effect on the MBL concentration in the bloodstream. Nevertheless, allele D in heterozygote variant (A/D), affect less for the MBL concentration than alleles A/B and A/C (Minchinton *et al.* 2002). The location of these three mutations is in the collagenous region of the MBL molecule (Figure 3). This leads to more unstable and distorted structure of MBL monomers, which are not able to form functional higher oligomers. These MBL variant proteins have reduced avidity to their ligands, they do not activate the complement and the protein has also shorter half-life (Super *et al.* 1992, Garred *et al.* 2003). The effect of polymorphism in the exon1 on the MBL concentration is most dramatic in the homozygote alleles O/O. This genotype causes total deficiency of MBL. In the heterozygotes alleles A/O, the MBL concentration is reduced. However, individual variation is high both in wild type A/A and in A/O genotypes; in which even levels similar to those found for the normal allele A/A can be detected (Garred *et al.* 2003). This inter-individual variation is affected by two SNPs in the promoter region of the *MBL2* gene and one SNP in the 5'-untranslated region, referred as H/L, Y/X and P/Q, respectively (Madsen *et al.* 1995, Madsen *et al.* 1998). At the position -619, calculated from the start codon of the mRNA, is the SNP H/L, in which G nucleotide is changed to C (H>L, rs11003125), at position -290 is X/Y where G nucleotide is changed to C (X>Y, rs7096206). At position -66 a P/Q site is present, in which C is changed to T (P>Q, rs7095891).(Madsen *et al.* 1998) These polymorphic sites are actually dimorphic sites, indicating that the two variants are equal and neither is defined as normal type (Heitzeneder *et al.* 2012). SNPs in the promoter region affect independently of the exon1 SNPs on the concentration of circulating MBL (Madsen *et al.* 1995, Madsen *et al.* 1995). Theoretically all these six SNPs could produce a high number of different haplotypes, but due to a strong linkage disequilibrium between the promoter and exon1 SNPs, only eight haplotypes have been identified; HYPA, HYPD, LYPA, LYPB, LYPD, LYQA, LYQC and LXPA. Haplotypes influence the promoter activity which affects the MBL production and haplotypes correlate with detected MBL levels. However, the molecular mechanism, how *MBL2* promoter SNPs affect serum MBL concentrations is unsolved (Garred 2008). Haplotype HYP is associated with high MBL level, LYQ and LYP with intermediate levels and LXP with the low level (Madsen *et al.* 1995, Madsen *et al.* 1998). The focus is here only on three SNPs in exon1 as these decreases the level of functional MBL ~90 % (Garred 2008).

Table 3. Summary of the *MBL2* gene polymorphisms.

Abbreviation	Position	Nucleotide change	Amino acid change	SNP rs code
H/L	-619 promoter	G/C		rs11003125
X/Y	-290 promoter	G/C		rs7096206
Q/P	-66 untranslated region	C/T		rs7095891
A/D	+154 exon1	CGT to TGT	Arg to Cys	rs5030737
A/B	+161 exon1	GGC to GAC	Gly to Asp	rs1800450
A/C	+170 exon1	GGA to GAA	Gly to Glu	rs1800451

Modified from Heitzeneder et al. 2012.

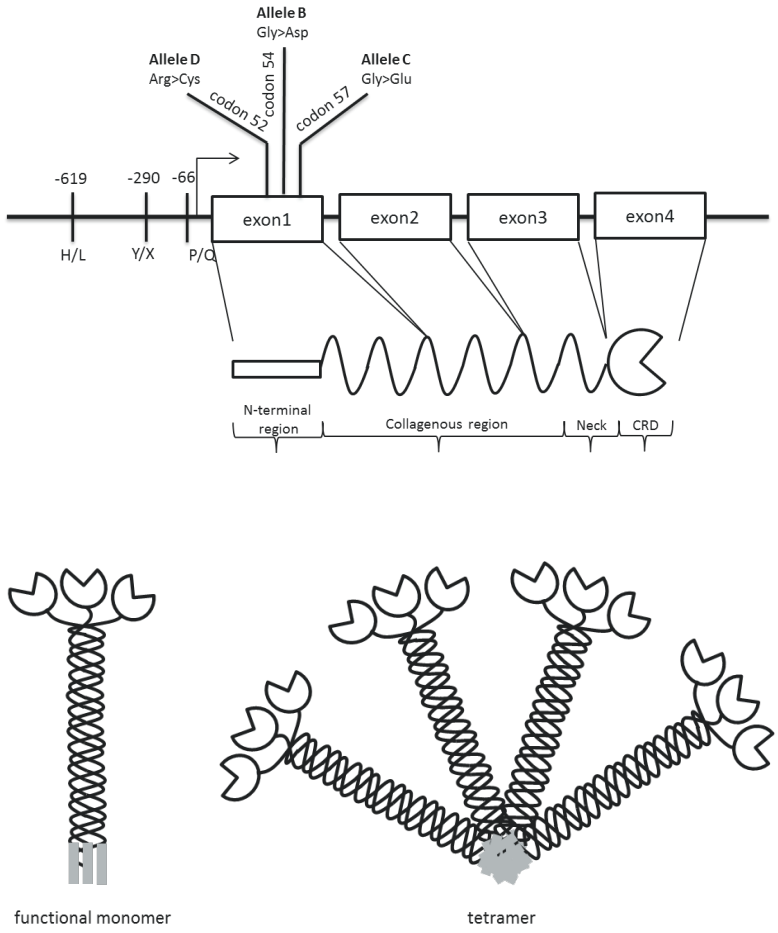

Figure 3. The location of gene polymorphisms in *MBL2* gene and the location of amino acid changes in the structural subunit of MBL protein. The length of the gene is not in true scale. The functional monomer of the MBL is illustrated in the lower part of the figure, on the left. Monomer assembles from three structural subunits. Higher oligomers such as tetramers (here on the right) forms when several functional monomers are combined. Modified from (Ip *et al.* 2009)

MBL polymorphism is common; however, the frequencies of variants differ significantly between the populations. The heterozygote variants A/O are very frequent in Caucasians, ~30 % of the population carries it, and of these the variant A/B is the prominent with frequency of 20 %. Similarly in Asian populations A/B is the dominating heterozygote variant comprising ~20 % of the population. Whereas in Sub-African populations the A/B is found only in 6 % of the population and instead A/C is the dominant heterozygote variant (20 – 40 %) contrary to Caucasians where the frequency is only 5 %. The most dramatic difference is in the frequency of homozygote variants O/O between Caucasian and certain Southern American Indian groups with frequencies of 5 % and 66 %, respectively (Garred 2008, Heitzeneder *et al.* 2012). It has been suggested that some populations may have selective advantage of low producing genotypes as the distorted MBL may reduce the uptake of certain intracellular microbes via the lectin pathway of the complement (Garred *et al.* 2006). Previous studies on Finnish population have detected the frequency of *MBL2* polymorphism similar to other Caucasians (Rantala *et al.* 2008).

Due to the different combinations of polymorphism in the gene and the promoter region, it is impossible to predict the MBL genotype based on the MBL concentration or vice versa. There is a wide heterogeneity in the limits used for defining normal, low and deficient MBL concentrations in the previous publications (Heitzeneder *et al.* 2012). Generally, the MBL concentration can vary between 0 and over 10 000 ng/ml. The median MBL concentration reported in Finnish population varies from 1100 to 4020 ng/ml based on studies in adult cohorts (Rantala *et al.* 2008, Rantala *et al.* 2009, Aittoniemi *et al.* 1996, Sajanti *et al.* 2015). Total MBL deficiency is commonly defined as a value under the detection limit of the method used (<100 or <50 ng/ml). However, also higher value (500 ng/ml) has been suggested to be used as a definition for deficiency (Eisen *et al.* 2008). The comparison of MBL concentrations between different studies should always be made with caution as methodology and study designs influence the results. The MBL concentration is also affected by the age. The concentration is highest at the age of one month, after which the concentration decreases slowly until at the age of 12 when it is in the level of adults (Aittoniemi *et al.* 1996). At the age of 49 MBL level starts to decrease further (Heitzeneder *et al.* 2012).

2.4.1.3. MBL2 polymorphism and MBL deficiency in infectious diseases

The total MBL deficiency is a consequence of *MBL2* gene polymorphism. However, no solid conclusion has been made on what cut-off should be used for the definition of MBL deficiency. In clinical studies, the concentration less than 100 ng/ml is commonly used, but there are studies using even 1000 ng/ml as a definition (Heitzeneder *et al.* 2012). The first *in vivo* model in mouse was able to proof the important role of MBL for host defence (Shi *et al.* 2004). In this study, all MBL null mice died after *Staphylococcus aureus* infection whereas over half of the wild type mice survived. In humans, numerous studies have shown an association between MBL polymorphism and susceptibility to

several infectious diseases. Also the clinical impact of MBL has been reviewed several times (Heitzeneder *et al.* 2012, Takahashi &Ezekowitz 2005, Ruskamp *et al.* 2006, Eisen &Minchinton 2003, Hoeflich *et al.* 2009). Only some examples of the association studies are referred here. Susceptibility to respiratory tract infections has been studied both in adults and infants. These investigations have shown that subjects with MBL variant genotypes or deficient MBL concentration are more prone for recurrent respiratory tract infections in infants and in adults (Rantala *et al.* 2008, Hoeflich *et al.* 2009, Summerfield *et al.* 1995, Kakkanaiah *et al.* 1998, Koch *et al.* 2001, Cedzynski *et al.* 2004, Chen *et al.* 2009). Nevertheless, the associations are not solid as contradictory results have also been published. In Denmark, a large adult cohort study failed to find any correlation between MBL deficiency and infectious diseases (Dahl *et al.* 2004). Questionnaire-based study in the Netherlands suggested that MBL deficiency is not contributing to the risk of respiratory tract infections in Dutch children (Ruskamp *et al.* 2008). Also a previous Finnish study could not find association between *MBL2* polymorphism and respiratory tract infections in children (Aittoniemi *et al.* 1998). In tuberculosis, MBL deficiency seems to be a protective factor which was shown in Danish and African cohorts. Low MBL concentration may inhibit the uptake of *Mycobacterium tuberculosis* by macrophages and prevents the intracellular activity of this pathogen (Hoal-Van Helden *et al.* 1999, Soborg *et al.* 2003). Increased risk of viral infections with MBL deficiency or MBL polymorphism have been reported with hepatitis B and C (Thio *et al.* 2005, Matsushita *et al.* 1998, Yuen *et al.* 1999), severe acute respiratory syndrome coronavirus (Ip *et al.* 2005) and human immunodeficiency virus (HIV) (Garred *et al.* 1997). In addition to healthy populations also association of MBL polymorphism and infectious diseases with some underlying condition such as HIV infection or chemotheraphy have been published (Heitzeneder *et al.* 2012).

2.4.2. Toll-like receptors

In 1996, a publication by Lemaitre *et al* described a significant, Nobel laureated finding, that Toll signaling pathway in *Drosophila* is not only responsible for the regulation of dorsoventral patterning in embryos but also was necessary for the antifungal response in adult flies (Lemaitre *et al.* 1996). It was one year later, the first mammalian toll-like receptor (TLR) was discovered (Medzhitov *et al.* 1997). These discoveries has considerably improved the knowledge on pathogen recognition and triggering of innate immune responses. TLRs are essential receptors of the human innate immunity, expressed on many cell types such as antigen presenting dendritic cells, monocytes and macrophages. They are transmembrane proteins with common structural features including a leucine-rich domain protruding outside the membrane, which mediates the PAMP recognition and an intracellular Toll-interleukin 1 receptor (TIR) domain that mediates the signal cascade downstream (Rock *et al.* 1998). In humans, ten TLRs have been identified (TLR1 – 10) and each has a distinct function in recognition of PAMPs. The variety of ligands recognized by TLRs is very wide including PAMPs from bacteria, viruses, parasites and fungi (Kawai &Akira 2010). TLRs are divided into two subgroups

based on their localization in the cell and the ligands they recognize. One subgroup consists of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10, which are located on the cell surface and sense the membrane components of extracellular pathogens. TLR2 appears both as a homodimer and as a heterodimer in combination with TLR1, TLR6 and TLR10. The partner of TLR2 defines the specificity of ligand recognition (Ozinsky *et al.* 2000, Guan *et al.* 2010). The PAMPs recognized by the subgroup attached on the cell membrane include bacterial lipopeptides, peptidoglycans, polysaccharides and flagellins (Kawai &Akira 2010). Nevertheless, the ligand of TLR10 is still unknown (Oosting *et al.* 2014). The other subgroup is intracellular, expressed on the surface of endoplasmic reticulum, endosomes and lysosomes; this group includes TLR3, TLR7, TLR8 and TLR9. These TLRs recognizes viral and bacterial nucleic acids including both RNA and DNA.(Medzhitov 2001, Kawai &Akira 2010) The TLR ligands are summarized in Table 4.

Table 4. The most common ligands of TLRs.

TLR	Ligand	Pathogen
TLR2	peptidoglycans phenol-soluble modulin glycolipids envelope proteins	Gram-positive bacteria *Staphylococcus aureus* *Treponema maltophilum* Measles virus, human cytomegalovirus and herpes simplex virus type I
TLR2/TLR1	triacyl lipopeptides	bacteria
TLR2/TLR6	diacyl lipopeptides, lipoteichoic acid, zymosan, GPI anchor	*Mycoplasma*, Gram-positive bacteria, Saccharomyces cerevisiae, Trypanosoma cruzi
TLR3	viral dsRNA, synthetic dsRNA (Poly(I:C))	
TLR4	LPS mannan F protein	Gram-negative bacteria Saccharomyces cerevisiae respiratory syncytial virus (RSV)
TLR5	flagellin	bacteria
TLR7 and TLR 8	ssRNA, synthetic imidazoquinoline derivatives (anti-viral drugs)	viral
TLR9	CpG DNA	bacterial and viral
TLR10	not identified, inhibitory receptor for TLR2 ligands*	

Modified from Bauer & Hartmann, 2008.
*(Oosting *et al.* 2014)

2.4.2.1. TLR signaling

The signaling cascade of TLR initiates with the recognition of the ligand. Although the signaling pathway and the response are characteristic to each TLR and its specific ligand, certain core pathway can be described. After having bound on the specific ligand,

the extracellular domains of the TLR are dimerized, which brings also the intracellular TIR-domains in close proximity. This TIR-TIR –attachment recruits specific signal mediators in the cytosolic site, called TIR-domain-containing adaptors which cause distinct downstream signaling. These adaptors include; myeloid differentiation primary response gene (MyD88), TIR-domain-containing adapter-inducing interferon-β (TRIF), TIR adaptor protein (TIRAP) and TRIF-related adaptor molecule (TRAM). Largely the TLR pathways can be divided on MyD88-dependent pathway; leading to the induction of proinflammatory cytokines and TRIF-dependent pathways which in addition to release of the proinflammatory cytokines, activates type I interferons (IFN). The MyD88-dependent pathway is common to all TLRs except TLR3. MyD88 activation recruits IL-1 receptor-associated kinases (IRAK) and eventually, after several signaling molecules involved, the protein complex regulating DNA transcription, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activates the proinflammatory gene expression. In the TRIF-dependent pathway, TRIF interacts with TNF receptor-associated factor1 (TRAF1) leading to cascade which eventually activates NF-κB. In addition to NF-κB activation, TRIF-dependent pathway activates interferon regulatory factor 3 (IRF3) causing type I IFN release. (Medzhitov 2001, Kawai &Akira 2010, Kawasaki &Kawai 2014, Bauer &Hartmann 2008) The MyD88 dependent and TRIF dependent pathways have similar activation kinetics, but the TRIF dependent pathway requires a time delay before it is activated (Covert *et al.* 2005). Figure 4 summarizes MyD88 and TRIF dependent pathways of TLR4 which are similar to what is observed in other TLRs.

2.4.2.2. TLR4

TLR4 was the first discovered human TLR (Medzhitov *et al.* 1997). Like all TLRs expressed on the cell surface, TLR4 is also expressed on the cytosolic membrane of several cell types such as DCs and macrophages. The main agonist of TLR4 is LPS, which is the characteristic component of the outer membrane of gram-negative bacteria (Poltorak *et al.* 1998). Other ligands of TLR4 include bacterial lipoteichoic acid and fusion protein of RSV (Bauer &Hartmann 2008). One of the virulence factors of *B. pertussis*, PT, which is also a component of all currently used aP vaccines, has also been reported to be a ligand of TLR4 (Wang *et al.* 2006). The lipid A part of the LPS structure is the main PAMP of the LPS (Saitoh *et al.* 2004). The immune response is not activated directly with LPS-TLR4 interaction, but it needs several accessory proteins. First, LPS-binding protein transfers LPS on CD14, the LPS receptor expressed on macrophages and soluble form in serum. Another molecule, MD-2 is also needed in the recognition complex of LPS. MD-2 is noncovalently associated with the extracellular domain of TLR4 and it is essential for TLR4 dimerization (Saitoh *et al.* 2004, Shimazu *et al.* 1999). TLR4 is able to activate both MyD88 and TRIF dependent signaling pathways. After activation, all four adaptor molecules mentioned above, MyD88, TRIF, TRAM and TIRAP, are involved in the signaling cascade, which is unique phenomenon only discovered with TLR4 (Figure 4) (Lu *et al.* 2008).

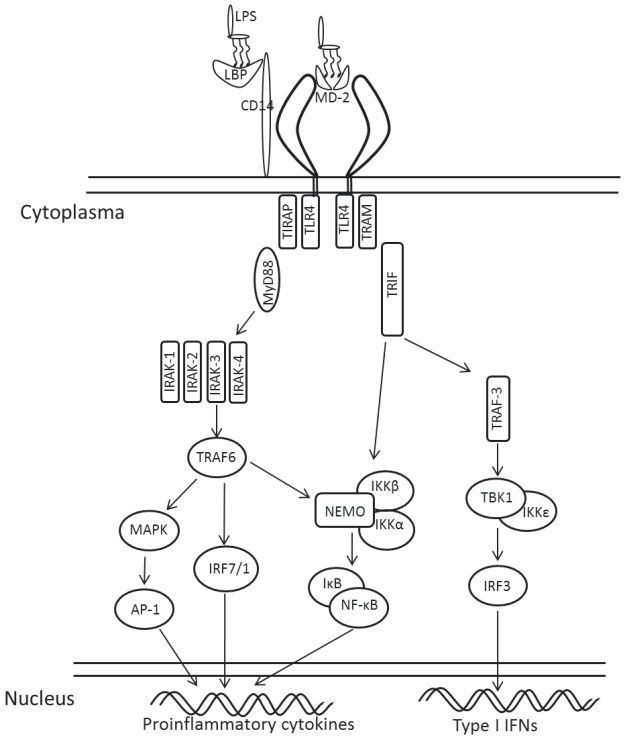

Figure 4. TLR4 mediated signaling pathways. Modified from (Lu *et al.* 2008, Lim &Staudt 2013).

2.4.2.3. TLR4 gene polymorphism

The gene encoding TLR4, *TLR4*, is located in the chromosome 9 and it consists of three exons (Smirnova *et al.* 2000). Soon after its discovery, also the first polymorphism sites were detected in *TLR4* gene at mRNA position +1063 and +1363, and these were reported to affect the LPS recognition by TLR4 as well as the susceptibility to sepsis due to infection with gram-negative bacteria (Arbour *et al.* 2000, Lorenz *et al.* 2002). Like other TLRs, also TLR4 is highly polymorphic (Smirnova *et al.* 2000). However, most of the SNPs discovered are rare with very low frequency in many populations (Smirnova *et al.* 2001). The first discovered two SNPs are still the most studied ones. Both of these SNPs are missense mutations, leading to an amino acid substitution. In the SNP at position +1063, transition A>G leads to substitution of aspartic acid (D) with glycine (G) in amino acid position 299 (D299G, rs4986790), and in position +1363 C>T change causes substitution of threonine (T) to isoleucine at amino acid position 399 (T399I, rs4986791) (Arbour *et al.* 2000). These two SNPs are cosegregating, which means these are commonly inherited together to the same daughter cell. Both SNPs are located in extracellular domain of TLR4 molecule, affecting the ligand binding. In the crystal structure of TLR4 complexed with MD-2 and LPS, the overall structure remained the same in D299G and T399I variants compared with normal protein structure. The

only difference was observed in the D299G region which is close to the MD-2 binding site, T399I had less effect on the structure (Ohto *et al.* 2012). Previously, a functional study also suggested that the D299G may have stronger functional effect compared with T399I (Arbour *et al.* 2000).

The frequency of D299G mutation in *TLR4* varies between the populations. In Caucasians and African populations the frequency of D299G polymorphism varies between 10 to 20 %, whereas in Asia and in certain American Indian populations the D229G mutation is practically missing (Ferwerda *et al.* 2007b). The infectious pressure in different environments as well as the so-called out-of-Africa migration routes has contributed the geographic difference in the SNP frequency. In addition, the lower mortality rate to malaria in Africans with the D299G mutation may have favored the high frequency of the SNP in these populations (Ferwerda *et al.* 2007b). In the Finnish population the frequency is ~15 % (Vuononvirta *et al.* 2011, Lofgren *et al.* 2010).

The clinical significance of D299G polymorphism in infectious diseases and other disorders has been studied in several populations (Schroder &Schumann 2005, Skevaki *et al.* 2015, Netea *et al.* 2012). The D299G polymorphism has been associated with higher risk of gram-negative bacterial infection and septic shock (Lorenz *et al.* 2002, Agnese *et al.* 2002). A study on HIV patients in Africa showed that subjects with D299G polymorphism had lower numbers of CD4+ T cells and also had increased susceptibility to active tuberculosis (Ferwerda *et al.* 2007a). RSV infection was significantly increased in children with the polymorphism compared with the normal allele (Awomoyi *et al.* 2007).

2.4.3. Cytokines

Cytokines are soluble glycoproteins of the immune system. They are essential signaling molecules involved in immune and inflammatory responses. Cytokines control the target cells function and initiation of signaling cascades. They can be generally divided based on their function to pro-inflammatory and anti-inflammatory cytokines. The main producers of the cytokines are T lymphocytes. T lymphocytes can be divided to two subgroups CD4+ and CD8+ cells, based on the CD-protein on their surface. CD4+ T cells, also known as T helper (Th) cells are the main producers of cytokines. Th cells can be further divided on sub-classes Th1, Th2, Th9, Th17, Th22 and regulatory T cells (Treg) (Geginat *et al.* 2014). Each T cell lineage has signature cytokines which they produce. Activation of adaptive immunity and T cell response initiates with pathogen recognition. TLRs are heavily expressed on antigen presenting cells (APC) such as dendritic cells (DC) and macrophages which support the role of TLR not only on pattern recognition but also in the initiation of adaptive immunity (Liu *et al.* 2010). TLR activation on the surface of DCs, triggers cytokine expression; especially IL-12 as well as chemokines and upregulation of major histocompatibility complex class II molecules (Brightbill *et al.* 1999, Banchereau &Steinman 1998). These events prime the maturation of DCs causing their migration to the lymph nodes, where DCs present antigens to naïve T

cells (Medzhitov 2001, Kawai &Akira 2010, Iwasaki &Medzhitov 2004). Secretion of TLR-induced cytokines, such as IL-12, directs CD4+ T cells to differentiate especially into Th1-type, whereas the generation of Th2 cells is TLR independent (Schnare *et al.* 2001). Signature cytokines of Th1 cells include IFN-γ, tumor necrosis factor (TNF) and IL-2. The generation of Th1 but not Th2 cells may result from the ligands recognized by TLRs. Ligands are derived from the same type of pathogens that are known to induce Th1 responses (Medzhitov 2001). However, at least in a mouse model, TLR4 ligand, LPS, seems to skew the Th1/Th2 activation in dose dependent way. Low dose of inhaled LPS induced the Th2 activation whereas increased LPS shifted the activation on Th1 side (Eisenbarth *et al.* 2002). Th2 cells are activated by IL-4 secretion which leads to Th2 cytokine production; including IL-4, IL-5, IL-6, IL-10 and IL-13. The Th17 cell lineage is activated by IL-1β, IL-6 and IL-23 secretion. These induce the production of Th17 cytokines IL-17, IL-21 and IL-22 (Geginat *et al.* 2014). Table 5 summarizes the main cytokines and their key functions.

Table 5. Examples of important Th1, Th2 and Th17 cytokines and their main functions.

Cytokine	Key Functions
IL-2	Development and division of T cells, cytokine release
IL-4	Th2 polarization, B cell maturation and antibody production
IFN-γ	Th1 polarization, macrophage activation
IL-12	Th1 polarization, stimulation of IFN-γ production
IL-10	Immune regulation, inhibition of IL-12 (suppression of Th1)
IL-6	Th17 polarization, B cell activation, antibody production, , acute phase response
IL-17	Th17 polarization, pro-inflammatory cytokine release, neutrophil recruitment
IL-23	Th17 activation, stimulation of IL-17 production
TNF-α	Pro-inflammatory response, innate immune response
IL-1β	Pro-inflammatory response, innate immune response

Summarized from (Smith &Humphries 2009, Geginat *et al.* 2014, Dinarello 2007)

2.4.3.1. IL-10 and the gene polymorphism

IL-10 is a critical cytokine with anti-inflammatory properties. Th2 cells are important producers of IL-10, however other immune cells such as Treg, Th1, Th17 and B-cells produce it as well. Nevertheless the production of IL-10 by Th1 and Th17 might be more a homeostatic mechanism to avoid activation of T cells in uncontrolled manner (Mosser &Zhang 2008). The anti-inflammatory role of IL-10 derives from its ability to inhibit the function of DC and macrophages. Expression of IL-10 inhibits the DC maturation and antigen presentation and the intracellular killing of pathogens by macrophages. IL-10 also inhibits the production of IL-12, the cytokine which activates Th1 response. This inhibition suppresses the Th1 response (O'Garra &Vieira 2007). In addition to its immunosuppressive role, IL-10 activates B-cells, natural killers and certain CD8+ T cells. Interestingly, IL-10 production may be distinct in different cell types against different pathogens or even same pathogens' different isolates.(O'Garra &Vieira 2007)

Like all cytokines, also *IL-10* gene is highly polymorphic. The gene is located in chromosome 1 (Eskdale *et al.* 1997). There are several polymorphism sites in the promoter region including five mostly studied; three are at the proximal promoter region -A592C (change of nucleotide A to C, in mRNA position -592, rs1800872), -C819T (change of nucleotide C to T, in mRNA position -819, rs1800871) and -A1082G (change of A>G, in mRNA position -1082, rs1800896). In the distal promoter region, two SNPs are discovered at positions -G2763T (change of G>T, in mRNA position -2763, rs6693899) and -A3575T (change of A>T, in mRNA position -3573, rs1800890). Haplotypes of three to five SNPs in the promoter region have been studied in association with the produced IL-10 level and it seems that the production is affected by the SNPs at some extent (Smith &Humphries 2009, Eskdale *et al.* 1998, Ouma *et al.* 2008). Total deficiency of IL-10 causes autoimmune diseases in mouse whereas overproduction may cause lethal infection due to the immunosuppressive ability of IL-10 (Mosser &Zhang 2008). In humans, the individual promoter SNPs or combinations of these SNPs have been studied in association with various disease conditions such as autoimmune diseases, cancer and infectious diseases. Both bacterial and viral infection association studies with IL-10 promoter SNPs have been reported. In the book by Kaslow *et al* the association studies are comprehensively reviewed (Kaslow *et al.* 2008). More severe infection has been reported with IL-10 polymorphism and meningococcal diseases severity, whereas the risk for *Chlamydia trachomatis* infection increased with IL-10 promoter polymorphism. Tuberculosis risk and IL-10 polymorphism has been studied in several populations but the results are contradictory. HIV infection risk is also increased with IL-10 polymorphism. (Kaslow *et al.* 2008)

2.5. Immunity after pertussis infection and vaccination

2.5.1. Innate immunity

In the beginning of *B. pertussis* infection, dendritic cells and macrophages are the first cells that recognize and respond to the bacteria. Neutrophils, natural killers and also Th cells follow to the infection site (Higgs *et al.* 2012). The recognition of *B. pertussis* is known to be mediated at least via TLR4. After recognition, most of the bacteria are phagocyted by macrophages but there is evidence, that some of the bacteria are able to evade the intracellular killing and can replicate intracellularily (Lamberti *et al.* 2010). Killing of *B. pertussis* is enhanced by IFN-γ and IL-17 secretion from Th1 cells (Higgs *et al.* 2012). Complement pathways are one of the first line defences against pathogens. However, many pathogens, such as *S. aureus*, *S. pneumoniae* and *Borrelia burgdorferi* and *B. pertussis* are able to evade complement recognition by producing proteins called regulators of complement activation (for example M protein of streptococci, SpA protein of *S. aureus*).(Lambris *et al.* 2008) *B. pertussis* is known to evade the alternative pathway of complement with use of proteins such as BrkA and Vag 8. The molecular mechanism of BrkA function is unknown; Vag8 inhibits complement protein C1.(Jongerius *et al.*

2015) After innate recognition, the adaptive immunity (both humoral and cell-mediated immunity) is activated.

Similarly to the immune response after infection, vaccination against pertussis induces both humoral and cellular immune responses. The observed immune response is affected by the vaccine type used; the response from DTwP resembles more the response of infection compared with DTaP. Initially, dendritic cells recognize the vaccine antigens, after DTwP vaccination it concerns the whole bacteria and after DTaP only the purified antigens. This different composition of DTwP and DTaP mediates distinct mechanisms of induced immunity. A sharp increase in humoral response is detected after DTaP vaccine, however it is limited to the vaccine antigens. Humoral and cell-mediated immune responses are described more detailed.

2.5.2. Humoral immunity

Infection with *B. pertussis* induces IgG and IgA secretion. Local mucosal secretions contain IgA before IgG is detectable in the serum. Antibodies are known to neutralize bacterial toxins, inhibit bacterial binding to respiratory tract and opsonize bacteria to help phagocytosis by macrophages (Mills 2001). IgG antibodies, produced after *B. pertussis* infection, are important for the protection but the protection is not lifelong (Storsaeter *et al.* 1998, Cherry *et al.* 1998). In other vaccine preventable diseases such as *Haemophilus influenzae* A, tetanus or diphtheria, certain antibody levels are known to be protective. After pertussis infection no solid level of protection has been defined.

Both DTaP and DTwP vaccinations induce high titers of IgG antibodies, but differences are observed as well. IgG antibodies against vaccine antigens such as PT, FHA and PRN are produced more extensively after DTaP vaccination (Gustafsson *et al.* 1996, Greco *et al.* 1996, Edwards *et al.* 1995, Ausiello *et al.* 2000). The most probable reason for the observed differences in antibody responses between DTwP and DTaP vaccines originates from the variable amount of antigens (Sheridan *et al.* 2014). Furthermore, as DTwP is composed of the whole bacteria, the antibodies are produced to a wider spectrum of antigens compared with DTaP. The IgG antibodies induced by both DTaP and DTwP vaccine decrease very sharply after vaccination, some studies observed low or undetectable level already within few years (Giuliano *et al.* 1998, Le *et al.* 2004). A review based on previous studies estimated the duration of vaccine derived immunity both from DTwP and DTaP to last from 4 to 12 years in children; however DTwP induced immunity declines more slowly (Wendelboe *et al.* 2005). A more systematic review suggested that the odds of pertussis increase by 1.33 times every year after the last dose of DTaP (McGirr &Fisman 2015). The authors predicted that after 8.5 years since the last DTaP vaccine, 10 % of children would be protected. Two large-scale assessments performed after epidemic in California 2010 indicated that the more time had elapsed since the last DTaP dose received, the higher the odds forpertussis infection (Klein *et al.* 2012, Misegades *et al.* 2012). The longevity of humoral immune responses may be affected by the vaccine administrated as a primary vaccine in combination with used

booster vaccine as well as by silent boosting effect from natural infection (Sheridan *et al.* 2014). Clinical trials in the 1990's indicated that IgG antibodies against DTaP vaccine antigens such as PT, Fim and PRN correlate with protection (Storsaeter *et al.* 1998, Cherry *et al.* 1998). Nevertheless, it is also known that children with diminished antibody levels are not totally susceptible to pertussis (Salmaso *et al.* 1998). Therefore it is difficult to estimate the level of antibodies that correlate with protection and no solid serological correlate for protection against pertussis has been established. However, the protection seems to be mediated by T-cell responses and B-cell memory in some extent after antibodies decayed (Hendrikx *et al.* 2011b, Mahon *et al.* 2000).

PT antigen is included in both types of vaccines, but only after DTaP vaccination it stimulates IgE response in addition to IgG (Ryan *et al.* 2000, Edelman *et al.* 1999). IgE activation is commonly discovered together with Th2 response after allergen stimulation. After DTwP vaccination, the production of IgE is downregulated, which is most likely caused by the LPS component in the vaccine when co-administrated with diphtheria and tetnanus antigens. LPS is known to suppress the Th2 response in favor of Th1 response (Gruber *et al.* 2001). IgA is produced after infection, but contrary neither DTaP nor DTwP vaccination induces the IgA production (Sheridan *et al.* 2014).

2.5.3. Cell-mediated immunity

After recognition of vaccine antigens or PAMPs present on the *B. pertussis* surface, DCs are activated. DCs play a critical role in antigen presenting and cytokine production, steps that are needed to activate the adaptive immune system and cell mediated immunity (CMI). The CMI after pertussis vaccination is mainly mediated by CD4+ T cells which consist of Th1, Th2 and Th17 cell types (de Gouw *et al.* 2011, Leef *et al.* 2000). Depending on the vaccine type used a distinct CMI response is detected. Figure 5 summarizes the differences.

DTwP includes a wide selection of antigens on the surface of *B. pertussis* that will activate response similar to pertussis infection. DTwP and infection induces the production of proinflammatory cytokines such as IL-6, IL-1, IL-12 and IL-23 by DCs and macrophages, stimulating the differentiation of naïve T cells to Th1 and Th17 cells (Mills *et al.* 2014). Th1 response is characterized by high level of IFN-γ production, which is needed for the production of opsonizing antibodies and activations of macrophages and neutrophils (Ausiello *et al.* 1997, Mascart *et al.* 2007). Th17 cells are activated by secretion of IL-23, IL-6 and IL-1 in response to DTwP. This induces IL-17 production by Th17 cells, promoting the recruitment and activation of macrophages and neutrophils (Higgins *et al.* 2006).

When DTaP is used as a primary vaccine, it stimulates macrophages to produce IL-10, which generates characteristic Th2 response with high secretion of IL-4 and IL-5 (Mills 2001, Ausiello *et al.* 1997, Ryan *et al.* 1998). Especially FHA stimulates the IL-10 secretion and inhibits IL-12 production, which transits the T cell balance to Th2 direction

(McGuirk &Mills 2000). However, in older children and adolescent also mixed Th1/Th2 response is detected (Ausiello *et al.* 1999, Meyer *et al.* 2007, Rieber *et al.* 2011). Th1 polarization may be explained by subclinical infections. PT is known to induce also Th17 responses in an IL-6-dependent manner (Chen *et al.* 2007). More recently, also induction of Th17 by DTaP has been proven in a mouse model (Ross *et al.* 2013). In this study, mice defective in IL-17A production were more prone to sustain *B. pertussis* infection after DTaP vaccination compared with wild type mice, demonstrating an important role of IL-17A for protective immunity after DTaP vaccination. A recent study has been able to show that even four years after a DTaP or DTwP booster vaccination, the T cell memory is still detectable (Smits *et al.* 2013). Moreover, the cytokine response was wider and proliferation capability sustained longer with previous DTwP vaccination compared with DTaP vaccination (Smits *et al.* 2013).

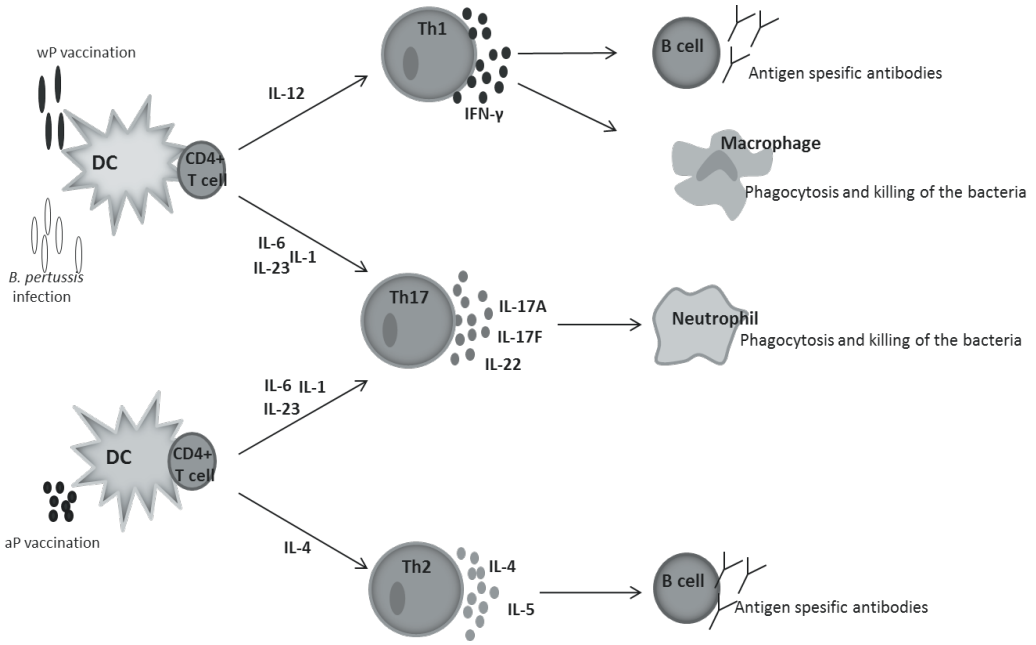

Figure 5. Differences in the Th cell vaccine responses induced by acellular (aP) and whole cell (wP) and infection. Modified from Mills et al 2014.

2.5.4. Human gene polymorphism in vaccine responses

Several factors influence the immune responses after vaccination, such as age, gender, number of doses given or environmental factors such as maternal antibodies or nutrition (Poland *et al.* 2011). Individual variation is always encountered with vaccinations, leading to variation in protective efficacy. Some vaccine recipients have no antibody response to vaccine antigens and these individuals are called non-responders. After measles vaccination on average 2 – 10 % of the individuals, and after hepatitis B primary vaccinations 5 – 20 % remains without response, respectively (Kimman *et al.* 2007). In

a Finnish adolescent study with DTaP booster vaccination, 5 – 8 % of the vaccinated individuals were nonresponders for at least one pertussis antigen (Tran Minh *et al.* 1999, Mertsola *et al.* 2010). However, the vaccine failure is dependent on the vaccine administrated as individuals seldom are non-responders to tetanus toxoid vaccine (Kimman *et al.* 2007). Immunogenetics is a field exploring the genetic heterogeneity in relation to human immune responses. The immune response genes are known to be highly polymorphic and it is already known that the effect of drug treatment may be affected by the genetic heterogeneity of the patients. During the last ten years, attention has been paid also to the role of gene polymorphism in vaccine responses against several bacterial and viral vaccines. (Poland *et al.* 2011) The vaccine response is composed of several immune response molecules and steps such as antigen recognition, processing, cytokine secretion and other signaling molecules; and that is why the polymorphism for these genes encoding the immune molecules may be involved directly or indirectly in vaccine responses. Only some of the association studies are reviewed here.

TLRs are highly polymorphic as was stated in the earlier section. The relation of TLR polymorphism on vaccine responses has also been studied. In a mouse model, after challenge with DTaP vaccine, anti-PT IgG level was lower in TLR4 deficient mice compared with wild type, confirming an important role of TLR4 in pertussis vaccine responses (Banus *et al.* 2008). During the same year an association study on 75 SNPs in TLR4 signaling pathway and DTwP vaccine responses in Dutch children was conducted (Kimman *et al.* 2008). Genes such as *CD14*, *TLR4, TOLLIP*, *IRAK3* and *IRAK4*, were shown to be associated with DTwP induced anti-PT IgG levels in the study cohort. This study indicates that there is a direct or indirect interaction of the TLR4 pathway with pertussis specific antibody production. Whether the link is via recognition of antigens, DC maturation or cytokine production or promoting the B-cell maturation by antigen presentation remains to be verified. Similarly, a wide selection of SNPs from TLR2 – 9 pathways was analyzed in association with measles vaccine responses (Dhiman *et al.* 2008). TLR3 was discovered to have an important role in immune responses after measles vaccination. Polymorphisms in the intracellular TLR pathway signaling molecules were further analyzed for 454 SNPs regarding a possible association with the response to measles vaccine. They discovered 8 associations with altered antibody responses and 52 associations with altered cytokine responses (Ovsyannikova *et al.* 2011). After hepatitis B vaccination, the responses are affected by polymorphism in TLR2, which is a known signaling molecule in hepatitis virus infection (Chen *et al.* 2011). In the same study also an association of polymorphism in cytokine genes IL-4Rα and IL-13 was observed with low response to hepatitis B vaccine. The relation of cytokine and cytokine receptor genes polymorphisms and altered antibody responses have been observed against several vaccines such as hepatitis B, measles, smallpox, pneumococcal conjugate vaccines, diphtheria and tetanus (Yucesoy *et al.* 2009, Wiertsema *et al.* 2007, Hohler *et al.* 2005, Haralambieva *et al.* 2011b, Haralambieva *et al.* 2011a, Dhiman *et al.* 2007). The Human Leukocyte Antigen (HLA) system is a group of genes that are necessary for antigen presenting. The HLA system is highly

polymorphic and several SNPs in HLA system have been associated with responses to viral vaccines against hepatitis A and B, influenza and measles (Kimman *et al.* 2007, Ovsyannikova *et al.* 2006). Few studies have also resolved the possible association of MBL with vaccine responses. A first report discovered that variant B decreased the risk of developing a poor antibody response after influenza vaccination compared with the normal allele (Tang *et al.* 2007). Another study failed to find any correlation between MBL polymorphism in adults with recurrent respiratory infections and pneumococcal vaccine responses (van Kessel *et al.* 2014). In addition, a study has been conducted with MBL null mice that were immunized with combine tetanus toxoid-conjugated Group B *Streptococcus* polysaccharide vaccine (Guttormsen *et al.* 2009). In this study it was discovered that mice lacking MBL had higher IgG response against *Streptococcus* polysaccharide when the vaccine was combined with tetanus toxoid. The effect was not seen if only Streptococcal polysaccharide was administrated to the mice. Based on the results, authors proposed that MBL might hinder IgG production and MBL deficiency might be beneficial for certain vaccine responses.

3. AIMS OF THE STUDY

Vaccination against pertussis has not been able to eradicate the *B. pertussis* from circulating and even large epidemics have been observed. In addition, recently it has been evidenced that the acellular vaccine currently used in Finland and many other developed countries does not provide a long-term protection.

The main objective of this study was to examine the association between host gene polymorphisms and altered immune responses after dTap booster or DTaP primary vaccination. Secondly, the purpose was to evaluate the risk of pertussis infection with polymorphisms in the innate immune system. Cell-mediated immunity accounts for the protection to some extent after the waning of pertussis specific antibodies. Another objective of this study was to evaluate the CMI responses after dTap vaccination.

The more specific aims of the present study were:

1. To investigate the role of *TLR4* (I) and *MBL2* (II) gene polymorphisms in antibody response and persistence after dTap or DTaP vaccination in Finnish subjects (I, II)

2. To analyse CMI responses in Finnish young adults with ten years interval between two dTap booster vaccinations (III)

3. To explore whether cytokine gene polymorphisms are associated with CMI responses after dTap vaccination (IV)

4. To analyse whether MBL deficiency is associated with pertussis infection (V)

4. MATERIALS AND METHODS

4.1. Subjects

dTap vaccine trial on Finnish adolescents (I, II, III, IV)

The initial study was performed in 1997, in Turku, Finland, when 510 adolescents, aged 11 - 13 years, were immunized with a single dose of dTap vaccine (Boostrix®, n=450) or with diphtheria and tetanus (dT) vaccine (n=60) followed with acellular pertussis (pa) vaccine one month after (Tran Minh *et al.* 1999). The same cohort was followed-up at 3- and 5-year time points when blood samples were collected. Ten years after the original vaccination, the cohort was invited to receive an additional dTap booster vaccine. Eighty-two subjects enrolled at the 10 year follow-up. Of the 82 study subjects, 75 (64 female, 11 male) had blood samples available for genomic DNA isolation and genotyping (I). For the analysis of *MBL2* polymorphism, additional DNA extraction was performed from serum samples of 280 subjects (II). For analysing the CMI at ten year follow-up (III), 57 subjects' peripheral blood mononuclear cells (PBMC) were available. All the subjects had received four doses of Finnish DTwP vaccine at the ages of 3, 4, 5 and 24 months. During the 10-year follow-up, there was no known clinically confirmed pertussis among the study subjects. Samples used from this cohort in studies I, II, III and IV are summarized in Table 6.

Infant cohort (II)

To study *MBL2* gene polymorphism and antibody responses after primary pertussis vaccination, 213 infants, with available DNA and serum samples, were selected (II). Infants were a portion of the larger cohort in study called the Steps to Children's Healthy Development and Wellbeing (STEPS). Serum samples were collected at the age of 2.6 months, 13 months and 2 years. Infants were vaccinated with DTaP vaccine at the age of 3, 5 and 12 months, according to the national vaccination program. Samples used from this cohort in study II are summarized in Table 6.

Pertussis patients and control group (V)

To study the possible association of MBL deficiency and pertussis infection susceptibility, 125 pertussis patients' serum samples were randomly selected from serologically diagnosed pertussis patients at the Department of Medical Microbiology and Immunology, the University of Turku in 2004. The control group included 430 individuals. The median age of the patients was 15 (range 1–71) and in the controls 15 years (0.5–97), respectively. These samples were randomly selected from the sera submitted for the diagnosis of celiac disease at the Department of Medical Microbiology and Immunology, the University of Turku, Turku, Finland. All control samples were

tested as negative for celiac disease and considered as healthy. There was no information concerning the vaccination history of the subjects. Samples used from this cohort in study V are summarized in Table 6.

Table 6. Summary of sample quality and number of selected subjects for each original publication. Time point of measurements are presented those methods where more than one time point was used.

Study	Methods used	N of subjects included	Time point of measurement
I	*TLR4* genotyping	75	
	Antibody measurement	75	Before and after original vaccination, 3 and 5 year follow-up, before and after second booster (10 year follow-up)
II	*MBL2* genotyping	355/ 213 (adolescents/infants)	
	MBL concentration	355 (adolescents)	Before and after original vaccination
III	Proliferation	57	Before and after second booster (10 year follow-up)
	IFN-γ / IL-17 ELISpot assay	21/17	Before and after second booster (10 year follow-up)
	Cytokine measurement	14	Before and after second booster (10 year follow-up)
IV	Proliferation	38	Before and after second booster (10 year follow-up)
	Genotyping	52	
V	MBL concentration	125/430 (patients/controls)	one measurement

4.2. Ethics

All the serum samples, used in the pertussis susceptibility study (V), were sent for diagnostic purposes to the diagnostic laboratory of Department of Medical Microbiology and Immunology, University of Turku. Prior to the inclusion into the study, all patient data, except for age and sex, were anonymized. Furthermore, no clinical data of the patients were handled at all. According to Finnish Medical Research Act, the opinion of the ethics committee is needed only in medical research involving intervention. Since this study was not a medical research involving intervention, ethical permission was not needed.

The study protocol for adolescent dTap vaccine trial and STEPS study has been approved by the joint commission on ethics of the Turku University and the Turku University Central Hospital and written informed consent was obtained from the study subject prior to enrolment (Vuononvirta *et al.* 2011, Tran Minh *et al.* 1999). The study was conducted

in accordance with Good Clinical Practice Guidelines and the Somerset West, 1996 version of the Declaration of Helsinki.

4.3. Antibody measurements (I and II)

All pertussis specific antibody measurements in the original publication (II) were performed with enzyme-linked double antibody immunoassay (ELISA) as described in the original publications. For these specific IgG antibody measurements, 96-well plate (Maxisorp, Greiner) were coated with purified antigens in concentration of 1, 2 and 2 µg/ml for PT, FHA and PRN, respectively. The standard curve was created using commercial Pertussis Antiserum (human) 1st IS - WHO international Standard serum (06/142, NIBSC, UK). As secondary antibodies, ReserveAP anti-human IgG (gamma) phosphatase labelled antibodies were used (Kirkegaard & Perry Laboratories, Maryland, USA). The absorbance was read at 405 nm. Positive and negative in-house controls were included in each run. The concentrations (international unit/ml) of anti-PT, anti-FHA and anti-PRN IgGs were calculated with UnitCalc-software (Uppsala, Sweden). For statistical analysis, subjects with undetectable concentration of IgG antibodies were given a value 0.1 IU/ml. The antibody measurements for the adolescent dTap vaccine trial have been published previously (Tran Minh *et al.* 1999, Mertsola *et al.* 2010, Edelman *et al.* 2007) and are presented for individual responses as IU/ml or per group as geometric mean concentration (IU/ml), as indicated. The detection limit is 5 IU/ml. A positive antibody response to pertussis antigens was defined as seroconversion, for initially seronegative subjects (<5 IU/mL), or as a ≥2-fold increase in antibody values, for initially seropositive subjects. This same definition has been used for the whole study period (Tran Minh *et al.* 1999, Mertsola *et al.* 2010, Edelman *et al.* 2007, Edelman *et al.* 2004).

4.3.1. Pyrosequencing (I, II)

Genotyping of SNPs in *TLR4* D299G (rs4986790) and *MBL2* codons 52 (rs5030737), 54 (rs1800450) and 57 (rs1800451) was performed using pyrosequencing. The methods are described in the original publications (I and II) and in (Vuononvirta *et al.* 2011, Roos *et al.* 2006). PCR is used for the amplification of the target sequence including the polymorphic site. One primer was biotin labelled. After PCR, the DNA strands are separated by heating and then extracting the one strand with biotin label using streptavidin-sepharose beads. The single strand of the PCR product is used for the pyrosequencing. Sequencing primer is annealed to the PCR product before the pyrosequencing reaction. In the pyrosequencing reaction, the Pyromark Q24 instrument (Qiagen) dispenses enzyme and substrate on the reaction plate that includes PCR product and the sequencing primer. Deoxynucleoside triphosphates (dNTP) are dispensed to the reaction in the predefined order. The results are presented as pyrograms. The known mutations were interpreted from the pyrograms manually based on the literature (Roos *et al.* 2006).

4.4. Measurements of cell-mediated immunity

4.4.1. Proliferation assay (III)

Proliferation assay was used to measure the activation of CMI in the adolescent dTap cohort and it is described in original publication (III). Peripheral blood mononuclear cells (PBMC) were isolated and cells were cultured immediately in supplemented RPMI medium as described before (Tran Minh *et al.* 1998). Shortly, RPMI included 10 % heat-inactivaed human AB-serum and 1 % glutamine and it was supplemented with penicillin (10 000 U/ml), streptomycin (10 mg/ml) and gentamycin (50 µg/ml). 10^5 cells/well were stimulated with heat-inactivated PT (hPT) 1 µg/ml, PRN 2.5 µg/ml and FHA 1µg/ml. PT was used as a heated form to avoid its mitogenic activity. Pokeweed mitogen (PWM) 1:400 dilution was used as a positive control. The cells were incubated for 6 days in +37°C and 5% CO_2. Sixteen hours before the end of incubation, ^{3}H-thymidine (0.5 µCi) was added to the cells. The cells were harvested and radioactivity was measured using Beta counter. Spontaneous response was measured from unstimulated cultured cells (medium, M). Proliferation results are expressed as the mean counts per minute (cpm) from triplicated wells and stimulation index (SI) which is the stimulation against antigen divided with spontaneous response. A positive proliferation was defined as more than four-fold increase in the stimulation against antigens compared to spontaneous response (stimulation index, SI ≥4).

4.4.2. IFN-γ and IL-17 ELISpot assay (III)

PBMCs were used for the ELISpot assays as described in the original publication (III). For both assays, $1x10^6$ cells were stimulated with hPT 1 µg/ml, PRN 2.5 µg/ml and FHA 1µg/ml, PWM 1:400 dilution was used as a positive control. Pokeweed mitogen (PWM) 1:400 dilution was used as a positive control. Unstimulated cells were used as a negative control. Both ELISpot assays were performed according to manufacturer's protocol (R&D systems, USA). It is known that IL-23 is critical to maintain stability of IL-17 in the cultured cells. Therefore, for IL-17 ELISpot assay, cells from 12 subjects, collected one month after the vaccination, were stimulated with PT supplemented with IL-23. The ELISpot plates were read by ImmunoSpot, CTL-Europe GmbH (Germany). The results are expressed as spot counts per 10^6 PBMCs.

4.4.3. Cytokine measurement (III)

PBMCs were used for the cytokine measurements performed in the original publication (III). PBMCs ($2x10^5$ cells/well) were incubated with the purified antigens: heat-inactivated pertussis toxin (hPT) 1 µg/ml, pertactin (PRN) 2.5 µg/ml, filamentous hemagglutinin (FHA) 1µg/ml. Pokeweed mitogen (PWM) 1:400 dilution was used as a positive control for the mitogenic activity. Spontaneous response was determined from unstimulated cultured cells (medium, M). Concentrations of the selected cytokines (IL1β, IL2, IL4, IL6, IL8, IL10, IL12p70, IFN-γ, IL17 and TNF-α) were measured with Bio-

Plex Pro human cytokine plex –kit (Bio-rad, CA, USA) as advised by the manufacturer. Measurements were performed from duplicated wells using 1:4 dilution and the results are expressed in pg/ml.

4.5. Cytokine gene polymorphisms (IV)

Cytokines and their SNPs (Table 7) were selected based on their known functions related to vaccine responses or infection susceptibility (Yucesoy *et al.* 2009, Hohler *et al.* 2005, Dhiman *et al.* 2007, Tang *et al.* 2007, White *et al.* 2012, Chen *et al.* 2010). The simultaneous detection of SNPs listed in Table 5, was performed using iPlex Gold technique (Sequenom). This was done in the University of Eastern-Finland, Kuopio, Finland. The iPlex Gold system is based on amplification of the target sequence using PCR following primer extension. The differences in the mass of the product due to the differing allele composition are detected with MALDI-TOF mass-spectrometer.

Table 7. Summary of the selected Cytokine Gene SNPs.

Molecule	SNP code	Nucleotide change	Position in gene
IL-10	rs1800871	G/A	promoter (-819)
IL-10	rs1800896	A/G	promoter (-1082)
IL-10	rs1800890	T/A	promoter (-3573)
IL-12RB1	rs372889	C/T	gene (+41157)
IL-12B	rs2546890	A/G	promoter (-634)
IL-17A	rs2275913	G/A	promoter (-197)
IL-23R	rs11209026	G/A	gene (+78790)

4.6. MBL concentration measurement (V)

MBL concentrations were measured from the serum samples of pertussis patients and controls using ELISA method described in the original publication (V). Microtitre plate was coated with mouse monoclonal IgG1 antibodies against human MBL (HYB 131-01, Statens Serum Institut, Copenhagen Denmark) in the concentration of 8 µg/ml. The secondary antibody was biotin labelled mouse monoclonal IgG antibodies against human MBL (BioPorto, Hellerup, Denmark). For creating the standard curve, commercial standard serum was used (~3200 ng/ml, Statens Serum Institut, Copenhagen, Denmark). Commercial MBL deficient serum (Statens Serum Institut, Copenhagen, Denmark) was used as a negative control in each run. The detection limit of the assay was 50 ng/ml. The concentration below the detection limit was given a value 25 ng/ml.

4.7. Statistics

Comparisons between different groups for the original publications I, II, III and IV were performed with GraphPad Prism software, version 4 (San Diego, CA). Non-parametric Mann-Whitney U test was used for comparison of two groups (I, II, III and IV) and Kruskal-Wallis test for applied for comparison of more than two groups in same analysis (II, V). In addition, the Fisher exact test was used (I, II). Differences were considered statistically significant when $P < .05$. For the original publication V, statistical analysis was performed with SAS for Windows version 9.4 (SAS Institute Inc., Cary, NC, USA). Values were log-transformed for statistical analysis due to positively skewed distributions. Paired t-test was used to test change in proliferation before and after the vaccination. Repeated measures analysis of variance (ANOVA) using unstructured covariance structure was used to examine proliferation and antibody responses. The model included genotype as between subject factor, time (before and after the vaccination) as within-subject factor and interaction between genotype and time. Tukey's adjustment was used in pairwise comparisons between genotypes. P-values less than 0.05 were considered statistically significant.

5. RESULTS

5.1. *TLR4* D299G polymorphism association with aP vaccine response (I)

Seventy-five subjects with available DNA samples who attended the ten year follow-up were included in this study. One subject's genotyping was unsuccessful. The normal homozygote allele AA was detected in 63 subjects (85.1%), 10 (13.5 %) subjects had heterozygote variant AG and one subject (1.4%) had homozygote variant GG. Due to the limited number of subjects in the homozygote variant group, the AG and GG variant groups were combined as a one D299G variant group. There was no difference in the frequency of TLR4 polymorphism between sexes ($P > 0.99$).

5.1.1. *TLR4* D299G and anti-pertussis IgG responses (I)

Geometric mean concentration (GMC) of IgG antibodies against all pertussis antigens present in the vaccine, PT, FHA and PRN, were compared between the subjects with TLR4 normal allele and D299G variant allele in all six time points; before and one month after the original vaccination, at 3 and 5 year follow-up and before and after the second booster vaccination (Figure 6). Before the original vaccination, no difference was observed between the genotypes for any anti-pertussis antibody concentrations. One month after the first booster vaccine, subjects with TLR4 normal allele AA had clearly higher antibody response against all three antigens. However, the difference was not statistically significant. At three year follow-up, individuals with normal allele AA had significantly higher GMC of IgG against all three pertussis antigens compared with D299G variant group (Figure 6). In subjects with D299G variant, the antibody concentration had decreased to the pre-vaccination level, which was observed three years before. The same trend of lower antibody levels in the D299G variant group was observed at 5 year follow-up and at 10 year follow-up before and after the vaccination. However, with FHA opposite effect was observed one month after the second booster vaccine but the difference was not significant.

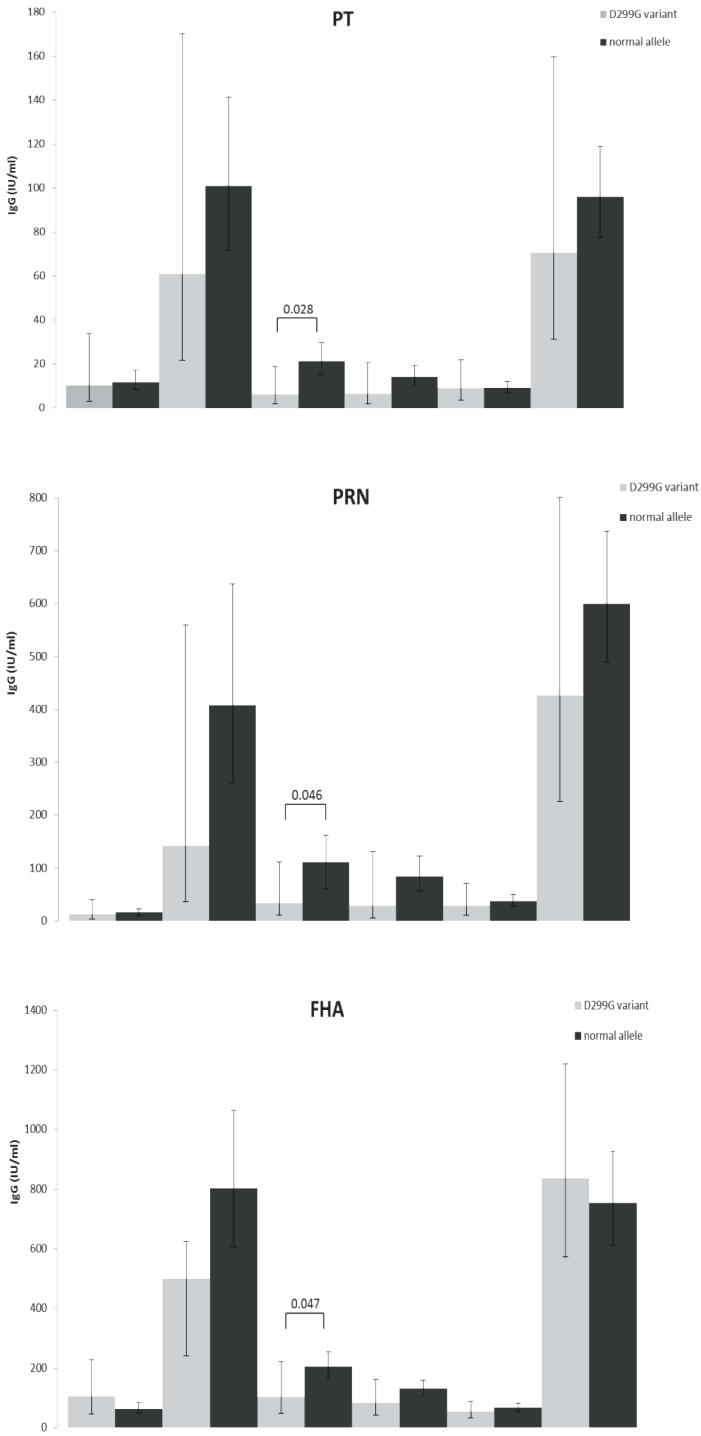

Figure 6. Geometric mean concentrations (GMC) of IgG antibodies with 95 % confidence intervals against pertussis antigens PT, FHA and PRN after booster doses of dTap vaccine in subjects with TLR4 normal allele (dark grey bars) and D299G variants (light grey bars). Significant difference ($p<0.05$) between genotypes is indicated in the figure. Modified from the original publication I.

Positive antibody response was defined as seroconversion for initially seronegative subjects (<5 IU/mL), or as a ≥2-fold increase in antibody responses for initially seropositive subjects. The seropositivity rate against PT at 3 year follow-up had decreased to 50 % in the D299G variant group, whereas for normal allele group it was still 90 %. For FHA and PRN seropositivity remained close to 100 % for the whole study period. After the original booster vaccination, positive antibody response to PT was detected from 60 subjects (81%), whereas 14 subjects (19 %) were nonresponders. After the original booster vaccination also the frequency of subjects with ≥2-fold increase in antibody response to PT differed in the study groups. In the normal allele group, 86 % (n=54) of the subjects had ≥2-fold increase, whereas only 55 % (n=6) of the subjects in D299G group had fold-increase equal or higher than two (P = 0.028, Figure 7). No significant difference in the fold increase to antibody responses to FHA and PRN was observed between the groups.

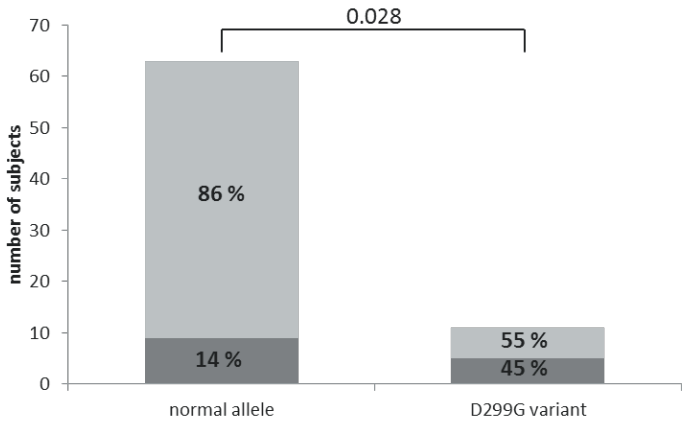

Figure 7. The percentages of subjects whose antibody response against PT increased ≥2-fold (light grey boxes) or <2-fold (dark grey boxes) after booster vaccination between subjects with normal allele and D299G variant allele. P-value was calculated using Fisher exact test. Modified from the original publication I.

5.2. *MBL2* gene polymorphism association with aP vaccine response (II)

This study aimed to search for the possible association of *MBL2* gene polymorphism and antibody responses after primary vaccination and booster vaccination with acellular pertussis vaccine. Total of 568 subjects' (355 adolescents, 213 infants) DNA samples were genotyped for *MBL2* exon1 gene polymorphism. Two infants' genotyping was unsuccessful due to technical error. The observed frequencies of *MBL2* genotypes are presented in Table 8. For the comparison of genotypes with antibody responses, heterozygote variants having one A allele were combined as one group A/O and other heterozygote variants and homozygote variants as one O/O group.

Table 8. The frequency of *MBL2* exon1 polymorphisms in infant cohort (n=211) and adolescent cohort (n=355).

MBL genotype	Infant cohort N(%)	Adolescent cohort N(%)
A / A	147 (69.7)	232 (65.4)
A / O	60 (28.2)	103 (29.0)
A / B	40 (18.8)	69 (19.4)
A / C	1 (0.5)	5 (1.4)
A / D	19 (8.9)	29 (8.2)
O / O	4 (1.9)	20 (5.6)
B / B	2 (0.9)	6 (1.7)
B / D	0	7 (2.0)
D / D	1 (0.5)	3 (0.8)
C / D	0	1 (0.3)
C / C	0	3 (0.8)
C / B	1 (0.5)	0

Modified from the original publication II.

5.2.1. MBL concentration in the adolescent cohort (II)

In addition to *MBL2* genotyping, the MBL concentration was measured from the serum samples of adolescents. Measurement was done from the serum samples collected one month after the first booster vaccination. The concentration was combined with the individual genotype data. The MBL concentration was found to be significantly higher in the group of subjects A/A, compared with A/O and O/O groups (Figure 8.). The concentrations of individual genotypes are presented in Figure 8. MBL concentrations were not associated with pertussis antibody responses in any time point measured.

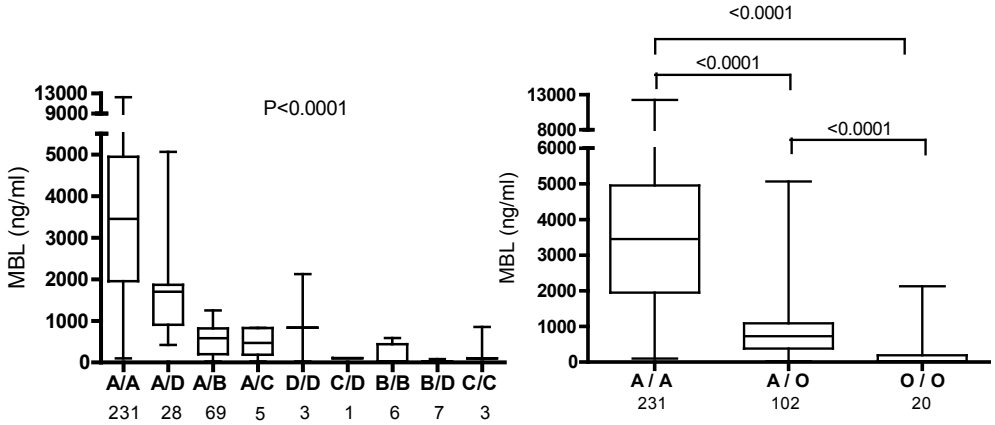

Figure 8. The MBL concentration distribution of individual *MBL2* genotypes and grouped genotypes A/A, A/O and O/O. Data are represented with the first and the third quartile and median line. The ends of the whiskers represent maximum and minimum values of the data. The number of subjects is indicated beneath each genotype. P-value for the overall concentration difference between the individual genotypes was calculated with Kruskal-Wallis test and for the combined genotypes with Mann-Whitney U test. Modified from the original publication II.

5.2.2. *MBL2* polymorphism and antibody responses after aP vaccinations (II)

MBL2 polymorphism was compared with antibody responses after primary DTaP vaccination from infants and after booster dTap vaccination from adolescents. The comparison after primary vaccination was performed at three time points; before the first vaccine dose at 2.6 months of age, after three primary vaccine doses at 13 months of age and one year after that at the age of 2 years. No association between *MBL2* genotypes and antibody concentrations were observed after primary vaccination (Figure 9).

In the adolescent cohort, association was evaluated in six time points; before and one month after the first booster vaccination, at 3 and 5 year follow-up and before and one month after the second booster vaccine (Figure 10). In addition to pertussis specific antibodies, also diphtheria (D) and tetanus (T) antibody concentrations were compared with different *MBL2* genotypes in the adolescent cohort. Anti-D and anti-T antibody responses were not available for the infant cohort. The only association was detected with FHA at the time point one month after the second booster. In that timepoint, subjects with O/O genotype had significantly higher GMC of antibodies against FHA than subjects with genotype A/A and A/O (A/A 585.8 ng/ml, A/O 450.5 ng/ml and O/O 1172.0 ng/ml, P=0.01). However the group included only three subjects. No association was observed for *MBL2* genotypes with anti-PT or anti-PRN antibodies. In conclusion, no association was detected between antibody responses after primary or booster vaccination and the *MBL2* polymorphism.

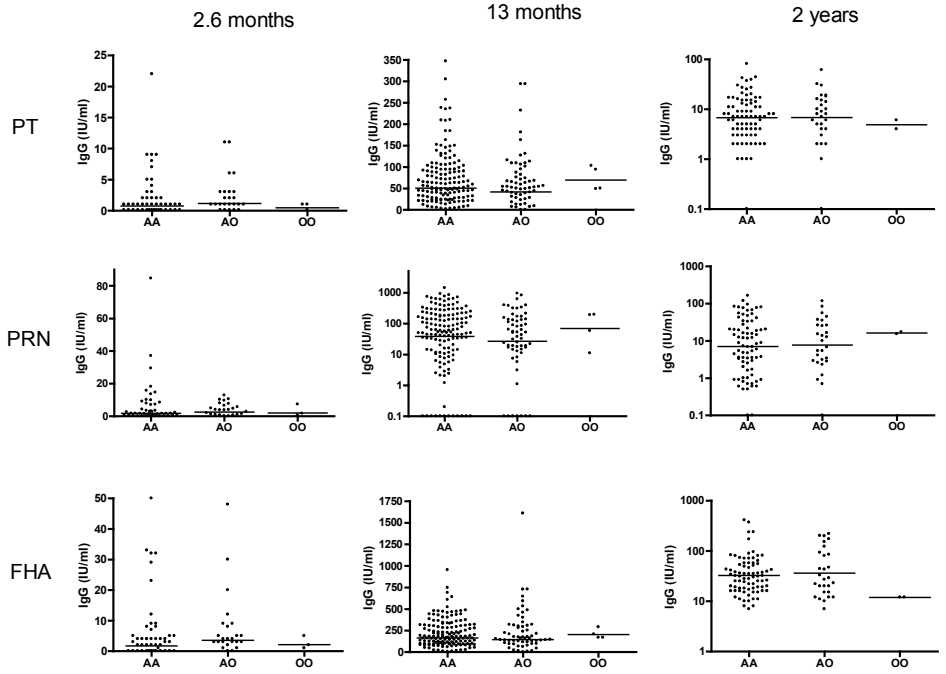

Figure 9. IgG antibody concentrations against PT, PRN and FHA in young infants at the age of 2.6 months, 13 months and 2 years. Subjects are divided according to their *MBL2* genotype to normal allele AA, heterozygotes variants AO and homozygous or combined heterozygote variants OO. Solid line indicates the geometric mean concentration. Modified from the original publication II.

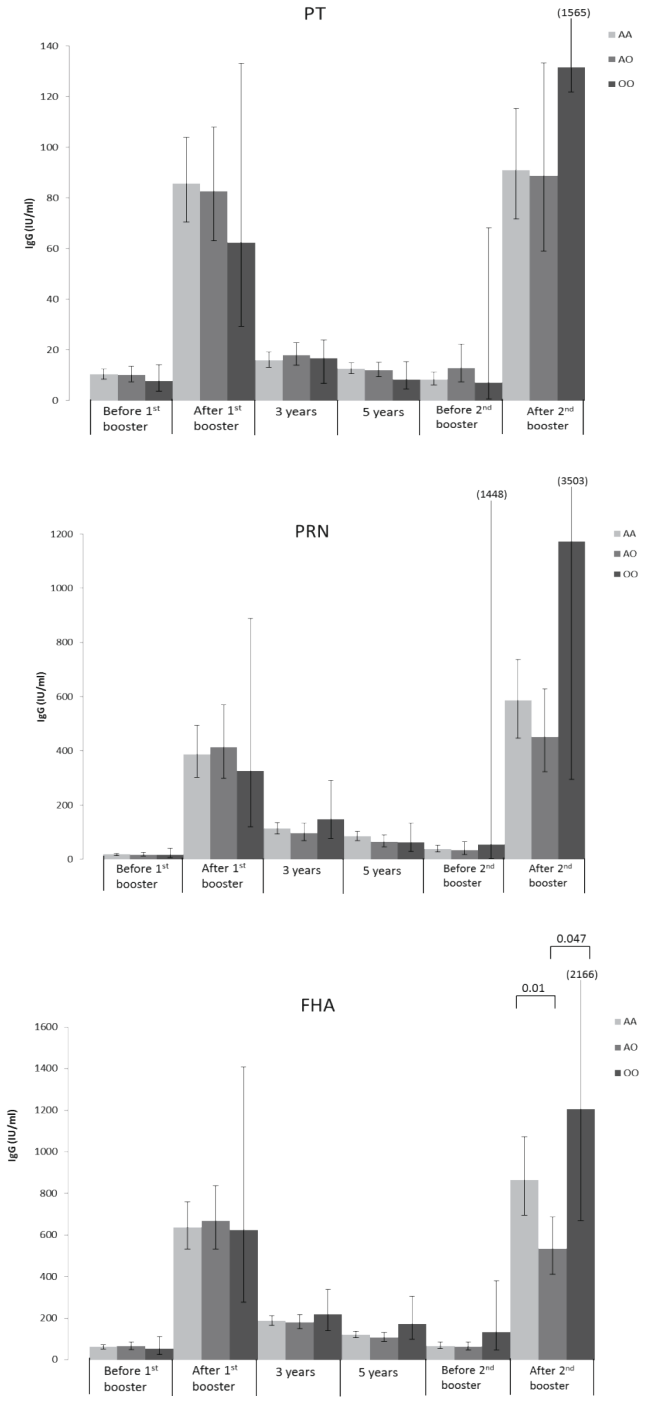

Figure 10. Geometric mean concentrations (GMC) of IgG antibodies with 95 % confidence intervals against pertussis antigens PT, FHA and PRN after booster doses of dTap vaccine in subjects with MBL genotypes AA normal allele, AO heterozygote variants and OO homozygous or combined heterozygote variants. Significant difference ($p<0.05$) between genotypes is indicated in the figure. Modified from the original publication II.

5.3. Cell mediated immunity ten years after an aP vaccination (III)

This study aimed to evaluate the CMI persistence ten years after a previous pertussis booster vaccination, as well as the CMI response after second acellular booster vaccination in young adults. From the adolescent's dTap vaccine cohort, 57 subjects with sufficient amount of PBMCs for CMI analysis were included. CMI immunity was evaluated 10 years after the first booster vaccination and one month after the second booster dose. CMI responses were evaluated by using proliferation assay, IFN-γ and IL-17 ELISpot and cytokine measurements.

5.3.1. Proliferation (III)

From 57 subjects included in the proliferation assay, before the second booster vaccination, positive response (SI≥4) against PT, PRN and FHA was detected from 51 %, 53 % and 89 % subjects, respectively. After vaccination the positivity rate was increased to 81 %, 81 % and 96 %, respectively. Both absolute cell count (cpm) and SI increased significantly against all pertussis antigens after the booster vaccine (Table 9). Subjects were further divided based on whether they had positive SI response (≥4) or did not have (SI<4) in the sample before vaccination. Those subjects with SI<4 before vaccination, had more clear increase in the proliferation after vaccination compared with subjects with SI≥4. Only proliferation against FHA was increased significantly in both sub-groups (Table 10).

Table 9. Summary of the proliferation results in young adults before the second booster vaccine and one month after.

	before the booster (N=57)		**1 month after the booster (N=54)**		
	cpm (95 % CI)	SI (95 % CI)	cpm (95 % CI)	SI (95 % CI)	P-value*
PT	334.8 (218.5 – 513.0)	6.6 (4.3 – 10.0)	1047.0 (653.1 – 1679.0)	20.2 (12.6 – 32.4)	0.001/ 0.001
PRN	256.5 (166.4 – 395.3)	5.1 (3.3 – 7.7)	1149.0 (770.6 – 1714.0)	20.1 (13.9 – 31.6)	<0.0001/<0.0001
FHA	1659.0 (1227.0 – 2242.0)	32.8 (23.2 – 46.2)	3144.0 (2337.0 –4229.0)	60.7 (42.1 – 87.5)	0.001/ 0.003

Given are geometric means (and 95% confidence interval) of absolute proliferation values in counts per minute (cpm) and geometric mean values (and 95% confidence interval) of stimulation indexes (SI). *P-values were calculated with Mann-Whitney U test. P-values are presented for the separate comparison of geometric mean cpm and SI values before and after vaccination, respectively. Modified from the original publication III.

Table 10. Comparison of the geometric mean value of stimulation indexes before and one month after the booster in subjects divided into two groups according to their SI value with 95% confidence intervals (CI). The first group consists of subjects without positive CMI response before vaccination (group SI<4) and the second group consists of subjects who had positive CMI response already before the vaccination (group SI≥4).

	SI<4				SI≥4			
	N of subjects	before	1 month after	P-value*	N of subjects	before	1 month after	P-value*
PT	28	1.6 (1.3–1.9)	14.0 (6.7–29.3)	<0.0001	29	26.3 (19.0–36.5)	29.2 (15.2–56.4)	0.67
PRN	27	1.2 (0.9–1.5)	16.4 (8.9–30.2)	<0.0001	30	19.0 (13.9–25.9)	26.5 (15.0–46.9)	0.18
FHA	6	1.9 (1.1–3.3)	8.6 (2.1–36.1)	0.015	51	45.7 (35.6–58.6)	77.5 (55.8–107.5)	0.0020

*P-values were calculated with Mann-Whitney U test. Modified from the original publication III.

Although the proliferation results observed did not correlate with the respective antibody responses, it was noticed that 20 subjects who did not have antibodies against PT (<5 IU/ml) in the sample before booster vaccine, 8 subjects (40 %) had positive proliferation response (SI ≥4). For PRN, only two subjects did not have any IgG antibodies before vaccination and from these one had a positive CMI response. For FHA, all subjects had antibodies against FHA already before the vaccination. All subjects became or remained seropositive after vaccination. However, ten subjects (18.5 %) were still without positive CMI (SI<4) against PT or PRN and only two against FHA, but they were seropositive for specific IgG antibodies.

5.3.2. IFN-γ and IL-17 ELISpot (III)

To evaluate the activation of IFN- γ and IL-17 secreting cells, ELISpot-assay was performed for PBMCs. For the IFN- γ ELISpot-assay 21 subjects were included and from these 14 subjects were further included for the IL-17 analysis. Due to the limited amount of available PBMCs for IL-17 assay, only samples after vaccination were available. For IFN-γ, an increase was observed in the median spot count against all vaccine antigens when before and after vaccination samples were compared (Figure 11). However, a significant increase was observed only with FHA. In addition, the detection rate increased for PT from 68 % to 81 % and for FHA from 95 % to 100%. For PRN the detection rate was stable at 69 %. The number of IL-17 secreting cells was much lower compared with IFN-γ secreting cells. The median spot count for IFN- γ was 16-fold, 6-fold- and 12-fold higher for PT, PRN and FHA, compared with the median spot count for IL-17 secreting cells. Supplementing PT with IL-23 increased the median spot count from 3 to 45 (p=0.0003). The number of IFN- γ secreting cells was found to correlate with SI from the proliferation assay both before and after the vaccination for all three pertussis antigens.

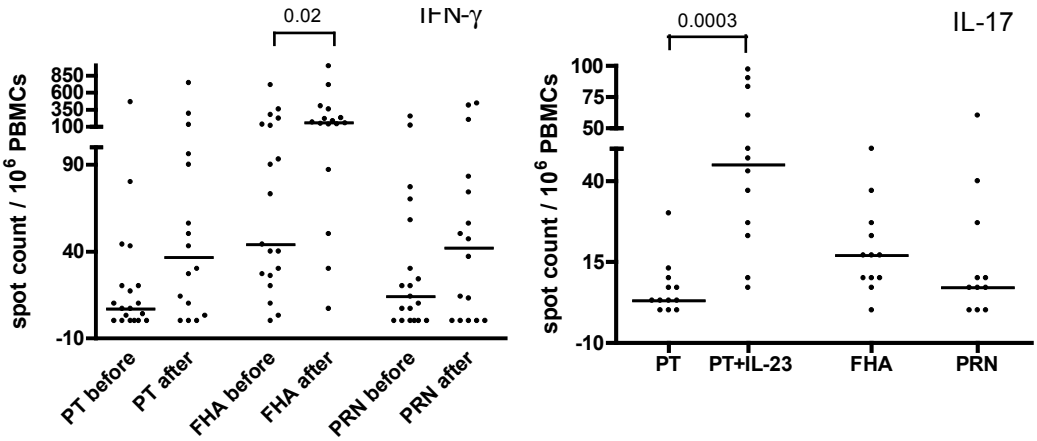

Figure 11. The spot counts in IFN-γ and IL-17 ELISpot assays. In the IFN-γ assay both before and one month after vaccination stimulation was measured. In the IL-17 assay only samples collected one month after vaccination were available. In the IL-17 assay, PT stimulation was performed with and without IL-23 supplement. Solid lines indicate the median values. Significant difference ($p<0.05$) are indicated in the figure. Modified from the original publication III.

5.3.3. Cytokine expression (III)

Cytokine expression was evaluated from the supernatants from stimulated PBMC taken before and one month after the second booster vaccine. Fourteen young adult subjects from the adolescent cohort were included. The selected cytokines were Th1-type (IFN-γ, IL-2, TNF-α, IL-12p70), Th2-type (IL-10, IL-4, IL-6), Th17-type (IL-17) and pro-inflammatory IL-8 and IL-1β. The absolute concentrations were increased for all measured cytokines; however a significant increase was noticed only for IL-8 after stimulation with PRN and FHA and for IL-6 after FHA stimulation (Figure 12). The unstimulated medium control was included for all subjects and this value was subtracted from the absolute cytokine concentrations of stimulated cells and the values were compared in the samples collected before and one month after the vaccination. Interestingly, only one subject (7 %) had an increase in all cytokine concentrations to PT stimulation whereas in six subjects' (42.9 %) samples all cytokine concentrations to PT decreased (Table 11). In addition, for two subjects all cytokine concentrations to PT, except IL-12p70, decreased. Similarly one subject had an increase in two cytokines (IL-4 and IL-8) and all others cytokines decreased. The other way around, six individuals had an increase in concentrations of at least one cytokine.

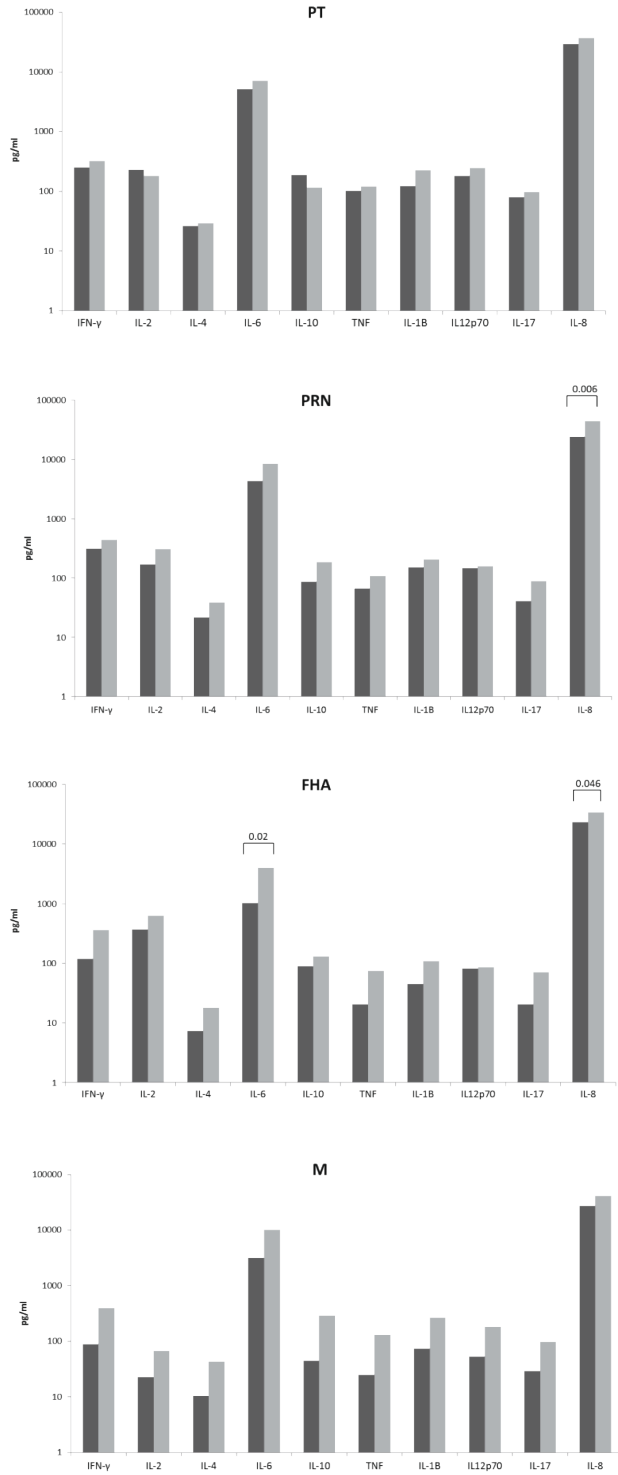

Figure 12. The GMC levels of cytokines in young adults before (dark gray bars) and one month after a second booster vaccination (light gray bars), against PT, PRN and FHA and medium (M) as a control. Significant differences ($p<0.05$) are indicated. Modified from the original publication III.

Table 11. The number of subjects (N=14) with decrease, increase or no change in cytokine concentrations one month after vaccination, after PT, PRN and FHA stimulation when medium control was subtracted from absolute values.

	all cytokines	IFN-γ	IL-2	IL-6	IL-4
PT					
Decrease	6	10	9	12	10
Increase	1	3	5	2	2
No change	7*	1			2
PRN					
Decrease	2	10	5	10	9
Increase	0	4	8	4	3
No change	12*		1		2
FHA					
Decrease	2	9	5	9	8
Increase	0	5	8	5	1
No change	12*		1		5

* Indicates number of subjects who had mixed change in concentrations of 10 tested cytokines before and after the vaccination. Modified from the original publication III.

When the individual results were combined, and the median values were compared in the samples before and one month after the vaccination, IL-2 was the only cytokine that remained positive whereas other cytokine concentrations decreased. When looking at the absolute concentrations, IL-6 was produced significantly more than IFN- γ, the signature cytokine of Th1 cells, against all pertussis antigens (Table 12). We also observed lower IL-12p70 production in FHA stimulated cells compared with PT stimulation (85.0 vs 244.6 pg/ml, P=0.05).

Table 12. The GMC of IL-6 and IFN-γ in 14 subjects before and one month after booster vaccination and after PT, PRN and FHA stimulation.

	Before	One month after	P-value*
PT			
IL-6	5204 (1950 – 13882)	7186 (3309 –15607)	0.60
IFN- γ	250.4 (56.1 – 1117)	322.9 (79.8 – 1307)	0.89
Ratio	21	22	
PRN			
IL-6	4342 (1654 – 11397)	8341 (4088 – 17020)	0.30
IFN- γ	311.4 (95.5 – 1015)	435.3 (129.3 – 1466)	0.75
Ratio	14	19	
FHA			
IL-6	1021 (395.6 – 2637)	4000 (1955 – 8187)	0.02
IFN- γ	119.1 (33.6 – 422.6)	361.0 (113.5 – 1147)	0.15
Ratio	9	11	

The GMCs between IL-6 and IFN- γ were significant in both time points and for all antigens. *P-values were calculated with Mann-Whitney U test and p-value <0.05 was considered significant. Modified from the original publication III.

5.4. IL-10 gene polymorphism association with proliferation in ten year follow-up (IV)

The purpose of this study was to investigate whether gene polymorphisms in selected cytokines and cytokine receptor genes (listed in Table 7.) are associated with CMI responses, measured with proliferation, after secondary booster vaccination with acellular dTap pertussis vaccine. Polymorphisms were analysed from 52 young adults whose DNA samples were available. However, the proliferation and genotyping data were available only from 38 of these subjects attending to the 10 year follow-up. The observed frequencies of the SNPs are listed in Table 13. Missing values in the number of subjects for each SNP are seen due to unsuccessful genotyping.

Table 13. The frequencies of IL-10 promoter SNPs and other cytokine related SNPs from 52 subjects.

Molecule	SNP code	Genotype	N (%)
IL-10 (-871)	rs1800871	GG	31 (63.3)
		GA	17 (34.7)
		AA	1 (2.0)
IL-10 (-1082)	rs1800896	AA	15 (31.3)
		AG	23 (47.9)
		GG	10 (20.8)
IL-10 (-3573)	rs1800890	TT	22 (42.3)
		TA	26 (50)
		AA	4 (7.7)
IL-12RB1	rs372889	CC	16 (31.4)
		CT	28 (54.9)
		TT	7 (13.7)
IL-12B	rs2546890	AA	18 (35.3)
		AG	23 (45.1)
		GG	10 (19.6)
IL-17A	rs2275913	GG	17 (32.7)
		GA	19 (36.5)
		AA	16 (30.8)
IL-23R	rs11209026	GG	46 (90.2)
		GA	5 (9.8)
		AA	0

Modified from the original publication IV.

Proliferation against pertussis antigens PT, PRN and FHA was compared with different genotypes and significant differences were only observed with the IL-10 SNP at position -1082 (A>G, rs1800896), and only for post booster responses. The comparison of subjects with the normal allele AA, heterozygote variant AG and homozygote variant GG with proliferation are shown in the Figure 13. One month after the vaccination, subjects with the heterozygote variant allele AG had higher proliferation responses (cpm) against PT compared with subjects having the normal allele AA (GMC 3209 vs 721.2, P=0.02). A

similar trend was observed with proliferation against PRN and FHA; however the difference was not significant (PRN 1908 vs 1185; FHA 4902 vs 3652). The lowest proliferation response against PT, FHA and PRN was detected with homozygote variant allele GG. After PT stimulation, the difference was significant between GG and AG responses (GMC 112.8 vs 3209, p=0.0003). A more modest difference was observed between the GG and normal allele AA (GMC 112.8 vs 721.2, p=0.078). After PRN stimulation, subjects with variant GG had significantly lower responses than subjects with variant AG (GMC 196.7 vs 1908, p=0.02). Whereas after FHA stimulation, response in subjects with variant GG was significantly lower than in normal allele AA (GMC 665.4 vs 3652, p=0.0085) and heterozygote variant allele AG (GMC 665.4 vs 4902, P=0.0008).

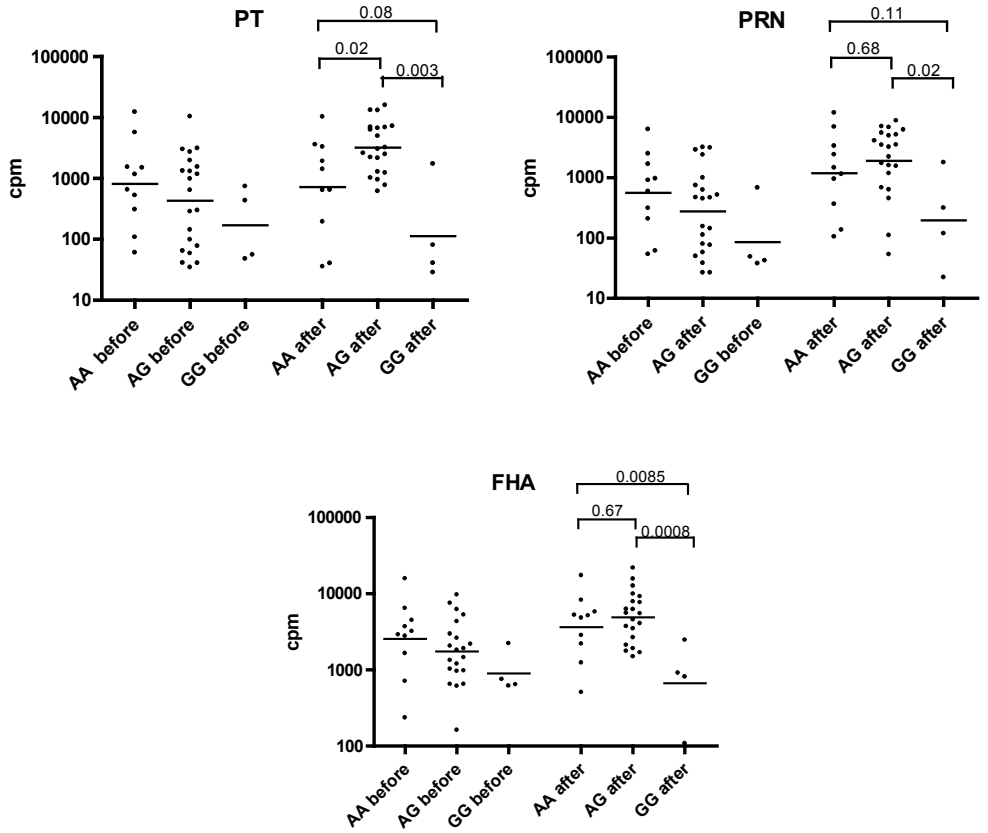

Figure 13. Absolute proliferation (cpm) of PBMCs stimulated by PT, PRN and FHA in different IL-10 -1082 genotypes; normal allele AA, heterozygote variant allele AG and homozygote variant GG, before and one month after the vaccination in 38 subjects. Solid line indicates the geometric mean. P-values are indicated in comparisons between genotypes after vaccination. Modified from the original publication IV.

Positive response in proliferation was defined as SI ≥4. Similarly to absolute counts after vaccination, also median value of SI after vaccination differed between the different IL-10 -1082 genotypes after stimulation with PT, PRN and FHA (Figure 14). Subjects with

heterozygote variant AG had significantly higher SI after PT stimulation compared with normal allele AA (63.7 vs 10.3, p=0.003) and compared with homozygote variant GG (63.7 vs 1.8, p<0.0001). Subjects with variant GG did not reach the positive SI response and had also lower SI compared with normal allele AA (1.8 vs 10.3, p= 0.08). A similar trend was observed with PRN and FHA, that heterozygote variant AG had the highest SI compared with AA normal allele and CC variant allele.

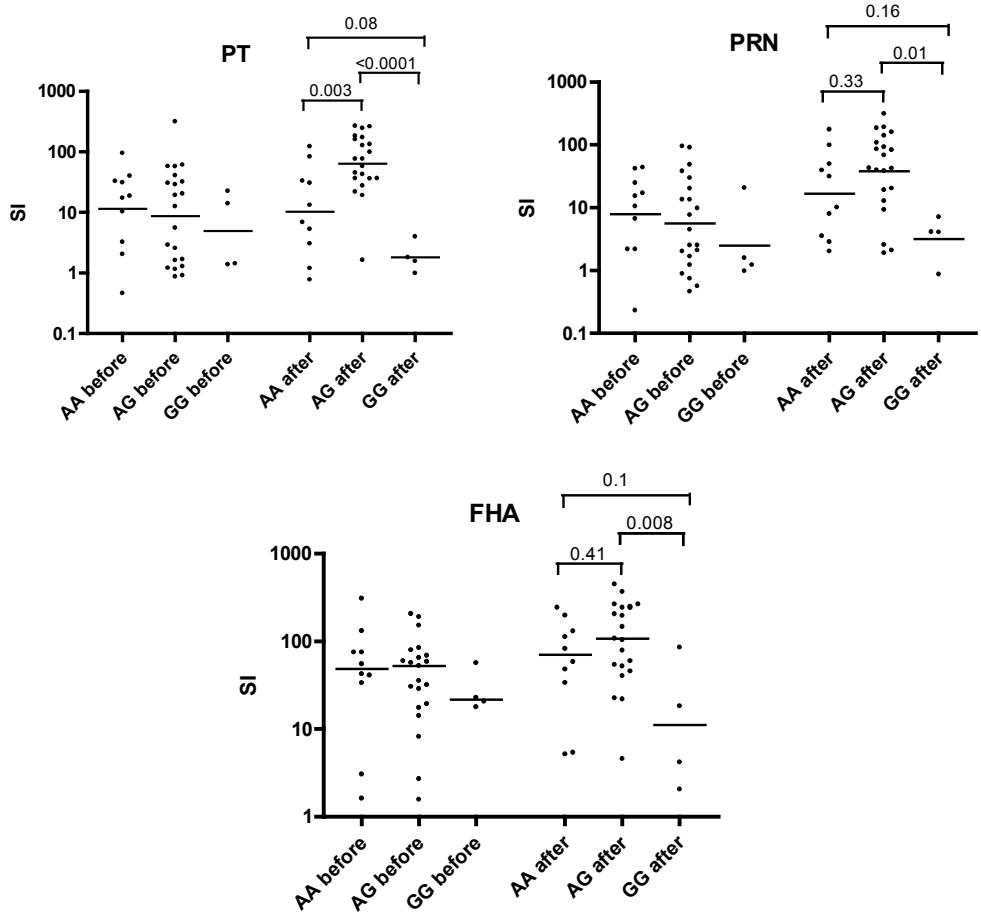

Figure 14. Stimulation indexes (SI) of proliferation of PBMCs stimulated by PT, PRN and FHA in different IL-10 -1082 genotypes; normal allele AA, heterozygote variant allele AG and homozygote variant GG, before and one month after the vaccination in 38 subjects. Solid line indicates the geometric mean P-values are indicated in comparisons between genotypes after vaccination. Modified from the original publication IV.

5.5. MBL deficiency in pertussis patients (V)

To evaluate the possible role of MBL deficiency in susceptibility to pertussis infection, MBL concentration was measured from 125 laboratory-confirmed pertussis patients' serum samples and 430 controls. High variation in MBL concentrations was observed

in patients (range 25–10407 ng/ml) and controls (range 25–8656 ng/ml). No difference was observed in the median MBL concentration between the patients and controls (2206 ng/ml and 1910 ng/ml, respectively). MBL concentration is known to decrease with age and both groups were divided according to their age for subjects older than 18 years of age and equal or younger than 18 years of age. Significantly lower concentrations were observed in the older age group both in patients and controls (Figure 15).

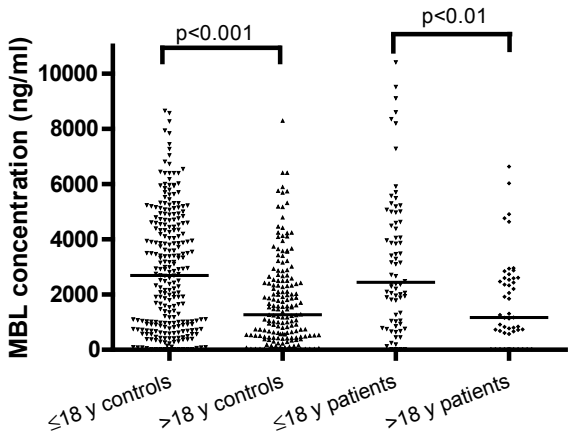

Figure 15. MBL concentration in controls and pertussis patients divided in two groups based on age; less or equal to 18 years and over 18 years. Solid line indicates the median. P-values were calculated with Mann-Whitney U test. Modified from the original publication V.

MBL deficiency is a consequence of polymorphism in *MBL2* gene. In this study, the definition for MBL deficiency was selected as the detection limit of the assay, which was 50 ng/ml. For statistical analysis, the values lower than the detection limit were given a value 25 ng/ml. In the control group, the frequency of MBL deficiency was 5.8%. In contrast, in the pertussis patients group MBL deficiency was observed significantly more often (11.2%, p=0.038). Moreover, in the patients group older than 18 years of age, the frequency of MBL deficiency was even higher (20.4%) compared with controls of the same age (8.6%, p= 0.021) (Table 14).

Table 14. The frequency of MBL deficiency (<50 ng/ml) in patients (N=125) and in controls (N=430). Subjects were also divided in two groups based on age; less or equal to 18 years and over 18 years.

	MBL deficiency (<50ng/ml)	Normal MBL	P-value*
All controls N (%)	25 (5.8)	405 (94.2)	0.038
All patients N (%)	14 (11.2)	111 (88.8)	
≤18 y controls N (%)	10 (3.9)	246 (96.1)	0.61
≤18 y patients N (%)	4 (5.3)	72 (94.7)	
>18 y controls N (%)	15 (8.6)	159 (91.4)	0.021
>18 y patients N (%)	10 (20.4)	39 (79.6)	

*P-values were calculated with Chi-square test. Modified from the original publication V.

6. DISCUSSION

6.1. Vaccination to control and prevent pertussis

Vaccination against pertussis has been in routine use for over a half-century in most of the developed world. After initiation of nation-wide vaccinations, the pertussis incidence decreased significantly. The first vaccines were DTwP vaccines that in many countries were produced by themselves. Nevertheless, the unfavorable side effects of the DTwP vaccine forced several countries to replace it with new, more tolerated DTaP. Reports on adverse effects were reduced. In the 1990's, coincidentally with the change of the vaccine, also the incidence of pertussis started to rise again. DTwP vaccine mimics the natural infection in its ability to induce same type of immune response to infective *B. pertussis*, whereas DTaP induces immune response only against the antigens selected in the vaccine. Currently used DTaP vaccines are a combinations of five antigens PT, PRN, FHA, Fim2 and Fim3 together with immune stimulatory adjuvant.

Immunity against pertussis is complex. *B. pertussis* produces several toxins and adhesins that are important for the pathogenesis of the bacteria. At the same time, the circulating strains are changing in order to adapt to vaccine-induced immunity. One of the extremes observed during the last years are the strains not expressing vaccine antigens such as PRN (Zeddeman *et al.* 2014, Lam *et al.* 2014, Martin *et al.* 2015). A study conducted with 753 *B. pertussis* isolates from case-patients, showed that individuals who were fully vaccinated according to the vaccine schedule, had 2 – 4 fold higher susceptibility to have an infection with a strain not expressing PRN than a strain expressing it (Martin *et al.* 2015). Even though the immunity is different when immunized with the DTaP vaccine compared to the infection, vaccination is the best strategy to prevent and control the circulation of *B. pertussis*.

6.2. Waning immunity after pertussis vaccination (I, II)

Vaccination with DTaP induces high titers of antibodies straight after vaccination. However, serological studies have revealed that protective immunity after DTaP vaccine wanes rather fast (Klein *et al.* 2012, Tartof *et al.* 2013). Odds for the pertussis infection increases significantly each year from the last dose of DTaP vaccine (Misegades *et al.* 2012). Contrary, a recent study showed that the infection severity and the rate of hospitalization are lower for children and adolescents ever vaccinated compared with totally unvaccinated ones, supporting that vaccine, regardless the waning immunity, prevents the most severe pertussis at individual-level (Barlow *et al.* 2014). Even the adequate serological correlate of protection has remained unsolved, antibodies against PT and PRN are protecting against the disease (Storsaeter *et al.* 1998, Cherry *et al.* 1998). In our infant cohort (II) very low antibody concentrations were observed already

12 months after the last of the three primary doses (GMC for PT, PRN and FHA 6.7; 4.4 and 32.9 IU/ml, respectively, unpublished). PT is the only *B. pertussis* specific antigen; however, also the anti-PRN and anti-FHA antibody levels were low in these low-responders (GMC IgG 2.9 and 13.0 IU/ml, respectively, unpublished). These low-responders may be vulnerable for the infection, although they have received the primary three doses.

In our adolescent cohort (I), before administrating the second booster vaccine at 19 - 21 years of age, the seropositivity rate had decreased to the level of prevaccination ten years previously. One month after the second booster, the overall seropositivity increased significantly, but still 19 % of the subjects did not reach the seropositivity (5 IU/ml) for PT. For PRN and FHA, antibodies persisted longer and seropositivity was 100 % after the second booster. It is commonly known that antibodies against PRN and FHA last longer than those against PT. However, it is known that there might be some cross-reactivity with other species such as other *Bordetella* species, *Haemophilus* species, *Mycoplasma pneumonia* and *Escherichia coli* (Guiso *et al.* 2011). This may also contribute to the better persistence of PRN and FHA antibodies. Pertussis is increasingly reported in adolescents and adults (Zepp *et al.* 2011, Clark 2014). In addition to waning immunity, one reason for the increased incidence in older age groups is the increased awareness and improved diagnostics. However, pertussis in adults is commonly lacking the classical symptoms, which causes unrecognition of the disease and this group serves as a big reservoir for the bacteria increasing the burden of pertussis (Hewlett & Edwards 2005). These adult patients with prolonged cough without solid diagnosis transmit the bacteria easily to more vulnerable infants. In case of an infant who has been immunized but is a non-responder, the transmission might be more plausible.

6.3. Genetic variation in vaccine responses

The reason for the variation observed in immune responses after pertussis vaccination is not studied in detail. It is acknowledged that several factors can contribute to this such as silent boosting, age or gender. Genes of the immune network are highly polymorphic and more evidence is being collected on genetic impact on immune responses. A first report showing the genetic heritability of antibody responses against measles vaccine was performed in a twin cohort (Tan *et al.* 2001). Twin studies are able to differentiate the environmental factors from genetic ones when exploring the variation in immune responses. Nevertheless, the method is a more general estimate of inheritance (Poland *et al.* 2008). More in-depth association studies have been performed on targeted genes and their influence on antibody responses after bacterial and viral vaccines such as tetanus, diphtheria, measles, mumps and rubella. Pattern recognition is the initial step of innate immune response. MBL and TLR4 are the soluble and membrane-bound actors of pattern recognition. Both are highly polymorphic and clinical association with susceptibility to pathogens has been proven.

6.3.1. Genetic variation of TLR4 and antibody responses after pertussis vaccination (I)

TLR4 is already known to recognize LPS and PT from *B. pertussis* and it has been shown to be involved in the DTwP induced vaccine responses (Wang *et al.* 2006, Banus *et al.* 2008, Banus *et al.* 2006). The role of TLR4 signaling pathway in pathogenesis after *B. pertussis* infection has been studied with a mouse model. TLR4-deficient mice had more severe infection than wild type mice and IL-10 production was inhibited, suggesting that TLR4 activation is needed for IL-10 production after pertussis infection (Higgins *et al.* 2003). Further study in mice verified the previous finding, that the bacterial clearance was delayed in the TLR4 deficient mice. This finding also indicated that TLR4 mediated TNF-α, IL-1β, and IFN-γ response enhances the bacterial clearance (Banus *et al.* 2006). Banus *et al* studied several SNPs in the TLR4 pathway genes such as *LBP*, *CD14*, *TLR4*, *TOLLIP* and *TIRAP* in association with PT-specific antibody responses after DTwP vaccination in Dutch children (Banus *et al.* 2008). They observed associations of several SNPs with antibody responses and the authors state that the results strongly support that TLR signaling is involved in pertussis vaccine responses. TLR4 is linking also between innate and adaptive immunity by activating the DCs and generating Th1 cell response (Schnare *et al.* 2001). These previous studies on TLR4 and immune responses against pertussis are based on DTwP vaccine and *B. pertussis* infection. Targeted analysis on TLR4 D299G polymorphism and DTaP vaccine responses was selected for our analysis (I). The selection was based on the previous studies on TLR4 and pertussis induced immunity as well as no published information on the association of TLR4 with DTaP vaccine responses. In addition, the frequency of D299G mutation is relatively high which enables to study the possible association. The frequency of TLR4 D299G polymorphism observed in our cohort was similar to those published previously in the Finnish population with frequency of 85.1% for normal allele AA, 13.5 % for heterozygote variant AG and 1.4 % for homozygote variant GG (Vuononvirta *et al.* 2011, Lofgren *et al.* 2010). Due to the limited number of subjects in the homozygote variant group (n=1), it was combined with the group of heterozygote variants and this group was defined as D299G variants. The study population included 64 females and 11 males. Unbalanced ratio of genders is observed because some of the males were dismissed from the ten year follow-up, as they had received a diphtheria-tetanus booster recently in the military service (Mertsola *et al.* 2010). However, we did not observe differences in the antibody responses between genders.

Our study indicated that subjects with TLR4 D299G polymorphism had decreased antibody responses after dTap booster vaccination in adolescents compared with normal allele. Our finding supports the previous finding that was observed with DTwP vaccine, that TLR4 is involved in immune responses after pertussis immunization. Although the cohort was rather small, the trend was clearly seen. After the first booster vaccination, the fold increase of antibodies against PT, PRN and FHA was over two times higher for subjects with TLR4 normal allele compared to those with the D299G variants. Subjects who had normal allele of TLR4 D299G, antibody GMCs were increased 8-fold for PT,

26-fold for PRN and 13-fold for FHA, whereas in subjects who had D299G variant, increase in antibody GMCs was 6-fold for PT, 12-fold for PRN and 5-fold for FHA. In addition, the antibody persistence was more short-term in subjects carrying the D299G variant. These latter subjects had pertussis specific antibodies already decreased to the levels of prebooster at 3 years follow-up, whereas in the subjects with the normal allele antibodies had decreased to the level of prevaccination only after 5 to 10 years. TLR4 D299G mutation is located on the extracellular domain of the protein and especially in the pattern recognition site (Ohto *et al.* 2012). This supports the hypothesis that there might be an altered recognition of vaccine antigens in subjects with D299G variant. It is also known that TLR4 recognizes PT (Wang *et al.* 2006). However, the recognition of FHA and PRN by TLR4 is not known. Our results indicate that in addition to PT, TLR4 might also recognize or interact with FHA and PRN. The difference observed in the persistence of pertussis specific antibodies between subjects with different D299G genotypes may be related to indirect involvement of TLR pathway with adaptive immunity and possible maintaining of the memory (Liu *et al.* 2010). However, we cannot rule out other possibilities such as TLR4 interaction with alum adjuvant present in the vaccine or altered interaction with other molecules of TLR4 pathway or SNPs in the other molecules of this pathway. Whether it is physical recognition or indirect association remains to be shown. The other common SNP in *TLR4* gene, T399I, was not investigated in this study. D299G and T399I are cosegregating and both are located in the ligand binging motif of TLR4. However, in the crystal structure, T339I seems to have less effect on the structure compared with D299G (Ohto *et al.* 2012). In addition, based on functional study, D299G may have stronger effect on TLR4 function compared with T399I (Arbour *et al.* 2000, Long *et al.* 2014).

6.4. *MBL2* polymorphism in association with pertussis vaccine responses (II)

Association studies on *MBL2* polymorphism and vaccine responses are limited. Positive association has been detected only with influenza A vaccine responses (Tang *et al.* 2007), whereas no associations were discovered with 23-valent pneumococcal polysaccharide vaccine and hepatitis B vaccine responses in humans (van Kessel *et al.* 2014, Osthoff *et al.* 2014). Previously the antibody responses to hepatitis B were shown to be affected by the *MBL2* polymorphism both in a mouse model and in the Indonesian population, while no association was observed with the Kenyan population (Ruseva *et al.* 2009, Davila *et al.* 2010). The study conducted in the Indonesian population was genome-wide SNP analysis and the only SNP affecting hepatitis B specific antibody responses was an intronic SNP and after correcting for non-genetic factors the significance was reduced (Davila *et al.* 2010). This implies the importance of considering the ethnicity of the study population, as the frequency of *MBL2* polymorphism varies dramatically in different populations (Garred 2008). Based on our finding with pertussis patients (V), that MBL deficiency was more prevalent in pertussis patients, it was interesting to look whether

MBL polymorphism or MBL deficiency affect the antibody responses after primary and booster vaccination with acellular pertussis vaccine (II).

MBL2 genotyping was performed for 355 adolescent from dTap vaccine trial cohort and 213 infants (II). Antibody responses were evaluated from adolescents' samples collected before and one month after the first DTap booster vaccine, at three and five year follow-up and before and one month after the second booster vaccine after 10 year follow up at the young adult age. From the infant cohort, pertussis specific antibody concentrations were measured before the first primary DTaP vaccination at 2.6 months of age, after three primary doses at 13 months and one year after that at 2 years of age. The frequency of *MBL2* exon1 polymorphisms in codons 52, 54 and 57 in both infant and adolescent cohort was similar to previous observations from Finnish and European populations (Garred 2008, Rantala *et al.* 2008, Vuononvirta *et al.* 2011). In addition to *MBL2* genotyping, MBL concentration was measured from the adolescent cohort and the definition for MBL deficiency was the same as defined in the study (V) (<50 ng/ml). The frequency of MBL deficiency (4.2 %) was rather similar to our previous study (V). MBL deficiency did not affect any of the IgG concentrations against the vaccine antigens, including D and T. Neither was the frequency of non-responders over-presented in the group of subjects with MBL deficiency. However, the overall variation of MBL concentrations correlated with genotypes; normal A/A allele presenting the highest median concentration, A/O moderate and O/O the lowest concentration. This is in line with previous studies (Minchinton *et al.* 2002). MBL concentration measurement was not performed for the infant cohort, as we did not observe any differences in antibody responses between adolescent subjects with MBL deficiency or normal MBL concentration.

The frequency of *MBL2* exon1 genotypes observed for adolescent and infant cohort were similar to previously observed in Finnish population (Rantala *et al.* 2008, Vuononvirta *et al.* 2011). When the genotyping data were compared with antibody responses, we could not find any differences in the antibody responses or antibody persistence and MBL genotypes; normal allele A/A, heterozygote variants A/O or combined heterozygote and homozygote variants O/O, neither in adolescents cohort (n=355) or infant cohort (n=213). It is clear that immunity after infection with *B. pertussis* is different from the immune reaction after vaccination. Acellular vaccine administrated to our cohort includes only three of the numerous antigens present on the surface of *B. pertussis*.

6.5. Cell mediated immunity in protection (III)

After diminished antibodies, CMI with T-cells and B-cells may still account for some of the protection against pertussis. This has been observed in several previous studies both in children and adolescents (Ausiello *et al.* 1999, Hendrikx *et al.* 2011b, Meyer *et al.* 2007, Guiso *et al.* 2007). We investigated the persistence of CMI ten years after the previous acellular booster vaccination in young Finnish adults (III). Our cohort included

57 young adults who attended the ten year follow-up of our dTap vaccine trial. Of the 57 subjects, 51 % and 53 % had positive proliferation response (SI≥4) against PT and PRN, ten years after the previous pertussis vaccination. For FHA the percentage was even higher (81 %), but it should be kept in mind that some cross-reactivity with other species such as *Haemophilus influenzae* or *Mycoplasma pneumoniae* may affect the result from FHA (Vincent *et al.* 2000, Hendrikx *et al.* 2011a). Booster vaccination improved the CMI response significantly when measured with proliferation. SI was positive for 81 %, 81 % and 96 % for PT, PRN and FHA respectively. The definition for positive proliferation response in this study is SI≥4. The geometric mean value of stimulation index increased considerably more in subjects who had SI<4 before the vaccination compared with subjects with SI≥4. This phenomenon might observe because the subjects with SI≥4 before vaccination already have reached the highest plateau of proliferation. From the whole ten year follow-up cohort, antibody responses have been published previously (Mertsola *et al.* 2010). From our sub-cohort of 57 subjects, 20 subjects had diminished antibodies against PT (<5 IU/ml). Interestingly, from these, seven subjects (40 %) still had positive CMI response. This supports the previous finding that CMI is detectable after waning of antibodies. Similarly to results published from a 5 year follow-up (Edelman *et al.* 2007), we did not find any correlation between antibodies and proliferation. Proliferation is mainly a measurement of the T lymphocyte function whereas the antibody production shows the function of memory B-cells that have re-encountered the antigens. The long-lasting memory observed in our cohort, even though ten years elapsed after the last vaccination might reflect the impact of the primary vaccine these subjects had received, which is DTwP. Currently observed outbreaks in developed countries are often seen in infants who are primed with DTaP vaccine. In addition, immunity in infants may be different from adults. Sharma and Pichichero recently reported that T cell responses after DTaP vaccination differ between adults and infants; with more of polyfunctional Th cells (TNF-α, IL-2 and IFN-γ producing) present in adults (Sharma &Pichichero 2012). In addition, Th cells were more differentiated in adults compared with infants and authors suggest that this might because of multiple vaccinations received in adults. It has been also suggested that after primary DTaP vaccine the T cells are more differentiated to effector memory T cells and these cannot be boosted effectively, compared with the larger amount of naïve T cells present after DTwP priming (de Rond *et al.* 2015).

Infection and DTwP vaccination result in secretion of IFN-γ and IL-2. Especially IFN-γ is critical for the protection against pertussis as it attracts macrophages to the local infection site and conducts the internalization and killing of the bacteria (Higgs *et al.* 2012, Ryan *et al.* 1997). Th2 cells, induced by DTaP vaccination have a more important role in the production of antibodies and stimulation of characteristic Th2 cytokines IL-4, IL-5 and IL-13. However, the response after dTaP vaccine in older age groups induces a mixed Th1/Th2 response. This mixed response may be partly caused by the subclinical infections in countries with high circulation of *B. pertussis* (Schure *et al.* 2012). A more recent discovery is the involvement of Th17 lineage in protection against pertussis and at least in a mouse model it seems to be activated both after infection and

aP vaccination (Ross *et al.* 2013). The difference observed in Th cell response after infection and DTaP vaccination has evoked the speculation whether this difference is one of the shortcomings of DTaP induced immunity (Mills *et al.* 2014). Our preliminary results indicate that a second dTap booster at the young adult age gives a rather modest induction of IFN-γ secreting cells against PT and PRN (III). Only stimulation with FHA caused a significant increase in the observed cell count. Even more modest was the induction of IL-17 secreting cells. To our knowledge, previous studies on Th17 cell secretion has been only reported in school children 4, 6 and 9 years of age who received a booster dose of aP vaccine (Schure *et al.* 2012). The trend observed was similar to ours with overwhelming IFN- γ secreting cells. IL-23 is known to induce and maintain the IL-17 secretion from Th17 cells (Park *et al.* 2005, Wilson *et al.* 2007). In our *in vitro* model, cells stimulated with PT in the presence of IL-23 increased the number of IL-17 secreting cells. The possible effect of IL-23 supplementation on induction of IL-17 production remains to be studied. The preliminary results presented in this study are promising in the sense that the dTap boosted immunity could be skewed to the direction similar to infection with proper adjuvant.

Cytokine responses were evaluated in samples collected from 14 young adult subjects before and one month after the second booster vaccine (III). Cytokines were selected from different Th subclasses; Th1-type (IFN-γ, IL-2, TNF-α, IL-12p70), Th2-type (IL-10, IL-4, IL-6), Th17-type (IL-17) and pro-inflammatory IL-8 and IL1β. The selection of the cytokines was done based on literature and the interest of the research group. We observed all absolute cytokine concentrations to increase in the stimulated samples one month after the vaccination. However, the limited number of subjects might have caused that significant difference was observed only in the concentration of IL-8 after PRN and FHA stimulation. Cytokine concentrations from unstimulated controls were subtracted from that stimulated by specific antigens. Interestingly we observed that many subjects had a decrease in their cytokine production in the samples collected one month after the vaccination. On the individual level, only one subject had increase in all cytokines measured whereas almost half of the subjects (n=6) had decreased concentration of all measured cytokines. When individual data were combined, only median value for IL-2 response remained positive. The repeated encountering of the vaccine antigens may have caused downregulation. This phenomenon is not new; it is known that Th cell response may be downregulated after *B. pertussis* infection. The production of IL-12 is suppressed by FHA is through upregulation of IL-6 and IL-10 (McGuirk &Mills 2000). In our cohort, we also observed lower IL-12p70 production in FHA stimulated cells compared with PT stimulation. IL-10 is known to be suppressed during the infection as it inhibits the IFN-γ secretion (Dirix *et al.* 2009). After vaccination, the repeated encountering of the vaccine antigens by PBMCs might cause downregulation in cytokine expression. It has also been shown that the primary pertussis vaccination affects the responses detected in adolescents and adults. Adolescents primed with DTwP in the childhood have been documented to produce a wider range of cytokines after dTap booster vaccination compared to adolescents with DTaP primary vaccination (Smits *et al.* 2013), whereas

another study observed that Dutch children primed with DTaP vaccine had more pertussis specific memory B-cell cells than DTwP primed children (Schure *et al.* 2013). It should be noticed that silent infection might also affect the observed differences in different vaccine groups (Schure *et al.* 2012, Rieber *et al.* 2008). In addition, it should be remembered that the phenomenon observed with our *in vitro* model might differ from the situation *in vivo*.

To summarize, our results show that dTap booster vaccine effectively induced CMI measured by proliferation and ELISpot assay in young adults. However, there seems to be decreased production of cytokines in our cohort.

6.5.1. IL-10 promoter polymorphism and cell mediated immunity (IV)

In addition to low antibody responses after vaccination, also low CMI response in some subjects can be detected. The phenomenon is less studied than the non-responsiveness measured with antibodies. Our aim in the original study (IV) was to search whether gene polymorphism in cytokine and cytokine receptors is more frequent in subjects with lower CMI responses, measured by proliferation after second booster dose of dTap. For this purpose, 38 young adults who attended the 10 year follow-up and whose proliferation results and genotyping data were available were included. SNPs selected for the study were present in genes encoding *IL-10* (–3575A/T rs1800890, -1082A/G rs1800896, -819G/A rs1800871), *IL-12B* (rs2546890), *IL-12RB1* (rs372889), *IL-17A* (rs2275913) and *IL-23R* (rs11209026). Gene polymorphisms in cytokines and cytokine receptors can change the expression level or the function of the cytokine and therefore the polymorphism may affect the observed immune responses. All selected genes are polymorphic and the selection criteria for the specific SNPs was based on previous studies on these SNPs. Variability has been previously observed between IL-10 polymorphism and vaccine responses against hepatitis A and B, influenza A and measles vaccine responses (Hohler *et al.* 2005, Dhiman *et al.* 2007, Tang *et al.* 2007). IL-12 and IL-12R gene polymorphisms have been observed to be associated with low responses after measles, hepatitis B and pneumococcal conjugate (7-valent) vaccinations (Yucesoy *et al.* 2009, White *et al.* 2012). IL-17A polymorphism has been associated with increased bacterial colonization in young children (Vuononvirta *et al.* 2015).

In our cohort, significant differences were observed only in CMI responses one month after vaccination and IL-10 promoter SNP at position -1082. In subjects with heterozygote variant AG, the proliferation responses was the highest when measured by both absolute counts from proliferation (cpm) and SI against PT, PRN and FHA, whereas in subjects with homozygote variant GG, the proliferation response was the lowest against all pertussis antigens. IL-10 is known as an immunosuppressive cytokine. It inhibits the antigen presentation to DCs (O'Garra &Vieira 2007). We did not measure the cytokine concentration from the subjects which could show the influence of the SNP on the level of IL-10. Polymorphism in the promoter region may cause alteration in the binding site of transcriptional factors, leading to inhibition

or activation of the transcription. Previously, it has been shown *in vitro* that -1082 homozygote variant GG correlates with high production of IL-10 (Turner *et al.* 1997). Similar observation has been reported from a study of healthy Caucasians from Spanish (Suarez *et al.* 2003). In our cohort, subjects with homozygote variant GG had the lowest proliferation responses against PT, PRN and FHA. The higher IL-10 concentration might lead to inhibition of the proliferation response. The highest observed proliferation was seen with heterozygote variant which might indicate the impaired regulation of IL-10 that is beneficial for CMI responses. The frequency of the heterozygote variant AG was 60 % in our cohort and it reflects the common frequency of the variant in Finnish population (Koponen *et al.* 2014, Pöyhönen *et al.* 2015) and Caucasians based on the international HapMap project. The AG variant allele is more frequently found than the normal allele AA. This may be an indication of evolutionary selection of this variant as a beneficial for the population.

Previous studies on polymorphisms of IL-10 promoter region and vaccine responses are mainly focused on antibody responses. One study performed with Northern-American cohort, on measles antibody and lymphoproliferative response after measles vaccination found three IL-10 promoter SNPs (at position -3584, -8537 and -626) to be associated with low vaccine response (Dhiman *et al.* 2007). Another study observed that antibody response to tetanus toxoid vaccine was lower in variant -1082GG than in AA allele and AG allele (Li *et al.* 2007). In a study on influenza A vaccine response, it was also found that the -1082GG variant with high IL-10 production is associated with low vaccine response (Corsini *et al.* 2006). Pneumococcal and diphtheria antibody concentrations after vaccination were affected by IL-10 SNP at position -819 (Yucesoy *et al.* 2009). Although it is common to analyse IL-10 promoter polymorphisms in haplotypes, the number of subjects in our cohort was limited and this hindered us to evaluate the possible effect of haplotypes.

6.6. MBL deficiency and pertussis susceptibility (V)

MBL functions in recognition of pathogens as part of the first line defence. Association of gene polymorphism in *MBL2* and MBL deficiency with several infectious diseases has been published with different populations and diseases conditions. This genetic variation in innate recognition may increase the susceptibility to infectious diseases (Heitzeneder *et al.* 2012). However, no previous studies on relation between MBL deficiency and pertussis infection have been published. In original study (V) this association was evaluated for the first time. MBL concentration was measured from 125 laboratory confirmed pertussis patients and 430 control subjects. The median concentration of MBL did not differ between the patients and controls (2206 ng/ml vs 1910 ng/ml, p=0.54). This is most likely due to different genotype background of the subjects. It is difficult to observe differences on high MBL concentrations as the SNPs in the exon1 and promoter region affect the MBL concentration (Minchinton *et al.* 2002).

MBL concentration is known to decrease slightly with age until the early adulthood (Aittoniemi *et al.* 1996). We divided the cohort to two age groups to avoid the possible interference of age for the MBL concentration. There was a clear difference in the MBL concentration in subjects ≤18 years of age and >18 years of age (in controls 2693 vs 1270 ng/mL, P< 0.001 and in patients 2443 vs 1172 ng/mL, P< 0.01, respectively). It is known that in people with O/O genotype, the MBL concentration is very low or undetectable. In this study, MBL deficiency (<50 ng/ml) was discovered to more frequent in pertussis patients cohort (11,2 %) compared with the controls (5.8 %) (P=0.038). The phenomenon was even more clear in adults (>18 years). In adult patients group, 20,4 % had MBL deficiency, whereas 8.6 % of the adult controls had the deficiency (P=0.021). It is not possible to estimate the MBL concentration based on genotype and vice versa due to the *MBL2* exon1 and promoter region polymorphisms that both affect the concentration. In addition, no general cut-off for MBL concentration has been defined, which would correlate with impaired MBL function. We assessed the limit for MBL deficiency as low as possible, which was the detection limit of the assay (<50ng/ml) to avoid possible confounding effect of functional MBL. With this definition, the frequency of total MBL deficiency (5.8 %) in the control group was similar to other studies conducted in Finland (Rantala *et al.* 2008, Huttunen *et al.* 2008). Recently in a study with Finnish cohort, it was evaluated that the limit for functional MBL can be defined to almost 10-times higher than the limit we used in our study (445 ng/ml vs 50 ng/ml) (Sajanti *et al.* 2015). The method is same in the study by Sajanti et al and ours. The methodological consideration is important when comparing the results of MBL concentration assays. Our method is so-called sandwich ELISA in which the capture molecules are monoclonal antibodies against human MBL. Many of the commercial kits use mannan as a coating molecule. ELISA using monoclonals as a coating allows detection of all MBL molecules not only the functional forms as in the case of mannan coating. Our method most likely detects even higher concentration of MBL than what is functional. It should be kept in mind that the control group used in this study was not randomly selected from totally healthy subjects as this type of subjects is difficult to recruit. Instead, control group consisted of samples sent for celiac diseases diagnostics but were reported as negative in the laboratory test. This may cause slighthly higher frequency of MBL deficiency compared to what would be observed from totally healthy controls which would further increase the difference observed between pertussis patients and controls. There are no studies on whether MBL recognizes *B. pertussis*. However, it is known that the repertoire of ligands of MBL is wide and it includes both gram-positive and gram-negative bacteria. *H. influenzae,* the identified ligand of MBL (Neth *et al.* 2000), is structurally similar to *B. pertussis*. However, it remains to be studied whether MBL is able to recognize *B. pertussis*.

6.7. Limitations of the study

It is acknowledged that this study has limitations. The polymorphism was studied in *TLR4*, *MBL2* and selected cytokines and cytokine receptors, as there are previous observations

that these SNPs in these genes are associated with infection susceptibility and altered vaccine responses. The association studies carried out in this study show preliminary results of their involvement in pertussis infection or vaccine responses. However, the functionality of these SNPs was not studied and remains to be shown. We did not study the *MBL2* promoter polymorphism as it is stated in the text that exon1 SNPs account for the most of the variation in circulating MBL protein. Also in *TLR4* and cytokines there are several other SNPs recognized. With high-throughput method, more SNPs could have been selected for the analysis. The number of subjects was also relatively low. The drawback of long follow-up studies is commonly the increased dropout rate. The experiments should be repeated with increased number of subjects and in different populations. However, with this limited cohort, the frequencies of the SNPs were similar to previous studies on the Finnish population or Caucasians.

6.8. Improvements for the current pertussis vaccines

The acellular pertussis vaccine has been under criticism during the last years. Several large and severe outbreaks of pertussis have been observed in countries with long history of nationwide vaccinations such as USA and UK. As stated earlier, waning immunity is one of the main reasons for the re-emergence of pertussis. Waning immunity was observed also in both of our cohorts; adolescents/young adults and infants. In addition to waning immunity, also low responders were observed when measured for both with pertussis specific antibodies and CMI responses. Vaccine failures and so-called non-responders are not recognized by the current system, as the commonly used vaccine strategy is the "one-size fits all". If an individual does not respond to the vaccine, it may increase the risk of secondary cases. However, high level of herd immunity provides some protection also for the unvaccinated individuals or non-responders, but only if the vaccine coverage is high enough. Nevertheless Mertsola has claimed that the herd immunity induced by the currently used acellular vaccines might not be good enough to protect the unprotected individuals (Mertsola 2014).

Poland *et al* have used a term "Immune response network theory" to define that the vaccine response is a comprehensive interaction of immune response molecules and the genetic variation and it may account for events such as differing recognition of antigens, processing and altered cytokine response (Poland *et al.* 2011). It is important to identify the initial steps in the antigen recognition, how the antigens are processed and how T-cells are activated to understand the generation of immune responses. Genetic variation may enlighten the steps in the immune response cascade. Our results in the original studies (I) and (IV) have shown that genetic variation in the initial steps of the recognition are related to the vaccine responses also after acellular pertussis vaccination. However, genetic studies should always be performed in different ethnicities and verified with large cohorts to confirm the results. The frequency of immune gene polymorphisms can be highly variable between different populations.

Improving the currently used acellular vaccines has been heavily discussed (Plotkin 2014, Allen & Mills 2014). Return to DTwP would most likely not be accepted by the public opinion in industrial countries even though many developing countries are still using it. The production of high concentrations of antibodies is characteristic for DTaP vaccines; however it might be that this is not the essential arm of the adaptive immunity as stated by Allen and Mills (Allen & Mills 2014). Cellular immunity is increasingly recognized to be important for the protection. Improving the immune responses of DTaP vaccines with new adjuvants that directs the response to Th1 direction might enhance the protection (Allen & Mills 2014). A natural adjuvant like LPS which is present in the DTwP vaccine induces the TLR pathway. TLRs are well recognized activators of adaptive immune system. When stimulated with LPS, TLR4 is known to transfer the Th cell response to Th1 direction. Th1 response would mimic more the immune response of infection and might improve the pertussis vaccine immunity. In the original publication (I) the importance of TLR4 was shown also in the dTaP vaccine responses. Substantial waning of antibodies after DTaP vaccination together with increased incidence of pertussis has resulted in the introduction of dTap booster vaccination in older children, adolescents and adults (Zepp *et al.* 2011). Nevertheless, Th2 and Th17 responses are recognized in hypersensitivity reactions, allergy or even in autoimmunity (Mills 2008). Repeated dTap boosting could lead to undesirable hypersensitivity against the vaccine (Mills 2008, Rennels *et al.* 2008). In the original publication (III) we observed that some important cytokines were downregulated. However, booster vaccination improved the CMI in other measurements.

The currently used aP vaccines have aluminum as an adjuvant to enhance the immune responses. Addition of alternative adjuvants to DTaP vaccine, such as TLR9 ligand dinucleotide CpG might be beneficial for enhancing the Th1 responses (Ross *et al.* 2013, Sugai *et al.* 2005). A non-toxic LPS, as a TLR4 agonist, was recently found to enhance the Th1 response after aP challenge in a mouse model (Brummelman *et al.* 2015). In addition to TLR4 and TLR9 activation, an artificial TLR2 agonist lipopeptide, was shown to activate Th1 and Th2 responses when administrated to mice in combination with aP (Dunne *et al.* 2014). Cytokines are one of the key regulators of the immune responses. Our result from CMI induction (III) showed that addition of IL-23 in PT stimulated PBMCs improves the induction of IL-17 secreting cells. This might also indicate a possible adjuvant possibility of cytokine. Administration of IL-12 with aP vaccine in mice has been shown to enhance the bacterial clearance compared with wP vaccinated mice (Mahon *et al.* 1996).

Other possible routes for improving the aP vaccine efficacy are also available. As it has been stated previously, *B. pertussis* is evolving and strains deficient in vaccine antigens are already visible. Probably the repertoire of antigens included in the vaccine should be increased. One candidate that has been suggested is ACT (Plotkin 2014).

If not changing the vaccine itself, change of the vaccine strategy is beneficial for the control of the disease. Vaccination of pregnant women in the last trimester may increase

the protection of newborns (Vilajeliu *et al.* 2015). In UK and USA, the large epidemics and the deaths in unvaccinated infants has already led to the recommendation to vaccinate pregnant women in the last trimester (Vilajeliu *et al.* 2015). Cocooning strategy is based on vaccinating the family members of newborns. However, it is difficult to reach high coverage and the costs of this strategy are quite high. In Finland, adults are currently recommended to receive diphtheria-tetanus booster vaccine in ten year interval. Changing the vaccine to dTap booster would increase the herd immunity in adults and especially those in the childbearing age. Acknowledging the genetic background of vaccinees could in the far future result in more personalized vaccine schedules including more doses in low responders.

7. SUMMARY AND CONCLUSIONS

Pertussis vaccination is the best tool to prevent and control *B. pertussis* circulation. However, neither infection nor the vaccination does provide a lifelong protection. Acellular vaccine induces high production of antibodies, but in addition to pertussis specific antibodies, CMI provides protection even after antibodies have decayed. Nevertheless, inability to produce antibodies or CMI response might be a consequence of genetic variation in immune system. Aims of this thesis were to examine the association of *TLR4* (I) and *MBL2* (II) polymorphism on antibody responses and persistence after dTap booster vaccination in adolescents and DTaP primary vaccination in young infants, and to evaluate possible associations of MBL deficiency with pertussis infection susceptibility (V). In addition, CMI responses were evaluated ten years after the previous dTap booster vaccination to study the effect of the second booster on CMI responses (III) in young adults. Association of cytokine and cytokine receptor polymorphisms and CMI responses was evaluated (IV).

I. This study supported the role of TLR4 in acellular pertussis vaccine induced immunity, which has previously shown with DTwP vaccine. Individuals with *TLR4* D299G variant type had lower antibody response after first booster vaccine and shorter duration of antibody derived immunity compared with individuals with *TLR4* normal allele. This finding supports the fact that genetic variation is an important factor in the induction and persistence of vaccine induced immune responses.

II. *MBL2* gene polymorphism did not affect antibody responses or persistence of antibodies neither after primary nor booster vaccination. This finding suggests that MBL is involved in *B. pertussis* recognition (V) but not with antigens present in the aP vaccine used in this study.

III. The results of the third study show that CMI is partly detectable even ten years after the previous dTap booster vaccination in young adults. In addition, a second booster dose enhanced the CMI responses. Interestingly, it seems that repeated dTap vaccination may downregulate production of certain cytokines.

IV. The results of the fourth study showed that *IL-10* -1082 promoter polymorphism was more frequent in subjects with low proliferation responses after second dTap booster vaccination in young adults. Individuals carrying heterozygote variant allele were able to produce highest proliferation responses compared to normal allele and homozygote variant allele.

V. In this study, MBL deficiency was more prevalent in pertussis patients than controls. Pertussis patients and especially adult patients had more often MBL deficiency compared with the controls. This indicates that MBL might recognize *B. pertussis* and the genetic variation may cause difference in susceptibility to infectious diseases such as pertussis.

To conclude, the genetic variation is observed in vaccine driven immune responses against *B. pertussis* antigens after acellular pertussis vaccination. The low vaccine response in some individuals may increase the vulnerability to pertussis infection. These subjects might benefit from additional booster doses of the vaccine. Similarly the susceptibility to pertussis infection in adults is affected by genetic variation of MBL. Pertussis is resurging in industrial countries. The findings presented in this thesis project stress the need for understanding not only the adaptation of *B. pertussis* to vaccine-induced immunity but also the host genetics and immune network in more detail.

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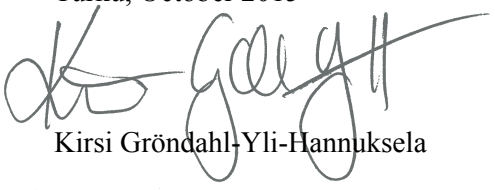

Kirsi Gröndahl-Yli-Hannuksela

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