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Impact of the inflammatory response on specific immunity in neurosurgical patients

ANNA LJUNGHILL HEDBERG

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Abstract

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Vaccination with a T-cell-dependent pneumococcal conjugate vaccine (PCV) followed by a T-cell-independent pneumococcal polysaccharide vaccine (PPSV) is recommended after basilar skull fracture to reduce the risk of meningitis. The optimal time frame for vaccination has not yet been established and varies widely in practice. Because the risk of meningitis is at its peak shortly after the trauma incident, early vaccination might be more desirable. After trauma and central nervous system (CNS) injury, T-cell defects leading to trauma and CNS injury-induced immune deficiency syndromes may affect the vaccine response. In light of the above information, the overall aim of this thesis was to investigate the impact of neurotrauma and neurosurgery on the response to T-cell-dependent and T-cell-independent vaccines.

In Paper I, we compared the antibody response to a T-cell-dependent conjugate vaccine in patients vaccinated within 10 days after neurotrauma or neurosurgery with those vaccinated after >3 weeks. To avoid interference with pneumococcal vaccination, a conjugate vaccine against *Haemophilus influenzae* type b (Hib) was chosen for the study. The majority of the patients responded to the vaccination, although the number of responders was significantly lower in patients vaccinated early.

In Paper II, we investigated the antibody response to the T-cell-independent vaccine PPSV in patients vaccinated within 10 days after neurotrauma or neurosurgery and in patients vaccinated after >3 weeks. Patients vaccinated early responded similarly to those vaccinated after the acute period, indicating that PPSV can be administered early after neurotrauma or neurosurgery.

In Paper III, we compared the response to Hib vaccine with the response to PPSV. We also examined whether individual clinical or immunological parameters might predict the response to T-cell-dependent vaccine and thereby help identify non-responders before vaccination. No correlation between Hib vaccine and PPSV responses was found, indicating that B-cell function is not similarly depressed as T-cell function. It was not possible to predict the T-cell-dependent vaccine response by standardized grading of the trauma or by parameters reflecting the innate immune response.

In Paper IV, we found a significant reduction in the *ex vivo* $CD4^+$ T-lymphocyte response to PCV in patients after neurotrauma or neurosurgery as compared with healthy controls.

Our results suggest that PPSV might be a viable alternative to T-cell-dependent PCV in early vaccination after neurotrauma.

Keywords: T-cell-dependent vaccine, T-cell-independent vaccine, CNS injury-induced immune deficiency syndrome, pneumococcal conjugate vaccine, pneumococcal polysaccharide vaccine, posttraumatic meningitis.

Anna Ljunghill Hedberg, Department of Medical Sciences, Infectious Diseases, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Hedberg A, Pauksens K, Ronne-Engstrom E, Lundberg M, Johansson B, Kayhty H, Sjölin J. (2015) Lower response to early T-cell-dependent vaccination after neurotrauma or neurosurgery in adults. *The Journal of infection*, 70(6):577-84

II Hedberg A, Pauksens K, Enblad P, Soderberg J, Johansson B, Kayhty H, Sjölin J. (2017) Pneumococcal polysaccharide vaccination administered early after neurotrauma or neurosurgery. *Vaccine*, 35(6):909-15

III Hedberg A, Pauksens K, Enblad P, Larsson A, Sjölin J. Relationship between T-cell-dependent and T-cell-independent vaccines after neurotrauma; Can the response be predicted? Submitted

IV Hedberg A*, Berglund S*, Wilske F, Enblad P, Lewen A, Winqvist O, Sjölin J. Decreased T-lymphocyte response to T-cell-dependent vaccines after neurotrauma and neurosurgery. Submitted

*authors contributed equally

Contents

Abbreviations

ACIP	Advisory Committee on Immunization Practices
APC	antigen-presenting cell
APR	acute phase response
CIDS	CNS-injury-induced immune deficiency syndrome
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
DAMP	damage-associated molecular pattern
ELISA	enzyme-linked immunosorbent assay
FASCIA	flow cytometric assay for specific cell-mediated immune response in activated whole blood
GCS	Glasgow Coma Scale
GMC	geometric mean concentration
Hib	*Haemophilus influenzae* type b
HLA	human leukocyte antigen
HPA	hypothalamo-pituitary-adrenal
IFN	interferon
IPD	invasive pneumococcal disease
ISS	Injury Severity Score
LPS	lipopolysaccharide
MHC	major histocompatibility complex
NISS	New Injury Severity Score
NS	neurosurgery
NT	neurotrauma
OPA	opsonophagocytic activity
PBMC	peripheral blood mononuclear cells
PCV	pneumococcal conjugate vaccine
PPSV	pneumococcal polysaccharide vaccine
PRR	pattern recognition receptor
PTX	pentraxin
PWM	pokeweed mitogen
SEB	staphylococcal enterotoxin B
TCR	T-cell receptor
Th-cell	T-helper cell
TIDS	trauma-induced immune deficiency syndrome
TNF	tumor necrosis factor

Introduction

The risk of meningitis after neurotrauma is well known. To reduce this risk, different actions have been taken. Prophylactic antibiotics are often administered, although at present there is little evidence to support such a regimen (1, 2). Increasing problems with antibiotic resistance heightens the need for the prudent use of antibiotics. Even if most of these cases of meningitis occur early in the course (3), the risk appears to persist for many years and almost half of the posttraumatic meningitis cases occur after 1 month (2, 4). *Streptococcus pneumoniae* is the most common causative agent and pneumococcal vaccination after neurotrauma is recommended in several national guidelines (5, 6). However, there are no recommendations regarding an optimal time of delivery of the vaccine. In clinical practice, vaccination is most often performed several weeks after the trauma. Because the risk of meningitis is at its peak shortly after the trauma incidence, vaccination within a few days would be preferable. Pneumococcal polysaccharide vaccine (PPSV) used to be the most common recommendation. During recent years pneumococcal conjugate vaccines (PCVs) have been introduced (7), offering long-term protection and is now recommended in both the USA and Sweden (5, 6).

Trauma, as well as surgery, activate the innate immune system (8) resulting in, among other things, decreased T-cell function (9-12). Patients with injuries of the central nervous system (CNS) may show signs of a specific CNS-injury-induced immune deficiency syndrome (CIDS), which is also characterized by impaired T-cell activity (13). Accordingly, it can be speculated that ongoing anti-inflammatory response after trauma, here referred to as trauma-induced immune deficiency syndrome (TIDS), and CIDS by impaired T-cell function could negatively affect the response to vaccines, especially to T-cell-dependent conjugate vaccines. In the present thesis, the main focus will be to observe the impact of TIDS and CIDS on the response to T-cell-dependent and T-cell-independent vaccines.

Background

The Immune System

The immune system is divided into two interactive spheres: the innate immune system and the adaptive immune system. The innate immune system, also known as the non-specific immune system, is phylogenetically older than the more specialized adaptive immunity. It is always present in healthy individuals, prepared to block the entry of microbes and to rapidly eliminate microbes entering host tissues. It is orchestrated mainly by cells of myeloid origin, i.e. neutrophils, monocytes/macrophages and dendritic cells, and functions as the first rapid line of defense against microorganisms. These immune cells induce phagocytosis of microorganisms, remove debris and activate the complement cascade and subsequently the adaptive immune system (14).

As a first line of defense, continuous epithelia form a mechanical barrier against microbes. Epithelial cells produce antimicrobial proteins that kill or inhibit growth of bacterial and fungal cells. One of these proteins is calprotectin that binds and sequesters metal ions needed by bacteria and fungi as nutrients (15, 16). Activation of the innate immune system rapidly causes inflammation by the recruitment of circulating blood leukocytes. Several cytokines are involved in the inflammatory response mediated by the innate immune system. IL-6 is a pro-inflammatory cytokine that is produced by monocytes, macrophages, endothelial and dentritic cells. This cytokine stimulates the liver to produce various proteins of the acute phase response (APR), such as C-reactive protein (CRP) and pentraxin 3 (PTX3). They both belong to the family of pentameric proteins called pentraxins and function as opsonins, enhancing phagocytosis, and activators of the complement pathway. Among APR proteins are also many components of the complement system, which contribute to both innate and adaptive immune responses. IL-6 is also a reliable marker of injury severity in the acute inflammatory response in surgery, trauma and critical care (17). Tumor necrosis factor-α (TNF-α), produced mainly by the same cell types as IL-6, has systemic effects, including inducing fever by acting on the hypothalamus. It also induces APR by acting on the hepatocytes. TNF-α is also one of the principal cytokines involved in recruiting blood neutrophils and monocytes to sites of infection by acting on vascular endothelium and causing increased vascular permeability, fluid loss and local blood clotting. TNF-α up-regulates the

expression of chemokines at sites of developing inflammation. Chemokines, like fractalkine, cause leukocytes to move into various tissue sites by inducing the adherence of these cells to the vascular endothelium (18). After migrating into tissues, leukocytes travel in the direction of increasing concentration of chemokines. The assembly of leukocytes at sites of infection, orchestrated by chemokines, is an essential part of mounting a focused inflammatory response.

Because uncontrolled innate and inflammatory responses can have adverse consequences, negative feedback mechanisms are activated to limit these responses. Macrophages and dendritic cells produce IL-10, causing reduction of co-stimulator and class II major histocompatibility complex (MHC) molecules expression as well as inhibition of cytokine and chemokine production, and thus is involved in regulation and suppression of inflammatory responses. IL-10 also promotes wound healing (15).

The adaptive immune system, also known as the acquired or the specific immune system, is formed by lymphocyte interactions to provide recognition of antigens with perfect specificity and diversity and provides immunological memory (14). The two main cell populations, T lymphocytes and B lymphocytes, are briefly described below.

T lymphocytes

T lymphocytes, or T cells, are required for almost all adaptive immune responses. The interaction between a naïve T cell and an antigen-presenting cell (APC) is the initiating of the adaptive immune response. Prior to this, the innate immune system has been activated at the site of infection or tissue damage. The APCs have been activated via their pattern recognition receptors (PRRs) and they have processed and presented peptides from extra- or intracellular pathogens in complex with surface MHC class I or II. APCs have moved to local lymph nodes and are now scanned by naïve T cells. Some T cells have committed to the $CD8^+$ cytotoxic T-cell lineage and will recognize MHC class I peptides. Others have committed to the $CD4^+$ helper T-cell lineage and will recognize MHC class II. When a naïve T cell binds to an MHC-peptide complex expressed on an activated APC, it becomes activated by signals generated through the T-cell receptor (TCR) and thereby stimulated to proliferate and differentiate into an effector cell that functions to eliminate microbes (15).

A central theme of adaptive immunity is diversity. Each lymphocyte expresses a different antigen receptor to allow a response against virtually any potential microbial invader. The molecular basis for antigen-specific responses derives from combinational TCRs that have almost infinite specificity. This system ensures that a great diversity of pathogens can be recognized, but because pathogen-specific cells are infrequent, it requires time for expansion of these cells to relevant numbers (19).

To initiate helper T (Th) cell differentiation, a migrant dendritic cell or another type of APC enters the lymph node with evidence it has encountered a pathogen in an infected tissue. To activate the Th cell, the MHC class II on the dendritic cell must bind to the glycoprotein CD4 expressed on the Th cell. When Th cells become activated, they proliferate and mature into different effector subtypes. Cytokines delivered by the activating cells, or by other cells in the environment, determine which subtype will predominate (20). Each effector subtype produces a different set of effector cytokines. The Th1 cells are mainly involved in the defense against intracellular microorganisms by activating macrophages, dendritic cells and cytotoxic T lymphocytes through secretion of various cytokines (e.g., IL-2, IL-12, interferon-γ (IFN-γ) and TNF-β). Th2 cells are involved in the defense against extracellular pathogens including parasites by secreting cytokines (e.g., IL-4, IL-5 and IL-13). It was once believed that only these two subsets existed. This Th1 cell and Th2 cell paradigm, first proposed by Mosmann and Coffman, has been used to explain how hosts elicit different adaptive immune responses to eradicate various pathogens (21). Later, other subsets than Th1 and Th2 have been identified. Thus, the former binary choice between Th1 and Th2 is now complicated by the additional choice of other subtypes. Th17 cells induce reactions that destroy extracellular bacteria and fungi. The major cytokines produced by this cell type are IL-17 and IL-22 (22). Another subset is the regulatory T cells, serving to modulate and deactivate the immune response (23).

Once a Th subset response develops, it seems to inhibit the differentiation of the other Th subsets by inducing different cytokines (19, 24). For example, IFN-γ not only activates macrophages to kill microbes but also promotes Th1 development and inhibits the development of Th2 and Th17 cells.

B lymphocytes

The main function of B lymphocytes (or B cells) is to produce antibodies, serum proteins that aid in the elimination of antigens. The antibodies secreted inactivate the microorganism by binding to it, the steric hindrance effect. They can also bind to the Fc receptor on phagocytic cells, opsonisation, or activate the complement system. They play an important role in humoral immunity, also called the antibody-mediated immunity.

When antigens are first introduced into the host, the initial antibody response is of the IgM class. As the immune response progresses, some B cells begin to produce IgG, which is the most plentiful type of antibody. IgG's smaller size allows it to move into tissues and it is the only type of antibody that is carried across the human placenta to the fetus. (25).

T-cell-independent antigens, such as polysaccharides, can stimulate short-lived B-cell responses by cross-linking the B-cell receptor, which drives the

differentiation of B cells to plasma cells. Then the production of antibodies, mainly IgM and IgG2, is initiated. No memory cells are produced.

T-cell-dependent antigens (e.g., proteins) activate the B cell with assistance of a Th cell, i.e. a more complex mechanism. After antigen activation of a Th2 cell, it starts communicating with the B cells. By producing IL-4, IL-5 and IL-6, the Th cell makes B cells of the right type migrate towards the activated Th cell. When it meets the cytokines and the antigen, the B cell is able to phagocyte the antigen and presents it as peptides in MHC II. When an activated Th cell recognizes the right combination of MHC II and antigen on the B-cell, the two cells bind to one another by CD40 and the CD40 ligand and the CD80/86/CD28 complex. The bindings send a signal to the B cell and represent the last step to full activation. The B cell now differentiates to a memory cell, enabling an immunological memory, or a plasma cell, producing large amounts of IgG1 and IgG3 antibodies (19, 25, 26).

Posttraumatic meningitis

Neurotrauma affects more than 10 000 patients in Sweden every year leading to substantial morbidity and mortality (27). Clinical outcome following neurotrauma is determined by the nature and severity of the primary injury as well as additional insults, such as hypoxemia, hypotension and posttraumatic infections (28).

Any tear or hole in the dural membrane that surrounds the brain and spinal cord may allow the cerebrospinal fluid (CSF) that surrounds those organs to leak. The basilar skull fracture is associated with CSF leak in 20-30% of the cases and visible leak is associated with higher mortality (29-31). In the majority of patients, the leak stops spontaneously within weeks. When a leak persists, the patient may receive a pharmacological agent to inhibit the production of CSF, (e.g., acetazolamide) and be fitted with an external CSF drainage device. Surgical intervention is indicated in patients with a persistent CSF leak lasting 10-14 days, or when there is a complication associated with the leak, such as progressive pneumocephalus or uncontrolled meningitis (32).

Trauma to the anterior skull base is frequently related to the paranasal sinuses, whereas trauma to the middle and the posterior skull base usually affects the petrous bone. Injury to the anterior fossa including the paranasal sinuses may produce CSF leak with rhino-liquorrhoea. Trauma to the petrous bone may cause CSF leak with otorrhoea or rhino-liquorrhoea (33). There are several clinical, chemical and radiological methods to identify CSF leak but no way to fully exclude it in clinical practice (34).

Acute bacterial meningitis is a well-known and serious complication to neurotrauma, especially after basilar skull fracture. It is thought that basilar skull fractures expose the CNS to contamination by bacteria from the nose

and throat, which increases the risk of meningitis (1). The incidence is up to 10-25% in the presence of basilar skull fracture and CSF leak (2, 3, 35). With persistent leak even higher figures have been reported (3). Reviews have not shown that antibiotic prophylaxis in order to prevent meningitis is beneficial in patients with skull fractures with or without CSF leak (1, 2). On the contrary, there is evidence that this strategy may be harmful because normal flora in the nasopharynx, nasal sinuses and external auditory canal, the sites from which the organisms causing meningitis are presumed to originate, is replaced by resistant bacteria that may cause infections that are more complicated to treat. Instead, patients should be monitored closely for signs and symptoms of early meningitis (2, 36). Despite these recommendations, many centers continue to use prophylactic antibiotics, most often a cephalosporin, for 5-7 days to protect this group of patients. Because the risk of meningitis appears to persist for many years and because almost half of the posttraumatic meningitis occurs first after 1 month (2, 4), vaccination against pneumococci, the most common causative agent, is generally recommended (5, 6, 37). Because the risk of meningitis is at its peak during the first posttraumatic weeks (3, 4, 38), patients might benefit from early vaccination to limit the unprotected period.

Vaccines against pneumococci

Invasive pneumococcal disease (IPD) remains a major cause of morbidity and mortality in adults ≥50 years of age (39). Diseases caused by pneumococci include bacteremic and nonbacteremic pneumonia, meningitis and septicemia (39, 40). Although the incidence of IPD is generally greatest at the extreme ages of life, the incidence of pneumococcal disease and associated hospitalization and mortality rates in younger adults is substantial.

Pneumococcal immunization dates back to 1882, 1 year after the pneumococcus was first isolated (41). Within a decade, it had been demonstrated that both active immunization and the protective transfer of antiserum could prevent pneumococcal infections. The first large scale clinical trial of a pneumococcal vaccine was conducted in 1911 (41). This trial was largely unsuccessful because of a lack of knowledge about pneumococcal serotypes, of which more than 90 are identified today (42). The polysaccharide capsules of pneumococci, composed of repeating saccharide units, define the pneumococcal serotypes. These capsules are also virulence determinants and their primary role in virulence is to shield the cell wall from reacting with host antibodies and host complement (43, 44).

Four pneumococcal polysaccharides were used for the development of a vaccine in 1945 (45). It was followed by a hexavalent vaccine, marketed in 1946, which was withdrawn because of the apparent ease of treating pneumococcal infections with penicillin. This lack of interest is in contrast to the

present show of enthusiasm in vaccine programs in the face of increasing antibiotic resistance among pneumococci. A pneumococcal vaccine that protected against 14 serotypes was licensed in the USA in 1977, later expanding to protect against 23 serotypes in 1983. This is a PPSV called Pneumovax® (Sanofi Pasteur MSD AB, Lyon, France), which is still available and in frequent use in clinical practice. Because it protects against 23 serotypes, it is referred to as PPSV23. Pneumovax® can be administered either by intramuscular or by subcutaneous injection with similar immunogenicity (46).

Because this type of vaccine is most effective in adults, and does not generate immunity in children younger than 2 years of age, a separate vaccine for children, Prevenar® (Pfizer Inc, New York, USA), was licensed in 2000. This is a PCV protecting against 7 serotypes, often referred to as PCV7. (41). Synflorix® (GlaxoSmithKline, Brentford, UK), protecting against 10 serotypes (PCV10), was licensed in 2009. In 2010, Prevenar® was expanded to include protection against 13 strains and renamed Prevenar 13® (PCV13). Recommended route of administration is by intramuscular injection.

The two types of vaccines, PPSV and PCV, work in different ways. Polysaccharides are T-cell-independent antigens that generally stimulate short-lived B-cell responses by cross-linking the B-cell receptor (Figure 1a). The repetitive structure and flexible backbone of polysaccharides allow them to interact with large numbers of B-cell receptors that aggregate into a single focus and deliver a potent signal to the B cell (25). This action drives the differentiation of mature B cells to plasma cells that produce antibodies. There are no polysaccharide-specific T cells because T cells only recognize peptides, not polysaccharides, displayed in MHC molecules. The fact that T lymphocytes are not involved leads to an absence of immunological memory and a lack of anamnestic response on rechallenge. New memory B cells are not produced in response to polysaccharide vaccines. Instead, terminal differentiation of memory B cells to plasma cells depletes the memory B-cell pool, resulting in hyporesponsiveness to future vaccine doses (47).

A conjugate vaccine is designed to fool T cells specific for other antigens into providing help to polysaccharide-specific B cells, inducing a T-cell-dependent response (Figure 1b). Chemical conjugation of the polysaccharide to a protein carrier (in Prevenar 13®, a non-toxic form of diphtheria toxoid is used as carrier) directs processing of the protein carrier by polysaccharide-specific B cells and presentation of the resulting peptides to carrier-peptide-specific T cells in association with MHC class II molecules. T cells will pair up with the polysaccharide-specific B cell to provide the necessary help to drive isotype switching to IgG-production and formation of memory B cells. Unlike the response to plain polysaccharide vaccines described above, responses to conjugate vaccines provide long-term immunity through this production of new memory B-cells and induces a memory response to a booster dose of the vaccine (25, 48).

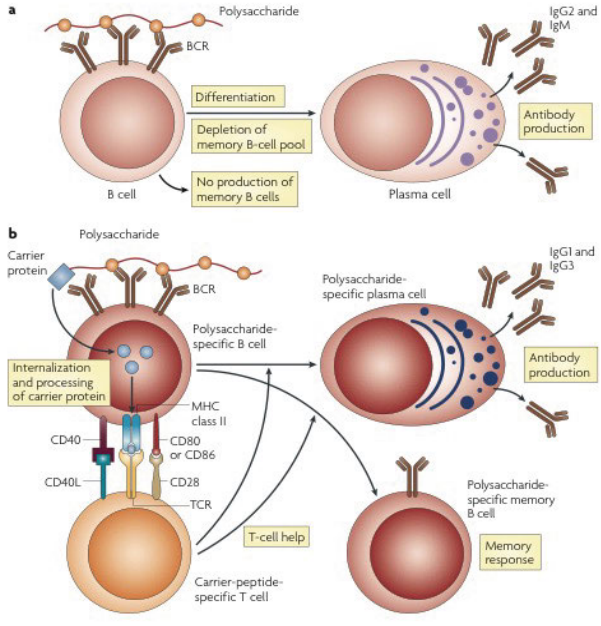


Figure 1. The immune responses to polysaccharide (a) and protein-polysaccharide conjugate (b) vaccines. From Pollard et al. Maintaining protection against invasive bacteria with protein-polysaccharide conjugated vaccines (47). Used with permission from Nature Publishing Group.

Vaccine recommendations after neurotrauma

In the USA, the Advisory Committee on Immunization Practices (ACIP) recommends that adults aged ≥19 years with CSF leak should receive pneumococcal vaccination with both PPSV23 and PCV13 (5, 49). In Sweden, the Public Health Agency recommended until 2016 that pneumococcal vaccination with PPSV23 should be offered to all patients with basilar skull fracture regardless of visible CSF leak (37). In current Swedish guidelines published in 2016, vaccination is now recommended only to patients with visible CSF leak. The combination of PPSV23 and PCV13 is recommended for this risk group (6).

None of the guidelines include recommendations about when the vaccine should be given relative to the onset of the trauma. In Sweden, current clinical practice is to vaccinate after the acute period has passed, leaving the patient without protection for several weeks. Because the risk of meningitis is at its highest within the first weeks after the trauma (3, 4, 38), vaccination within days would be preferable as the onset of the vaccine protection is expected at earliest 2 weeks after vaccination (50). Because of difficulties to

completely rule out CSF leak, vaccination should probably be offered to all patients with basilar skull fracture regardless of visible CSF leak.

Serotype replacement

Following the introduction of PVC7 in routine infant immunization programs worldwide, rates of IPD caused by PCV7 serotypes have declined dramatically among children in recent years. Because PCV7 also prevents transmission of PCV7 serotypes, rates of IPD among unvaccinated groups have also declined (51). Whereas the nasopharyngeal carriage of vaccine serotypes in children decreased, previously rare serotypes became prevalent in the nasopharynx, altering the epidemiology of pneumococcal prevalence (52). This phenomenon, referred to as serotype replacement, is associated with PCVs because PPSVs do not alter carriage (53, 54). As a result of serotype replacement, the protective coverage of PCVs has gradually diminished. In the extreme, serotype replacement eroded the benefits of PCV7 in certain geographic areas (55). A similar decrease in coverage after introduction has been observed with PCV13. The difference in serotype coverage by PPSV23 and PCV13 was small in the USA and Europe before PCV13 introduction: PPSV23 covered only 15% more of IPD incidence than PCV13 (56). In the USA, more recent analysis showed that the difference has increased from 19 to 37% following the introduction of PCV13 (57).

In the light of serotype replacement caused by PCVs, the relative benefit of PPSV23 in risk groups increases.

Systemic and local inflammatory response after neurotrauma and surgery

Not only microbes but also trauma and surgery activate the immune system. Following trauma or surgery, a number of mediators, also called damage-associated molecular patterns (DAMPs), are released in the blood stream. DAMPs are recognized by APCs such as dentritic cells and neutrophils via PRRs and trigger cytokine relapse that includes TNF-α, IL-1, IL-6, IL-8 and IL-12. These mediators activate both the innate and the adaptive immune system by amplifying the activation, maturation and recruitment of immune cells to the site of trauma (58). In this systemic inflammatory response syndrome, pro-inflammatory and anti-inflammatory responses occur early and simultaneously (8). Pro-inflammatory cytokines play a central role in wound healing and bacterial defense. However, excessive production may lead to shock and multiple organ failure (9-12). Auto-regulatory immune cells orchestrate the anti-inflammatory response, here referred to as TIDS, resulting

in, among other things, decreased T-cell function (9-12). Th-cell lineages are suppressed after trauma in general (8). In addition to systemic immune cells, the CNS plays an important role in the development of TIDS via the hypothalamo-pituitary-adrenal (HPA) axis and the sympathetic and parasympathetic nervous systems (13, 59).

The brain was long thought to be immunoprivileged because of the presence of the blood-brain barrier, the lack of a lymphatic system and the shielding of neural antigens from peripheral immune surveillance. More recently, these assumptions have been challenged and revised (60). Microglia are considered the primary immune cells of the CNS but other glial cells (astrocytes and oligodendrocytes) as well as neurons display immune-competent functions (61, 62). Activated resident cells of the CNS, in combination with migrating inflammatory cells from peripheral blood, form an intricate immune network resulting in neuroinflammation. Although this local immune response may be initiated to protect the brain, it is becoming evident that it can result in harmful outcomes for the CNS as it contributes to increased edema, cellular death and blood-brain barrier disruption (63).

Although the initial local response to neurotrauma, measured as cytokine levels in cerebral microdialysis, is mainly pro-inflammatory (64, 65), patients also show signs of downregulation of the systemic inflammatory response to prevent post-injury autoimmune aggression (13). Patients with injuries of the CNS may consequently show signs of CIDS, a condition characterized by impaired T-cell activity (13, 66, 67) and thus CIDS may add to TIDS after neurotrauma. The mechanisms by which CNS injury triggers the systemic anti-inflammatory response in CIDS remain to be elucidated. The autonomic system of CNS is hard wired with secondary lymphoid organs and thus it has been proposed that these signals may be interrupted in addition to intracerebral cytokine-induced activation of the HPA axis and the autonomic nervous system (13). Defects in immune function in CIDS include reduced peripheral blood lymphocyte counts and peripheral blood T cells show reduced mitogen-induced cytokine production and proliferation (66, 67). Humoral immune responses seem less affected (13, 68-70), although a reduced number of B-cells have been described after CNS injury (71). In general, changes in immune responses correlate with the severity of brain injury (13). Chronic inflammation with persistently elevated cytokines has been reported (72).

Both TIDS and CIDS are characterized by defect T-cell function (13, 66, 73), which may be strong enough to affect the antibody response to a T-cell-dependent vaccine. Given that the humoral immune response, including ability to produce antibodies, is less affected by TIDS and CIDS (13), the response to a T-cell-independent vaccine such as the PPSV might theoretically be less affected. This possibility might be of special interest for the choice of pneumococcal vaccine after neurotrauma.

Aims of the thesis

The overall aim of the thesis was to investigate the impact of neurotrauma and neurosurgery on the response to T-cell-dependent and T-cell-independent vaccines.

The specific aims were to:

Paper I investigate the antibody response to a T-cell-dependent conjugate vaccine in patients vaccinated within 10 days from the onset of possible TIDS and CIDS after neurotrauma or neurosurgery and to determine whether these patients had a similar or lower response compared with patients vaccinated after the acute period according to our standard regimen.

Paper II study the antibody response to a T-cell-independent pneumococcal polysaccharide vaccine in patients vaccinated within 10 days from the onset of possible TIDS and CIDS after neurotrauma or neurosurgery and to compare the response with that in patients vaccinated after the acute period.

Paper III compare the response to a T-cell-dependent vaccine with that to a T-cell-independent vaccine in order to investigate whether there are similar responses, only varying magnitude, and thus investigate whether there is a more general immunosuppression in neurotrauma patients also affecting B-cell function. A secondary aim was to explore whether individual clinical or immunological parameters might predict the T-cell response and thereby enable the identification of patients that would respond to a T-cell-dependent vaccine.

Paper IV investigate the *ex vivo* T-cell response to the pneumococcal conjugate vaccine in neurotrauma and neurosurgery patients and compare that response to healthy controls.

Patients

Paper I-III

From 2001 to 2008, patients admitted to the Department of Neurosurgery, University Hospital of Uppsala were prospectively included in the study resulting in Paper I-III. Patients with basilar skull fracture, with or without known CSF leakage, were enrolled in the neurotrauma (NT) group; patients scheduled for elective, transsphenoidal pituitary gland surgery were assigned to the neurosurgery (NS) group. The control group in Paper I-II consisted of patients referred from the Department of Neurosurgery to the Department of Neurological Rehabilitation, Uppsala University Hospital, or to a local hospital in the patients´ home county. All control patients had suffered from neurotrauma with or without basilar skull fracture, or had neurosurgery at least 3 weeks earlier. Patients with neurotrauma without skull fracture were allowed in the control group, since the reported important factor for CIDS and impaired T-cell response has been the traumatic brain injury irrespective of skull fractures (13, 66, 67).

The study protocol was approved by the Regional Ethical Review Board at Uppsala University (reference number 00-254). Informed consent was obtained from the patients or, in unconscious or incompetent patients, from the patients´ legally authorized representative. Consecutive patients were screened and included when vaccination and follow-up were possible to perform and consent had been obtained.

Paper IV

Trauma patients admitted to the neurosurgical intensive care unit at the Department of Neurosurgery, Uppsala University Hospital from 2016 to 2018 were eligible to be assigned to the NT group. Patients scheduled for elective, transsphenoidal pituitary gland surgery, constituting an intermediate control group with a more limited neurotraumatic inflammatory insult compared with the NT patients, were enrolled in the NS group during the same period. Healthy volunteers from the hospital staff served as controls. Our goal was to collect samples from 15 individuals from each group in this pilot study. Consecutive patients were screened and eligible to participate after informed con-

sent had been obtained. The study protocol was approved by the Regional Ethical Review Board at Uppsala University (reference number 2015/217).

Methods

Paper I-III

Vaccination and sera collection

A conjugate vaccine against *Haemophilus influenzae* type b (Hib) was chosen as the T-cell-dependent antigen. All patients received a single subcutaneous injection of 0.5 ml Act-HIB® (Sanofi Pasteur MSD, Lyon, France) in the upper right arm. A 0.5 ml dose of this vaccine contains 10 μg of Hib polysaccharide conjugated to 24 μg of tetanus protein.

At the same time, all patients received a single subcutaneous injection of 0.5 ml Pneumovax® containing 25 μg of purified capsular polysaccharide from each of the 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) in the upper left arm.

Patients in the NT and NS groups were vaccinated within 10 days after trauma or elective surgery. Control patients were vaccinated according to the local routine for pneumococcal vaccination at least 3 weeks after trauma or elective surgery. Adverse reactions to the vaccine were recorded in the case report form.

Pre-vaccination sera were collected just before vaccination and post-vaccination sera were obtained 3 and 6 weeks after vaccination. Samples were stored at -70°C pending analysis. The laboratory was blinded to group assignment of the patients.

Paper IV

Sera collection

Blood samples were collected from patients in the NT group as soon as possible after trauma but at least within the first week of hospitalization. If the patient were still an inpatient at Uppsala University hospital 1 week later, a second sample was collected. Blood samples from patients in the NS group were collected before and after surgery. Healthy controls were sampled once.

Analysis

Paper I

IgG antibody concentrations to Hib polysaccharide were determined by enzyme immunoassay (74).

An anti-Hib polysaccharide antibody concentration of 0.15-1.0 μg/ml has been associated with long-term protection against invasive Hib infection after vaccination of children with Hib polysaccharide vaccine (75). Based on previous experience in children (74, 76), a post-vaccination concentration of 10 μg/ml, 10 times above the proposed protective concentration, was chosen as the target level for a good response to the vaccination in this study.

Paper II

Serotype-specific anti-polysaccharide binding IgG antibody levels to serotypes 4, 6B, 9V, 14, 18C, 19F and 23F were determined by enzyme immunoassay (77).

A serotype-specific IgG >0.35 μg/ml has been defined as the correlate of protection for invasive disease in infant recipients of PCV (78). The true correlate of protection for adults after vaccination with PPSV23 is not known (79). The value of 1.0 μg/ml was chosen as the target level for a good response to the vaccination in this study (80).

Paper III

In pre-and post-vaccination sera, serotype-specific anti-polysaccharide binding IgG antibody levels to serotypes 4, 6B, 9V, 14, 18C, 19F and 23F as well as IgG antibody concentrations to Hib polysaccharide were determined by enzyme immunoassays (74, 77). Responders to Hib were defined as in Paper I.

Concentrations of CRP and calprotectin were measured with particle-enhanced turbidimetric assay (PETIA) technology using a Mindray BS380 chemistry analyzer (Mindray, Shenzhen, China) and CRP reagents (6K2602) from Abbott Laboratories (Abbott Park, IL, USA) and calprotectin reagents from Gentian AS (Moss, Norway).
Concentrations of IL-6 (DY206), IL-10 (DY217B) and PTX3 (DY1826) were analyzed with commercial sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN, USA). Concentrations of fractalkine (DY365) were measured with Human CX3CL1/Fractalkine DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). The ELISAs had a total coefficient of variation of approximately 6-7%.

In patients in whom this was possible to perform, heparinized whole blood was collected just before vaccination for lipopolysaccharide (LPS) stimulation. Samples were incubated for 4 hours at 37° C with a standardized stimulation solution containing cell culture medium with LPS in a concentra-

tion of 500 pg/ml (Milenia *ex vivo* stimulation kit). Concentrations of IL-6, IL-10 and TNF-α in the supernatant were determined by commercial sandwich ELISA. For practical reasons this analysis could only be performed in 30 patients (20 NT, 10 NS).

The Glasgow Coma Scale (GCS) (81), Injury Severity Score (ISS) (82) and New Injury Severity Score (NISS) (83) were used to define injury severity in the NT group. A GCS $\leq$ 8 was defined as severe head injury, ISS $\geq$16 as severe injury and NISS $\geq$16 as major trauma (84). Data to calculate these scores were collected from patient charts.

Paper IV

Samples were stimulated with two conjugate vaccines, PCV13 and Act-HIB®. PCV13 contains 13 *Streptococcus pneumoniae* serotypes (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F) individually conjugated to a non-toxic mutated diphtheria protein. One dose of 0.5 ml contains 2.2 µg polysaccharide from each serotype, except 4.4 µg of the 6B serotype and 32 µg of the carrier protein.

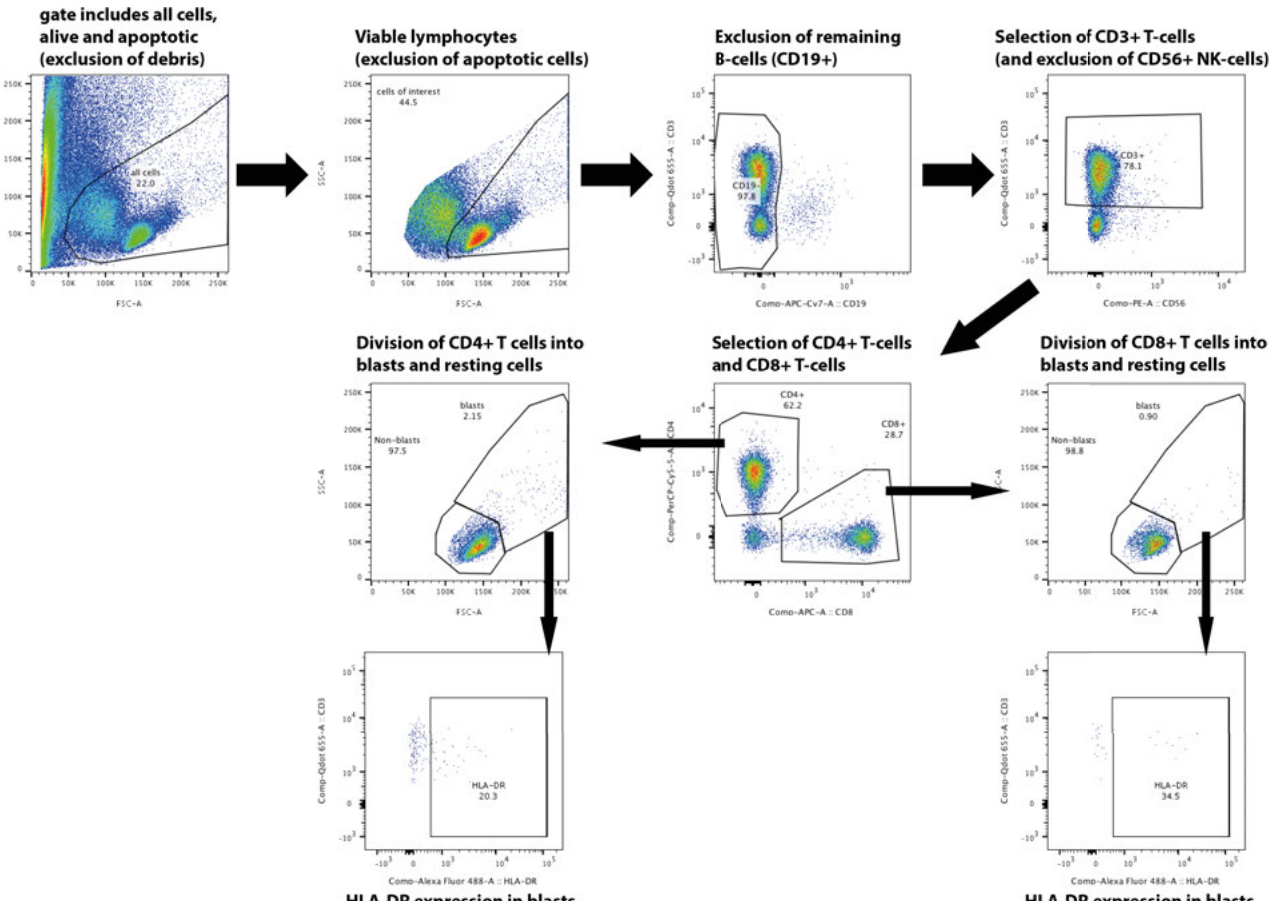
Information about age, type of injury, patient data to calculate injury severity scores and levels of CRP and leucocyte counts on the day of vaccination was collected from patient charts. The GCS and ISS were used to define injury severity in the NT group.

An adapted version of the flow cytometric assay for specific cell-mediated immune response in activated whole blood (FASCIA) was performed (85). Briefly, blood samples were centrifuged and plasma samples collected. Density gradient separation was performed using Ficoll-Paque™ (GE Health Care Life Sciences) to obtain peripheral blood mononuclear cells (PBMCs). PBMCs were seeded at 0.5 million/ml in complete RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/mL penicillin G, 100 mg/mL streptomycin (all from ThermoFisher Scientific, Gibco) and 2 mM L-glutamine solution (Sigma Aldrich Inc) or AIM-V medium (ThermoFisher Scientific, Gibco). The cells were transferred to culture plates, rested at 37^{o} C and 5% CO_2 for 1-2 hours and then stimulated with either staphylococcal enterotoxin B (SEB, 1 µg/mL medium), Pokeweed mitogen (PWM, 5 µg/mL medium) or 2, 10 or 20 µL/mL medium of either of the two vaccines. Afterwards, stimulated cells were cultured for 7 days at 37^{o}C and 5% CO_2, followed by flow cytometric analysis using a BD Fortessa instrument (BD Biosciences). The antibodies used are listed in Supplementary Table 1. Representative flow cytometry plots displaying the gating strategy are shown in Figure 2.

The calculation of the number of blast-transformed $CD4^+$ T lymphocytes after stimulation was performed in two steps. First, the number of blasts in the $CD4^+$ T-cell gate was recorded and correlated with the number of events (viable and dead cells) from the "all cells" gate to get a blast count/total cell

count for each culture sample. After that, these numbers were adjusted to a number of blasts/10 000 total cells to equalize the samples and thus permit a valid comparison. In the analysis of cells stimulated with PCV and the Hib vaccine the cultures stimulated with the amount of vaccine leading to the best response for that particular sample were used for the analysis. The rationale for this selection was that the response kinetics to antigen presents inter-individual variations and, consequently, the amount of antigen causing the "peak" stimulation after 7 days of culture will vary between individuals. The best recorded response is therefore a better measurement for a response comparison between individuals.

Figure 2 (next page). Representative flow cytometry plots displaying the gating strategy. The first gate selects all events with a size and granularity meeting the criteria of cells: live, dead and apoptotic. The second gate excludes the dead and apoptotic cells to select living cells. The next two gates are used to select T cells. The first gate excludes $CD19^+$ B cells. The second selects $CD3^+$ (T-cell lineage-specific marker) events and excludes $CD56^+CD3^-$ NK cells and $CD56^-CD3^-$ events. The $CD3^+$ T-cell population is then further divided into $CD4^+$ and $CD8^+$ T-cell populations. Out of these, proliferating lymphoblasts are selected according to their characteristic size and granularity signature in forward and side scatter. The percentage of HLA-DR^+ events in the blast gate is then measured. Abbreviations: SSC-A: side scatter area, FSC-A: forward scatter area, APC: allophycocyanin, Cy: cyanine (used conjugated to other fluorophores in tandem conjugates such as APC-Cy7), PE: phycoerythrin, PerCP: peridinin chlorophyll protein complex, FITC: fluorescein isothiocyanate, HLA-DR (human leukocyte antigen-DR).


gate includes all cells, alive and apoptotic (exclusion of debris)
all cells
22.0
FSC-A
Viable lymphocytes (exclusion of apoptotic cells)
cells of interest
44.5
SSC-A
FSC-A
Exclusion of remaining B-cells (CD19+)
CD19-
97.8
Comp-Qdot 655-A :: CD3
Como-APC-Cv7-A :: CD19
Selection of CD3+ T-cells (and exclusion of CD56+ NK-cells)
CD3+
78.1
Comp-Qdot 655-A :: CD3
Como-PE-A :: CD56
Division of CD4+ T cells into blasts and resting cells
blasts
2.15
Non-blasts
97.5
SSC-A
FSC-A
Selection of CD4+ T-cells and CD8+ T-cells
CD4+
62.2
CD8+
28.7
Comp-PerCP-Cy5-5-A :: CD4
Como-APC-A :: CD8
Division of CD8+ T cells into blasts and resting cells
blasts
0.90
Non-blasts
98.8
FSC-A
HLA-DR
20.3
Comp-Qdot 655-A :: CD3
Como-Alexa Fluor 488-A :: HLA-DR
HLA-DR expression in blasts
HLA-DR
34.5
Comp-Qdot 655-A :: CD3
Como-Alexa Fluor 488-A :: HLA-DR
HLA-DR expression in blasts

Calculation and statistics

A p-value of <0.05 was considered statistically significant. Values are given as mean ±SE, unless otherwise indicated. Statistical calculations were performed using Prism (v6.0, GraphPad Software, San Diego, CA, USA), unless otherwise specified.

Paper I

The NT group was to be compared with the control group while the NS patients constituted an intermediate group with a more limited neurotraumatic inflammatory insult.

Patients who were able to mount an antibody response leading to the target post-vaccination titer were defined as responders. The proportion of responders in the NT group was to be compared with that in the control group in the primary analysis. Patients with pre-vaccination concentrations of >10 μg/ml had already achieved the vaccination target and were excluded from this analysis. With a power of 0.8, an α-error of 0.05 and a detectable difference of at least 35%, 25 patients were required in the NT group and 25 in the control group. Considering the possibility of high pre-vaccination concentrations, a minimum of an additional four patients in each of the groups was deemed necessary. Therefore, enrollment to the study was terminated once the number of patients reached 29 in both the NT and control groups.

Secondary analyses were the response rates in patients without underlying diseases, possibly affecting the antibody response, and pre- and post-vaccination antibody concentrations in the groups.

Differences between the groups in the proportion of patients achieving the target concentration of 10 μg/ml were analyzed by Fisher's exact one-tailed test. Geometric mean antibody concentrations (GMCs) are given as $\log_{10}$ values. For the analysis of differences between groups Student's t test was employed. Pearson's product-moment correlation was computed to analyze the correlation between pre- and post-vaccination concentrations. The relationships between post-vaccination concentrations and age and time from trauma/surgery were analyzed by the Spearman rank correlation.

Paper II

In the analyses, the antibody levels of the NT and the NS groups were to be compared with those in the control group. Antibody levels were logarithmically transformed and expressed as GMCs. Linear mixed models were employed to analyze differences in antibody levels. The Chi-square test, using Akaikes IC and the degree of freedom from the fitted models, was performed to determine the best covariance structure. The covariance structure that best fitted the models was the unstructured one. The maximum likelihood method

was applied to estimate the parameters for study group and time point for collected sera: both were treated as fixed effects in the models. Differences in proportion of patients with antibody levels $\geq$1.0 μg /ml before and after vaccination were analyzed by Wilcoxon matched-pairs signed-rank test and differences in the number of responders by Fisher's exact two-tailed test. To analyze the relationship between post-vaccination concentrations and age and time from trauma/surgery, Pearson's product-moment correlation was computed.

Paper III

The vaccination response was calculated as the difference between the logarithmically transformed titers in the post-immunization and the pre-immunization sera, which signifies fold antibody increases. Post-immunization titers were obtained from the 6-week samples when available; if not, the 3-week post-vaccination titers were used. The Spearman rank correlation test was performed to analyze the relationship between the different serotype-specific anti-pneumococcal polysaccharide responses. The same test was employed to analyze the association between the pneumococcal response and that caused by the Hib vaccine. To restrict the number of analyses in the primary analysis a mean pneumococcal response was to be calculated for each individual if there were fair correlations between the responses to the different pneumococcal serotypes.

In the prediction of the T cell-dependent response to the Hib vaccine in the NT group a comparison of ISS, NISS and GCS levels as well as pre-vaccination concentrations of the inflammatory parameters in responders and non-responders was made using the Mann-Whitney U test. Because the response/non-response is a dichotomous variable, Spearman rank correlation coefficients were also calculated between these parameters and the Hib antibody response.

Paper IV

Lymphocyte function, assessed by the capacity to mount a blast transformation response, was the measurement used for analysis of the FASCIA data. The main focus was on the $CD4^+$ T-cell compartment. Accordingly, $CD4^+$ T-cell blast transformation in response to the PCV antigens constituted the primary analysis. Blast transformation in response to stimulation in T cells from samples obtained early after trauma in the NT patients was compared with that seen in T cells from healthy individuals and to the response seen in T cells obtained from the same NT patients 1 week later. For the NS patients, the individual blasting responses to stimulation in T cells sampled after surgery were compared with those seen in T cells from healthy controls. In addition, a comparison was performed between responses in T cells

sampled before and after surgery. Analyses of $CD8^+$ T-cell blast transformation responses were performed as a secondary objective and the same inter-group comparisons were made for this data set. As an indicator of activation, expression of HLA-DR on blast-transformed $CD4^+$ T cells after stimulation with PWM, SEB and PCV was analyzed and comparisons made between the patient groups. For the comparison between study groups, the Mann-Whitney U test was used and for the differences in individual patients at varying time points the Wilcoxon rank sum test. The relationships between the responses to the PCV and those to the Hib vaccine were analyzed by the Spearman rank correlation test. The same test was used to analyze the relationships between vaccine response and ISS and GCS and for the relationships between concentrations of CRP and the number of blast-transformed $CD4^+$ T cells after stimulation with PCV, SEB and PWM.

Results

Paper I

Eighty-five patients (33 NT patients and 23 NS patients vaccinated early and 29 control patients vaccinated late) were enrolled in the study. None of the patients reported previous vaccination against Hib. Three patients in the NS group and one in the NT group had pre-vaccination anti-Hib concentrations of >10 μg/ml. According to the protocol, they were excluded from further comparisons between the groups. Characteristics of the patients are shown in Table 1.

Table 1. Patient characteristics, cause and type of neurotrauma, underlying conditions and time between trauma/surgery and vaccination, Paper I-III

	Neurotrauma **(Paper I-III)**	Neurosurgery **(Paper I-III)**	Controls **(Paper I-II)**
Number of patients	32*/33**	20*/23**	29
Age median (years) (range)	41 (22-85)* 40 (22-85)**	59 (29-82)* 59 (29-82)**	39 (17-78)
Sex male/female	29/3* 30/3**	12/8* 14/9**	20/9
Cause of neurotrauma			
Assault	0	0	4
Traffic accident	18*/19**	0	16
Explosion	2	0	1
Falling accident	10	0	5
Other or uncertain	2	0	1
Type of neurotrauma			
Skull fracture	32*/33**	0	21
Intracranial hemorrhage	21*/22**	0	22
Transsphenoidal pituitary gland surgery	0	20*/23**	2
Underlying diseases possibly affecting the immune response			
Diabetes mellitus	1	1	2
Malignancy	1	1*/2**	2
Corticosteroids or other immune-suppressive drugs	0	0	1
Median time from trauma/surgery to vaccination (days) (range)	6 (1-10)	3 (1-6)	91 (23-303)

*Paper I
**Paper II-III

Proportion of non-responders

The correlation between the two follow-up samples was high and the differences were limited. For this reason, individual mean values were calculated and analyzed together with those collected from patients in whom only a single sample was obtained.

In the control group, 26/29 patients responded to vaccination. The corresponding figures were 22/32 in the NT group and 15/20 in the NS group. We found significantly more non-responders in the NT group vaccinated early than in the control group vaccinated late (the proportion of non-responders is listed in Figure 3A). When patients with underlying diseases affecting outcome were excluded from the calculations, there were significantly more non-responders both in the NT (9/30) and NS (5/18) groups than in the control group (1/24) (Fig 3B).

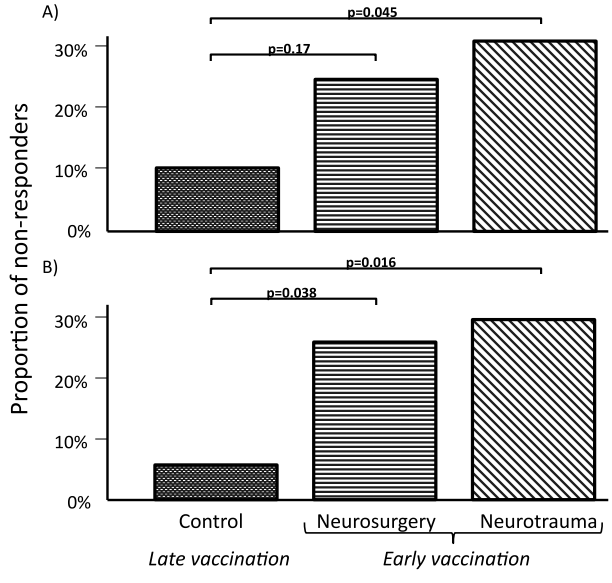


Figure 3. Proportion of non-responders in neurosurgical patients vaccinated against Haemophilus influenzae. A) All patients (control n=29, neurosurgery n=20, neurotrauma n=32). B) Patients without underlying diseases (control n=24, neurosurgery n=18, neurotrauma n=30). Comparison was done using Fisher's exact one-tailed test.

Geometric mean concentrations before and after vaccination

After vaccination, the NS group had significantly lower antibody levels, expressed as GMCs, than the control group (Figure 4). Expressed as $\log_{10}$ concentrations, anti-Hib concentrations in the NT, NS and control groups were 1.52±0.15, 1.38±0.15 and 1.81±0.12 μg/ml, respectively.

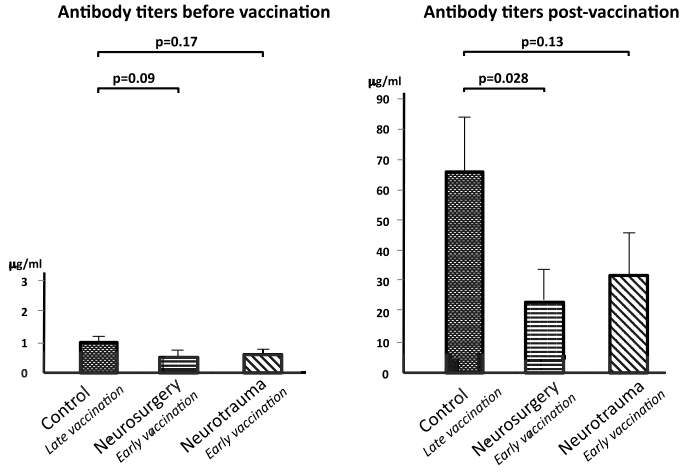


Figure 4. Geometric mean antibody concentrations (+SE) before and after vaccination in the neurosurgery and neurotrauma groups in comparison with the control group in all patients (control n=29, neurosurgery n=20, neurotrauma n=32). Comparisons between goups were done by the t test for independent samples.

No association between post-vaccination concentrations and time for vaccination in relation to trauma or surgery was observed within groups (NT group: r=-0.13, NS group: r=-0.03, control group: r=-0.12). The impact of age was limited (NT group: r=-0.05, NS group: r=-0.50, control group: r=-0.05).

No major adverse effects occurred. In 17 patients, minor adverse effects from the vaccine, such as local redness or swelling at the injection site, were observed during the first week after vaccination.

Paper II

Eighty-five patients (33 NT patients and 23 NS patients vaccinated early and 29 control patients vaccinated late) were enrolled in the study. Three- and 6-week post-vaccination sera were obtained on a median of 21 (range 13 to 32) and 42 days (range 34 to 266), respectively. Characteristics of the patients are shown in Table 1. For various reasons only one follow-up sample was obtained from 23 patients while two follow-up samples were collected from 62 patients. None of the patients reported previous vaccination against pneumococci.

Geometric mean concentrations before and after vaccination

Antibody responses, expressed as GMCs, are shown in Figure 5. Antibody levels were slightly higher in the control group than in the other groups before vaccination. There was a highly significant antibody response against all serotypes tested. No differences between groups or in the time by group interactions were observed against any of the serotypes tested, indicating that the response to the vaccine was similar in all three groups (Table 2).

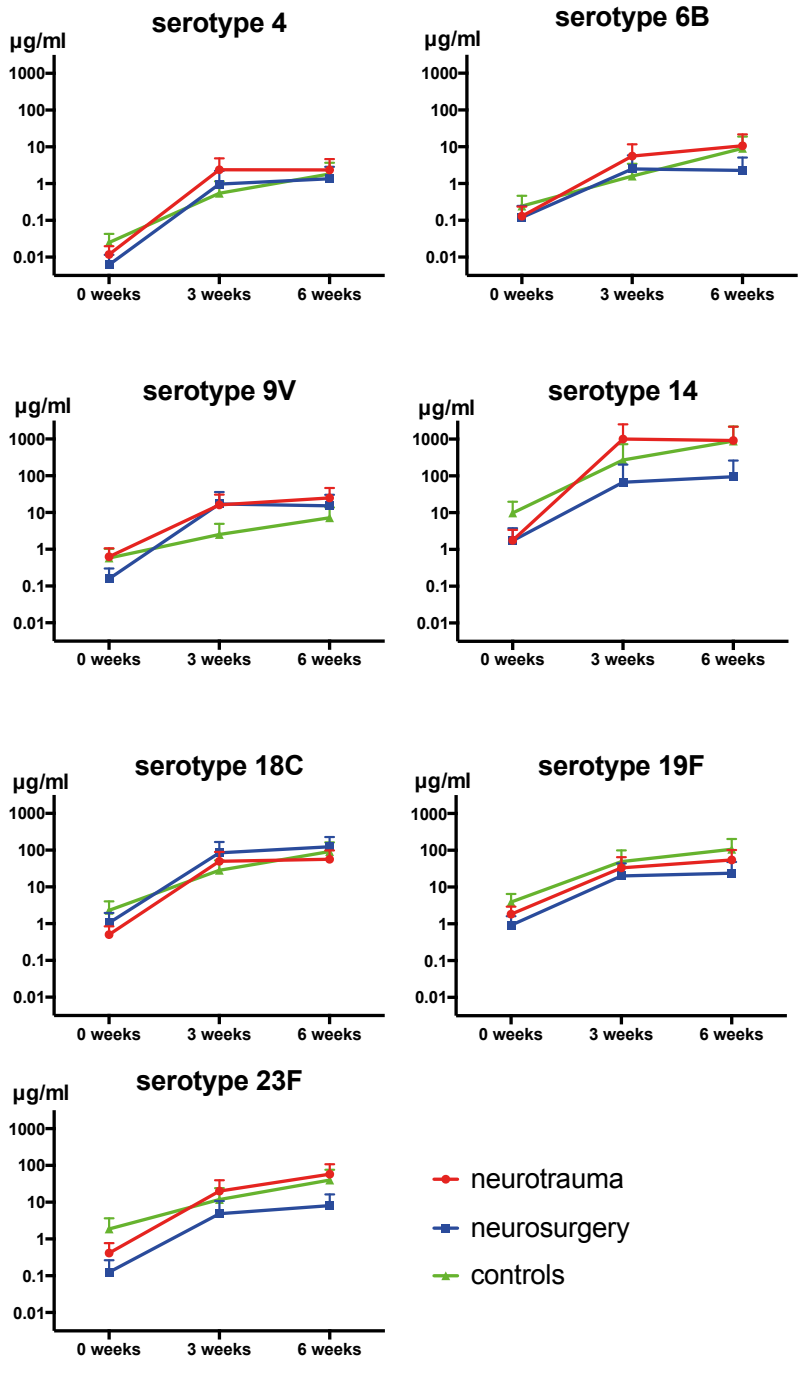


Figure 5. Serotype-specific IgG geometric mean concentrations before and after pneumococcal polysaccharide vaccination

Table 2. P-values from the linear mixed model analysis regarding differences in antibody responses against serotypes 4, 6B, 9V, 14, 18C, 19F and 23F after vaccination with pneumococcal polysaccharide vaccine over time and between groups.

Serotype	Difference over time	Difference between groups	Time x group interaction
4	<0.001	0.67	0.34
6B	<0.001	0.67	0.09
9V	<0.001	0.32	0.06
14	<0.001	0.24	0.07
18C	<0.001	0.58	0.16
19F	<0.001	0.27	0.91
23F	<0.001	0.12	0.10

Proportion of patients reaching target concentrations

There was varying protection against the different serotypes before vaccination. More than half of the patients reached target concentration (antibody levels $\geq$1.0 μg/ml) against serotypes 14, 18C and 19F, whereas this was found in only a limited number of patients against serotype 4. After vaccination, target concentration was observed in approximately 80% or more of the patients against all serotypes except serotypes 4 and 6B, against which target concentration was observed in a little more than half of the patients for serotype 4 and two thirds for serotype 6B. There were no differences between the control and the NT and NS groups in this aspect (Table 3).

Table 3. Antibody concentrations ≥1,0 ug/ml against serotypes 4, 6B, 9V, 14, 18C, 19F and 23F in neurotrama (NT) patients (n=33), neurosurgery (NS) patients (n=23) and control (C) patients (n=29) before and after vaccination with a pneumococcal polysaccharide vaccine

Serotype	Patient group	Before vaccination, number of patients (proportion; 95% CI)	After vaccination, number of patients (proportion; 95% CI)	P-value*
4				
	NT	1 (0.03; 0-0.09)	20 (0.61; 0.44-0.77)	<0.001
	NS	0 (N/A)	12 (0.52; 0.32-0.73)	<0.001
	C	4 (0.14; 0.01-0.26)	15 (0.52; 0.34-0.70)	0.01
6B				
	NT	9 (0.27; 0.12-0.42)	23 (0.72; 0.56-0.87)**	<0.001
	NS	6 (0.26; 0.08-0.44)	16 (0.70; 0.51-0.88)	0.01
	C	9 (0.31; 0.14-0.48)	20 (0.69; 0.52-0.86)	0.01
9V				
	NT	12 (0.36; 0.20-0.53)	26 (0.79; 0.65-0.93)	<0.001
	NS	7 (0.30; 0.12-0.49)	19 (0.83; 0.67-0.98)	0.003
	C	9 (0.31; 0.14-0.48)	23 (0.79; 0.65-0.94)	<0.001
14				
	NT	18 (0.55; 0.38-0.72)	30 (0.91; 0.81-1.01)	<0.001
	NS	11 (0.48; 0.27-0.68)	18 (0.78; 0.61-0.95)	0.01
	C	21 (0.72; 0.56-0.89)	25 (0.86; 0.74-0.99)	0.29
18C				
	NT	14 (0.42; 0.26-0.59)	26 (0.79; 0.65-0.93)	0.001
	NS	13 (0.57; 0.36-0.77)	22 (0.96; 0.87-1.04)	0.02
	C	17 (0.59; 0.41-0.77)	26 (0.90; 0.79-1.01)	0.04
19F				
	NT	19 (0.58; 0.41-0.74)	27 (0.82; 0.69-0.95)	0.01
	NS	12 (0.52; 0.32-0.73)	18 (0.78; 0.61-0.95)	0.07
	C	20 (0.69; 0.52-0.86)	27 (0.93; 0.84-1.02)	0.07
23F				
	NT	13 (0.39; 0.23-0.56)	27 (0.82; 0.69-0.95)	<0.001
	NS	8 (0.35; 0.15-0.54)	19 (0.83; 0.67-0.98)	0.002
	C	18 (0.62; 0.44-0.80)	26 (0.90; 0.79-1.01)	0.02

* Wilcoxon signed-rank test

** Results from one patient is missing, n=32

Underlying diseases had a limited effect on the vaccination response and there were no statistically significant differences between the groups (data not shown).

A negative correlation between antibody levels and age could be demonstrated for serotypes 19F and 23F but not for the other serotypes. No association between antibody levels and time point of vaccination in relation to neurotrauma or neurosurgery was observed.

No major adverse effects occurred. In 14 patients (NT group n = 7, NS group n=4, control group n=3) minor adverse effects, such as local redness and swelling at the injection site, were observed.

Paper III

Fifty-six patients (NT=33, NS=23) were enrolled in the study. In the NT group 11 patients demonstrated a GCS of $\leq$8, 24 patients an ISS of $\geq$16 and 31 a NISS of $\geq$16. Patient characteristics are listed in Table 1. None of the patients reported previous vaccination against pneumococci or Hib. Six-week post-vaccination sera were not available in 11 patients (10 NT, 1 NS patients) and in these patients the 3-week titer was used. Three- and 6-week post-vaccination sera were obtained on a median of 21 (median, range 13 to 27 days) and 42 days (range 34 to 52 days).

Analysis of the uniformity of the responses to different pneumococcal serotypes

Significant correlations between the seven serotype responses were seen in all but 4 of the 21 analyses: mean correlation coefficient for all seven serotypes was 0.33, with serotype-specific coefficients ranging from 0.27 to 0.39 (Table 4). Serotypes 4 and 23F demonstrated somewhat weaker associations than the other serotypes. The correlation coefficients between the calculated mean pneumococcal response and the different serotype responses exceeded 0.5 for all serotypes, indicating a good relationship between the mean pneumococcal response parameter and that to each of the serotypes included. Accordingly, the mean pneumococcal titer could be used in the primary analysis of the relationship to the Hib response.

Table 4. Correlation coefficients between the responses to the different pneumococcal serotypes and the calculated mean pneumococcal response in the NT and NS groups (n=56).

Pneumococcal serotype	4	6B	9V	14	18C	19F	23F	Mean pneumococcal response[1]
4								0.56
6B	0.39**							0.64
9V	0.29*	0.43**						0.68
14	0.31*	0.31*	0.34*					0.71
18C	0.17	0.30*	0.44**	0.45*				0.66
19F	0.23	0.34*	0.41**	0.41*	0.41**			0.66
23F	0.25	0.30*	0.45**	0.30*	0.19	0.32*		0.59
Mean ±SD	0.27±0.08	0.35±0.05	0.39±0.06	0.35±0.06	0.33±0.13	0.35±0.07	0.30±0.09	0.64±0.05

[1]represents the mean of all serotype-specific responses in each patient
*p<0.05; **p<0.01

Relationship between the pneumococcal response and the response to Hib

In the NT+NS group, the mean pneumococcal response was 0.87 ±0.42 and the Hib response 1.61 ±0.87, consistent with 7- and 40-fold increases of the antibody titers, respectively. Corresponding responses in the NT group alone were 0.92 ±0.41 and 1.65 ±0.95. In the primary analysis there was no correlation between the T-cell-independent mean pneumococcal response and that to the T-cell-dependent Hib vaccine, with a correlation coefficient of only 0.13. This result is given in Table 5, which also shows the correlations between different pneumococcal serotype responses and that to Hib in the NT+NS group and in the NT and NS groups alone. The absence of covariation is most marked in the NT group, with a correlation coefficient between the mean pneumococcal response and that to Hib of 0.01, whereas this coefficient was 0.32 in the NS group, a difference that proved to be statistically significant in a post-hoc analysis ($p<0.05$).
Of the 15 patients that did not reach the criteria for response to the Hib vaccination, 9 (60%) demonstrated a more than four-fold increase in the mean response to the pneumococcal antigens.

Table 5. Correlation coefficients between mean pneumococcal and different serotype responses and that to Hib in the combined and separate NT and NS groups.

Pneumococcal serotype	**NT+NS groups n=56**	**NT group n=33**	**NS group n=23**
Mean pneumococcal response	0.13	-0.01	0.32
4	0.27	0.09	0.54
6B	0.05	-0.10	0.29
9V	0.18	-0.03	0.51
14	-0.10	-0.18	0.06
18C	0.10	0.12	0.03
19F	0.11	0.04	0.21
23F	0.01	-0.05	0.18

Prediction of non-response to the Hib vaccine

In the NT group, there were no differences in GCS, ISS and NISS: median scores (with interquartile range) of 11 (8-15), 17 (13-26) and 27 (22-43) in responders and 12.5 (6-14.25), 18 (16.75-21) and 27 (23.5-32) in non-responders for GCS, ISS and NISS, respectively. Pre-vaccination concentrations of CRP, IL-6, IL-10, PTX3, fractalkine and calprotectin in the NS+NT group are shown in Figure 6. There were no differences between responders and non-responders; nor were there any differences between responders and non-responders in the concentrations of TNF-α, IL-6, IL-10 and after *ex vivo* LPS stimulation. In addition, when analyzed with the Spearman rank correlation coefficient no significant correlations were found in the NS+NT group between pre-vaccination concentrations of inflammatory cytokines or LPS *ex vivo* responses and the response to the Hib vaccine. There were also no significant correlations between GCS, ISS and NISS and the response to the Hib vaccine in the NT group (r=0.11-0.06).

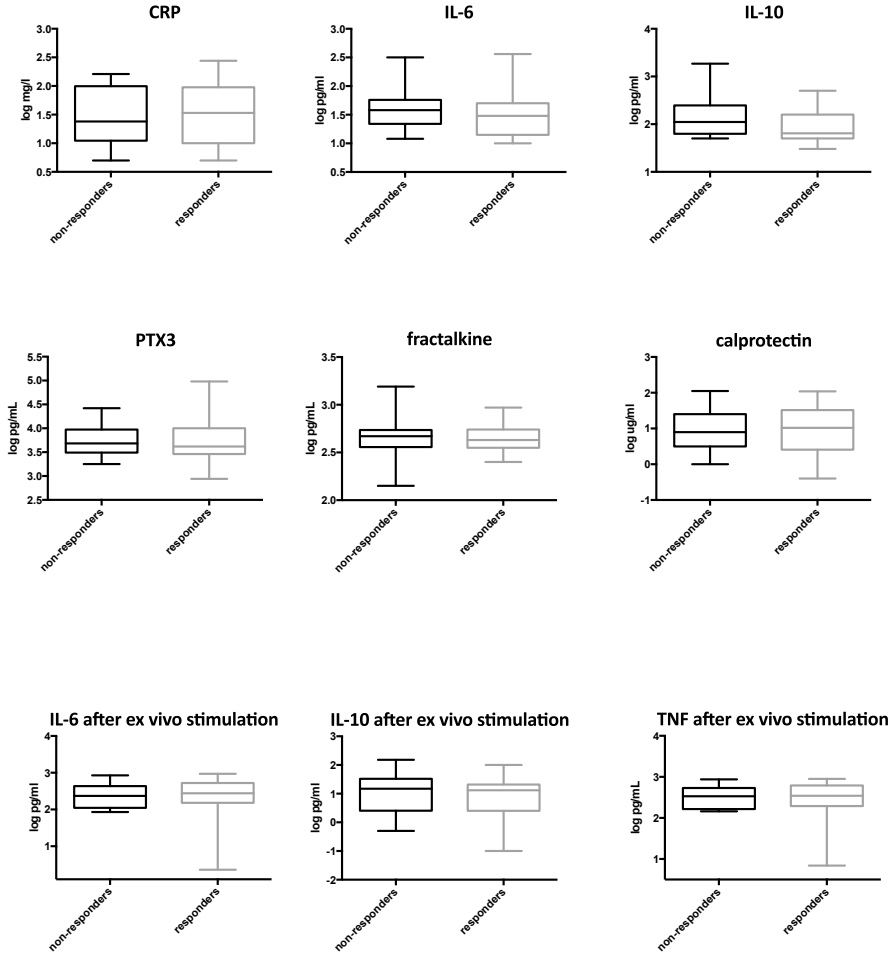


Figure 6. Pre-vaccination concentrations of selected parameters in the neurosurgery and neurotrauma groups. The line represents the median, the box extends from the 25th to the 75th percentile and whiskers range from min to max values.

Paper IV

In all, 15 NT patients, 15 NS patients and 14 healthy controls were enrolled in the study. Samples from 11 NT patients arrived to the laboratory in time for analysis. Of these 11 patients, 6 submitted a second sample as they were still inpatients at Uppsala University Hospital. Samples from 14 NS patients were analyzed and 10 had a complete series of two samples. Both samples from one patient and one of the two samples from three patients did not arrive to the laboratory in time. In one NS patient and one healthy control one sample was of insufficient quality for analysis. Characteristics of the patients with at least one sample are shown in Table 6.

Table 6. Characteristics of the patients and healthy controls

	Neurotrauma patients	**Neurosurgery patients**	**Healthy controls**
Number of patients	11	14	13
Age median (years) (range)	51 (19-69)	58 (26-69)	50 (33-64)
Sex (male/female)	8/3	7/7	3/10
Median time from trauma/surgery to sampling, days (range)	4 (2-6)	1 (1-3)	
CRP median, mg/L (range)	151 (51-315)	0.9 (0.2-7)	
Leucocyte count, $*10^9$/L (range)	9.8 (3.1-19.6)	6.9 (3.2-12)	
ISS median (range)	21 (10-30)		
GCS median (range)	8 (6-15)		
Intracerebral contusions	4		
Traumatic subarachnoid hemorrhage	5		
Subdural hematoma	4		
Skull fracture	9		

Blast-transformed $CD4^+$ and $CD8^+$ T cells after stimulation with pneumococcal conjugate vaccine

T-lymphocyte blast responses towards the PCV components evaluated by FASCIA are presented in Figure 5. Healthy controls responded well to PCV activation. In comparison with healthy controls, $CD4^+$ T lymphocytes from NT patients, harvested within a week after trauma, responded with significantly lower blast levels (p=0.04) (Figure 7A). Similarly, in the elective NS group the numbers of PCV-responding $CD4^+$ T lymphocytes decreased after surgery in comparison with the numbers observed before surgery (p=0.03) and were lower compared with healthy controls (p=0.004). As expected, the numbers before surgery did not differ from those in the healthy control group. Although not reaching the same levels as in the healthy controls, the number of blast-transformed $CD4^+$ T-lymphocytes in the NT group significantly increased in the second sample obtained 1 week later (p=0.02).

Although a trend to lower levels during the first week after trauma could be observed, the number of blast-transformed $CD8^+$ T-lymphocytes did not differ significantly between the groups (Figure 7B).

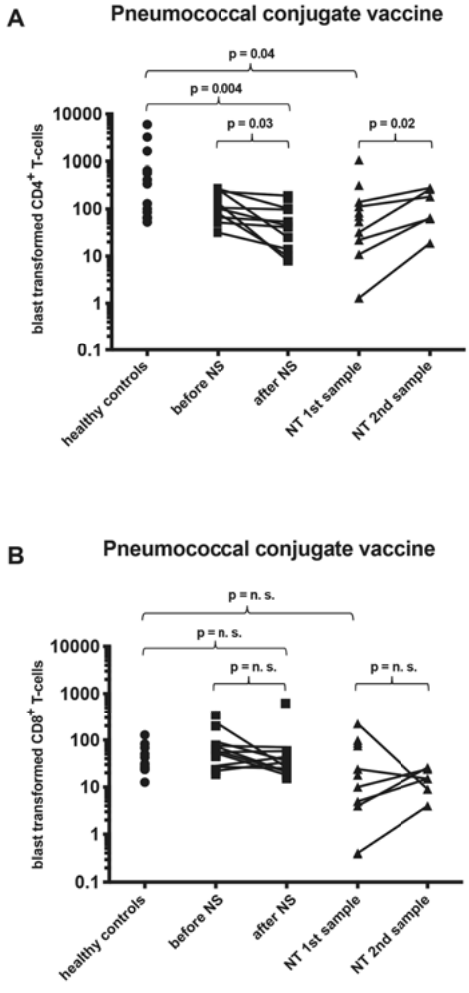


Figure 7. Number of blast-transformed $CD4^+$ T cells per 10 000 cells after stimulation with pneumococcal conjugate vaccine in healthy controls, neurosurgery patients (NS) and neurotrauma patients (NT). For NT, the first sample is obtained within 1 week after neurotrauma and the second sample 1 week later. In the NS and NT groups samples from the same patients are interconnected with lines.

Blast-transformed $CD4^+$ T cells after stimulation with pokeweed mitogen and staphylococcal enterotoxin B

The $CD4^+$ lymphocyte proliferative responses to the non-specific mitogens PWM and SEB are displayed in Figure 8. In contrast to the responses seen to PCV, there were no significant changes in blast transformation between samples from healthy controls and posttraumatic or post-surgery patients; nor were there any differences when comparing the time points within the NS and NT groups.

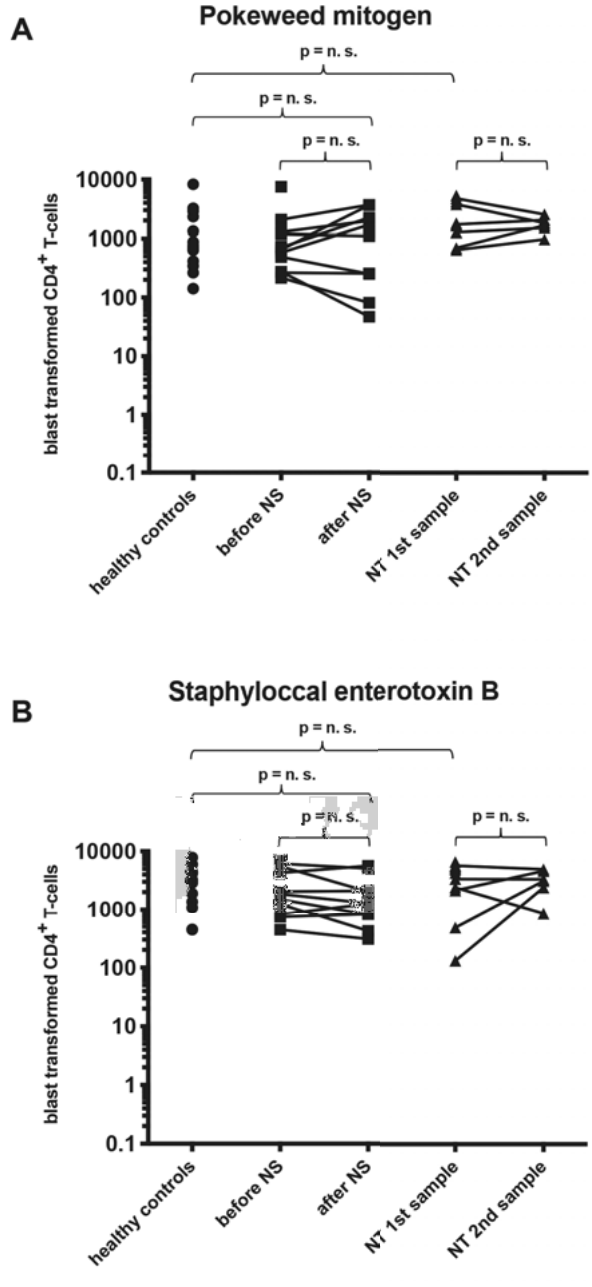


Figure 8. Number of blast-transformed CD4+ T cells per 10 000 cells after stimulation with pokeweed mitogen and staphylococcal enterotoxin B in healthy controls, neurosurgery patients (NS) and neurotrauma patients (NT). For NT, the first sample is obtained within 1 week after neurotrauma and the second sample 1 week later. In the NS and NT groups samples from the same patients are interconnected with lines.

Proportion of blast-transformed $CD4^+$ T cells expressing HLA-DR

To explore the level of T-cell activation one step further, the proportion of blast-transformed $CD4^+$ T cells expressing HLA-DR was assessed after stimulation with PCV, SEB or PWM. These results are depicted in Figure 9. The fraction of activated HLA-DR-expressing $CD4^+$ T lymphocytes after PCV stimulation was significantly lower in samples obtained after elective NS compared with samples from healthy controls (Figure 9A, $p=0.04$). Similarly, although failing to reach statistical significance, there was a trend towards a reduced PCV response the first week after NT. HLA-DR expressing $CD4^+$ T lymphocytes in samples stimulated with SEB and PWM did not differ between the groups.

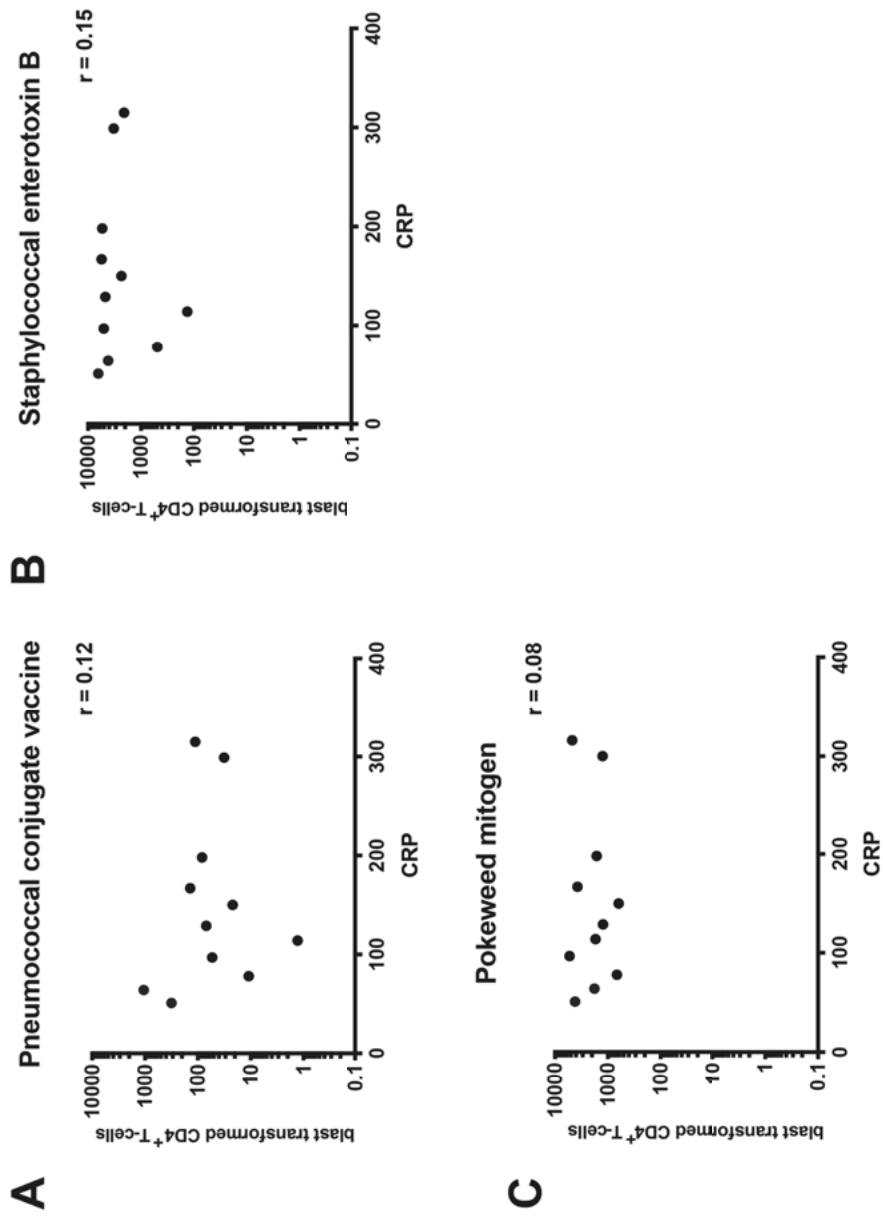


Figure 9. Proportion of blast-transformed $CD4^+$ T cells expressing human leukocyte antigen-DR after stimulation with pneumococcal conjugate vaccine, staphylococcal enterotoxin B and pokeweed mitogen.

Relationship between T-cell responses to different conjugate vaccines

To investigate whether there were similar responses to stimulation with two different conjugate vaccines, the relationships between the number of blast-transformed $CD4^+$ T lymphocytes after stimulation with PCV and those after stimulation with Hib vaccine were analyzed. In the sample obtained within 1 week after NT, there was a high and significant correlation ($r=0.74$, $p=0.01$); this correlation was weaker in the NS group after surgery ($r=0.39$, ns) and absent in the healthy controls ($r=0.08$, ns).

Relationship between clinical parameters and the T-cell response to pneumococcal conjugate vaccine

Finally, clinical parameters were analyzed in relation to PCV-induced blast-transformed $CD4^+$ T lymphocytes. No significant correlations were found between the clinical parameters age, ISS, GCS or days between trauma and sampling and the number of blast-transformed $CD4^+$ T lymphocytes after stimulation with PCV, SEB and PWM for the first sample in the NT group (data not shown). Even if the two patients with the lowest increase in CRP demonstrated the highest number of PCV-induced $CD4^+$ blasts, there was no overall correlation between these cells and CRP (Figure 10). Comparable results were seen after stimulation with SEB and PWM. Similarly, no correlation was observed with leucocyte count on the day of vaccination.

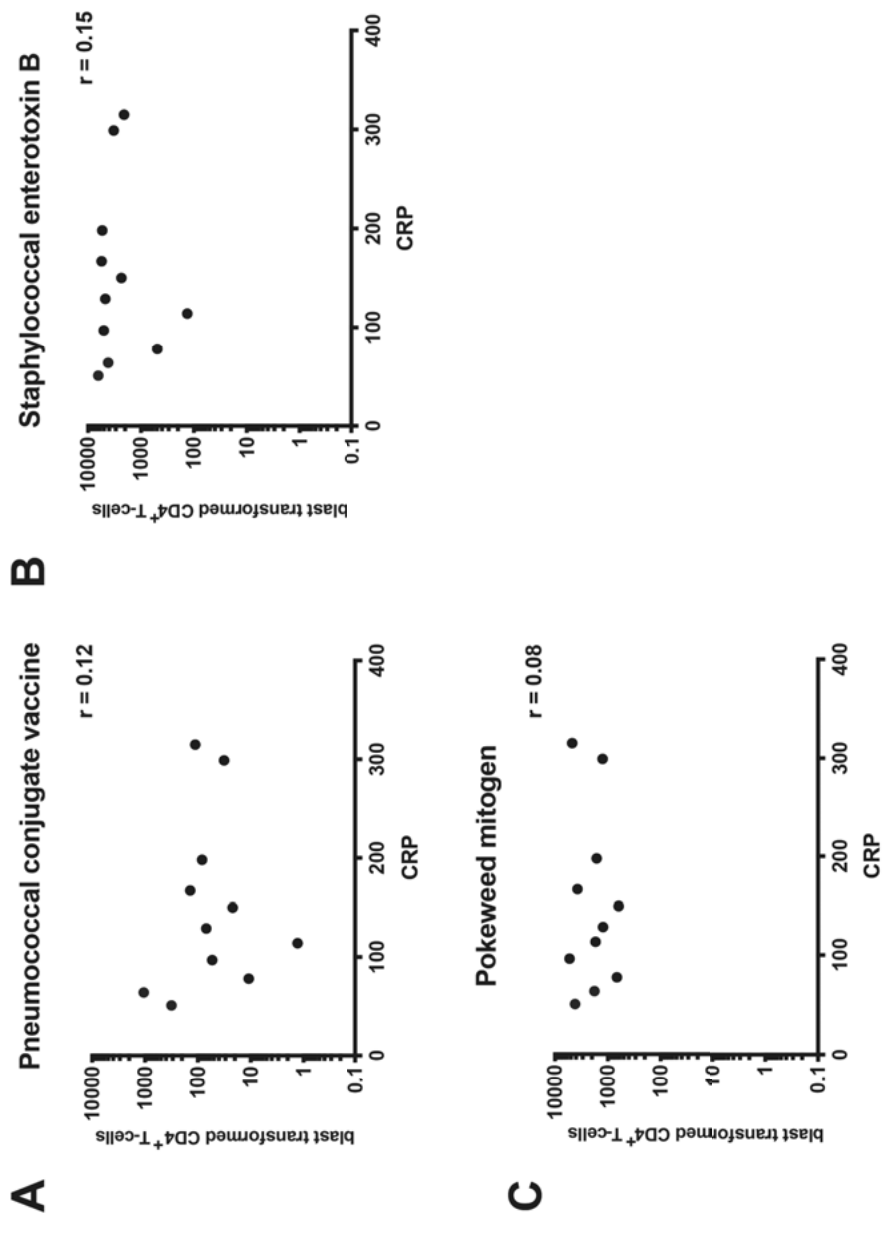


Figure 10. Correlation between concentrations of C-reactive protein (CRP) and the number of blast-transformed $CD4^+$ T cells per 10 000 cells after stimulation with pneumococcal conjugate vaccine, staphylococcal enterotoxin B and pokeweed mitogen in neurotrauma patients in the sample obtained during the first week post-trauma (sample 1).

Discussion

Paper I-II

Our investigation was conducted to explore the necessity of a larger trial for the study of the response to a T-cell-dependent PCV or of a trial comparing a T-cell-dependent PCV with a T-cell-independent PPSV early after neurotrauma. The aim was to ascertain that early vaccination would not affect antibody responses. To our knowledge, such a study of the active response to vaccination has not previously been carried out in this patient group with their risk of developing both TIDS and CIDS.

The choice of the Hib vaccine (instead of PCV) allowed simultaneous vaccination with the PPSV, which enabled us to study the response to a T-cell-dependent and a T-cell-independent vaccine in the same individual at the same time. The choice also facilitated analysis and calculations. The reason is that the Hib vaccine, in contrast to the pneumococcal vaccines that contain several serotypes, elicits an antibody response directed only against one serotype.

Paper I

The post-vaccination target concentration of IgG anti-Hib was set to >10 μg/ml and the protocol stated that patients who had reached this target before vaccination should be excluded in the primary endpoint analysis. For this reason, four patients were not included. They responded with less antibody increase and post-vaccination titers were in the magnitude of others, which would lend some support to the speculation that these patients might have had a recent exposure to Hib or cross-reacting organisms.

The results of the remaining patients demonstrated an impaired response to the T-cell dependent vaccine in the NT group vaccinated within 10 days after trauma. When patients with underlying diseases possibly affecting the immune response were cleared, this result was even more evident, showing a significant lower response even in the NS group. As also shown in Figure 4, the antibody concentrations were lower in the NT and NS groups than in the control group, although not significantly so in the NT group.

Although safe, patients with immunosuppressive diseases, such as malignancies or patients with immunosuppressive treatment, may respond more poorly than healthy individuals to this type of vaccine (86-88). Several stud-

ies have shown that patients with type 1 or type 2 diabetes mellitus exhibit an inferior response after both primary and supplementary vaccinations (89-91). Not to confound the analysis by diseases known to affect the immune response, the protocol stated that a secondary analysis should be performed in which patients with such diseases were excluded.

After other types of trauma, patients have been found to mount an adequate antibody response to a recall antigen in the form of a T-cell-dependent vaccination against tetanus (92). Therefore, we hypothesized that patients in the NT and NS groups might be able to mount an adequate immune response to a T-cell-dependent recall antigen. However, the present results cannot exclude the possibility that the vaccination response is affected by the CNS injury.

Already before vaccination, a trend towards lower antibody concentrations was seen. It is well recognized that immunoglobulin concentrations in patients suffering from trauma are reduced during the first days and that the extent of the reduction is associated with the magnitude and severity of the trauma (93-96). The mechanism responsible for this reduction is not known. Loss due to hemorrhage or a dilution effect is not responsible because the decrease affects IgG concentrations more than other immunoglobulin classes (95, 96). In the present study, there was a moderate correlation between pre- and post-vaccination antibody concentrations. Therefore, reduction in antibody concentrations before vaccination and the underlying mechanisms to this might have partly contributed to the final result in the form of lower post-vaccination antibody concentrations in the NT and NS groups.

The majority of the patients responded to the vaccination, but the present results still suggest that an impaired response after vaccination with a T-cell-dependent vaccine during the first posttraumatic week cannot be excluded.

Paper II

Given the results in Paper I and the fact that B-cell function seems to be less affected in CIDS, it was relevant to study the response to a T-cell-independent vaccine (such as PPSV23) and whether this was left unaffected by the alterations of immune function in CIDS and TIDS.

We found significant responses to PPSV23 against all serotypes tested in the three study groups (Table 2). The responses in the NT and NS groups were not inferior to those in the control group when comparing antibody levels after 3 and 6 weeks (Figure 5, Table 2). In Table 2, the time by group interaction approached significance for the responses to serotypes 6B, 9V, 14 and 23F, indicating different time courses in these responses. This effect was not caused by an inferior but by a better response in the trauma group in comparison with the other two groups. Differences in age might affect the immune response (97) but there was no age difference between the NT group and the controls. The patients in the NS group were older and, to some

extent, this might have affected the response, especially in relation to our results in serotypes 19F and 23F. The limited effect of age is consistent with the results from the study by Lee et al (97). Whereas underlying diseases are of importance in the response to the T-cell-dependent Hib vaccination, the effect of these conditions on the response to PPSV23 was small and differences in underlying diseases did not affect the overall result. Thus, taken together, the data suggest that the antibody response to PPSV23 in patients vaccinated within 10 days from the onset of possible TIDS and CIDS after neurotrauma or neurosurgery is not inferior to that in patients vaccinated >3 weeks after the acute period. Together with the results in Paper I that indicated a reduced response to a T-cell-dependent vaccine, the results in Paper II support the notion that B-cell function seems to be more preserved in CIDS and TIDS after neurotrauma and neurosurgery.

Although patients had target concentrations against several serotypes already before vaccination, the response rates were similar among the groups in patients with antibody levels below target concentrations before vaccination. Other studies have reported similar findings on prevalence of target concentrations before vaccination (80, 97). After vaccination, the proportion of patients with target levels was 80% or more for serotypes 9V, 14, 18C, 19F and 23 F, 70% for serotypes 6B and 50% for serotype 4 (Table 3). These results are in agreement with those in patients without TIDS and CIDS (79, 80, 98). The majority of those patients were older than ours, except in one study that comprised adults <45 years; however, even in this age group the results were similar to ours (97). This similarity indicates that delayed TIDS or CIDS did not affect our control group.

A limitation of our study is that only one post-vaccination serum was obtained from 23 patients. However, as shown in Figure 5, the differences in serotype-specific anti-polysaccharide binding IgG antibody concentrations between 3 and 6 weeks were limited and the correlations between the two concentrations were high (r=0.79-0.92 in different serotypes, data not shown). Furthermore, in the statistical analyses, linear mixed models, which have the advantage of handling missing values, were employed. Another limitation of the study is that only antibodies against serotypes included in PCV7 were tested. The broad T-cell-independent responses to the serotypes tested, including those with low response in other studies (97, 99), suggest, however, that this might be the case for the majority of the 23 serotypes in PPSV23.

Elderly patients may have antibodies that lack avidity to induce opsonophagocytosis (97). Antibody concentrations after PPSV23 were determined by enzyme immunoassay in the present study. This might be a limitation because the effect of neurotrauma and neurosurgery on opsonophagocytic activity (OPA) of the antibodies is unknown. An analysis of OPA would be interesting, but the method chosen for our study is well established and represents accredited methodology (77). Antibodies measured by this

method generally correlate with OPA (100, 101) in the absence of B-cell malignancies (102) and, to our knowledge, the true OPA cut-off level for protection is not fully established, although suggestions have been made (103).

A recent study on neurosurgical patients (104) found a more limited response to PPSV23 than we did and another suggested that PPSV might result in better responses if delayed until 14 days after splenectomy (105). However, the first-mentioned study included patients of higher age and mainly with subarachnoid hemorrhage and no trauma and antibody responses were measured already after 2 weeks; in the latter study, a central lymphoid organ was removed that might offer an explanation to results that differ from ours.

Paper III

No correlation was found between the T-cell-dependent and the T-cell-independent responses. The significant correlation between the investigated serotype-specific anti-pneumococcal polysaccharide antibody responses makes it reasonable to hypothesize that there were similar responses to the other serotypes included in PPSV23. This finding is in accordance with earlier studies, although not investigated in the posttraumatic setting (106, 107).

After trauma, the immunosuppression has been characterized by decreases in the capacity of monocytes to produce inflammatory cytokines after Toll-like receptor stimulation and of APCs to prime antigen on type II major histocompatibility complex molecules as well as a reduced number of $CD4^+$ and $CD8^+$ lymphocytes (9, 58). Studies of CIDS have shown that after severe head injury Th cells expressed less IL-2 receptor, transferrin receptor and HLA-DR molecules, whereas the proliferative response to the a B-cell mitogen stayed unaffected (108) indicating that CIDS affects APCs and T cells more than B cells (13, 66, 108, 109). The published results from Paper II agree with these findings, supporting a more preserved B-cell function in neurotrauma. Yet, even if there were similar responses to PPSV23 in neurotrauma patients as in the controls, only 57-80% reached protective levels against the different pneumococcal serotypes. If non-responders to the T-cell-dependent vaccine were also those with the most inferior response to the T-cell-independent vaccine, there would be limited benefit in changing a T-cell-dependent vaccine to an independent one. However, our study shows that there was no evident association in the two responses and the complete absence of correlation in the NT group might exemplify a more pronounced disturbance in the relationship between T and B cells than in the NS group with only a more moderate neurotrauma. This finding confirms that in CIDS the B-cell response is not affected similarly to the T-cell response. Moreover, among the non-responders, 60% had at least a four-fold increase in their antibody response to PPSV23. However, a drawback of PPSV23 was the

lower magnitude of the antibody response, a finding in concert with previous data comparing PPSV23 and PCV (79). An alternative to vaccinations with either PCV13 or PPSV23 for the initial immunization of NT patients would be to use the PCV13 vaccine only in the responders and the PPSV23 in the presumed non-responders, provided non-responders are possible to identify before vaccination. This might be done by an *ex vivo* PCV13 stimulation of the patient's lymphocytes but such analyses are laborious, need several days for lymphocytes proliferation (85) and are not available in clinical practice. However, we speculated that the downregulation of lymphocyte function might be associated with the magnitude of the trauma or more easily measured parameters of the innate immune system. Regrettably, none of the clinical scores representing trauma and neurotrauma or the parameters of the innate inflammatory reaction used in the present study could separate non-responders from responders.

The major strength of our study is the design, which involved simultaneous administration of two vaccines to enable investigations of the T-cell-dependent and T-cell-independent responses in the same patient under the same conditions. In addition, expressing the antibody response as fold increases eliminates differences in pre-vaccination levels. Still, there are several limitations. First, post-vaccination sera at 6 weeks were replaced by 3-week sera in 11 patients in whom 6-week sera could not be to obtained. Even if there were about 10% higher antibody levels in the 3-week sera, they were of equal magnitude for the two vaccines and the correlation between 3- and 6-week sera in patients sampled at both time points were very high (r=0.8-0.95), suggesting that this change in sampling time point did not appreciably affect the results. Second, it would also have been desirable to measure monocyte expression of HLA-DR as a marker of monocyte activation. However, at the time of planning the study, this analysis could only be performed on fresh samples and the method was not available in-house. Nevertheless, TNF-α production from LPS-stimulated monocytes, which has been shown to correlate well with the HLA-DR expression, might serve as a useful substitute (110-112). Finally, antibody concentrations in our study were determined by enzyme immunoassays and the effect of neurotrauma and neurosurgery on OPA is not known. Antibodies analyzed by enzyme immunoassay, however, generally correlate with OPA and therefore the effect on the present results should be limited (100, 101).

Paper IV

This study investigated T-cell responsiveness in patients who suffer from NT by exploring the ability to mount T-cell responses to T-cell-dependent conjugated vaccines. The rationale behind this study was that TIDS and CIDS might negatively influence the outcome of vaccination with PCV, which is

recommended after neurotrauma to prevent pneumococcal posttraumatic meningitis (5, 6). This study showed that the $CD4^+$ T-cell responses to PCV early after neurotrauma or neurosurgery were significantly lower than those in healthy controls. This finding indicates that the protective effect of vaccination with PCV early after neurotrauma might be reduced. The $CD8^+$ T-cell responses, in comparison with the $CD4^+$ T cells, were generally lower, implying that there was little cross-presentation of exogenous antigens in the assay used, most probably because specialized cross-presenting dendritic cells are rare in PBMCs. Some of the NT patients demonstrated a reduced $CD8^+$ T-cell response in comparison with the controls but differences between the groups did not reach significance. Thus, even if an effect on $CD8^+$ T cells cannot be excluded, the effect on the $CD4^+$ T cells is more evident. HLA-DR expression on $CD4^+$ blasts is a marker of activation and associated with B-cell differentiation and proliferation (113, 114). The lower values seen in both the NS and NT groups compared with the controls, although significant only in the NS group, might further reduce $CD4^+$ T-cell function.

Non-specific stimulation with the superantigen SEB and the mitogen PWM did not result in reduced $CD4^+$ responses in comparison with the controls, which, to some extent, diverges from the results in an older study in which the PWM response was somewhat reduced in patients with head injury (108). However, in that study ISS was higher and GCS lower, which might have affected the results. Specific antigen stimulation is a controlled TCR-dependent activation in the presence of costimulatory molecules and cytokines, whereas non-specific activation using SEB and PWM are supraphysiological stimuli, where PWM even bypasses the TCR. Thus, the absence of a reduced SEB and PWM response in our study might indicate that the T-cell suppression after PCV stimulation in patients with TIDS and CIDS is mainly at the TCR level of antigen-specific activation.

The $CD4^+$ T-cell response to Hib in the NT group demonstrated a high correlation to that induced by PCV, indicating that the *ex vivo* responses to the two T-cell-dependent conjugate vaccines were affected in a similar way. A trend towards correlation was found in the NS patients with a more limited neurotraumatic inflammatory insult, whereas no correlation was found in the healthy controls. A possible explanation for this finding might be that the $CD4^+$ T-cell responses to the PCV and Hib vaccine in healthy individuals mainly reflect recent exposures to pneumococci or Hib, whereas in patients with neurotrauma, exposure seems to be of less importance in relation to the CIDS- and TIDS-induced downregulation. The lower $CD4^+$ T-cell responses to the PCV and Hib vaccine in our study supports the relevance of our finding of a lower *in vivo* response to the conjugate Hib vaccine when administered early after neurotrauma or neurosurgery (Paper I). Moreover, the correlation between the responses to Hib and PCV in the current study supports the argument that the reduced *in vivo* response to Hib might also be relevant for the response to PCV.

A wide variation in the responses in the NT patients was observed. Although the initial local response to neurotrauma, measured as cytokine levels in cerebral microdialysis, is mainly pro-inflammatory (64, 65), patients also show signs of downregulation of the systemic inflammatory response (13). It seems plausible to hypothesize that downregulation is associated with the severity of the neurotrauma and the initial inflammatory response. However, even if the two patients with the lowest CRP increase demonstrated the lowest downregulation of the $CD4^+$ T-cell responses, no overall correlation was observed, neither to CRP nor to the ISS and GCS severity scores. These results are in accordance with the results from Paper III in which clinical parameters could not predict the *in vivo* response to Hib vaccination early after neurotrauma or elective neurosurgery.

General discussion and future perspectives

American as well as updated Swedish guidelines recommend a first dose of PCV followed by a dose of PPSV23 at least 8 weeks later (5, 6). Similar to the Swedish recommendations, ACIP recommendations omit information about the time point for the first vaccination in relation to the trauma. Although most patients seem able to achive an adequate response, our data indicate that vaccination with a conjugate vaccine during the first 10 days might lead to low post-vaccination concentrations in a substantial number of the patients compared with the control group that were vaccinated at least 3 weeks post-trauma.

The data from our studies underline the need for a randomized study to determine the optimal pneumococcal vaccination schedule after neurotrauma. In such a study, the current ACIP recommendation of PCV13 followed by PPSV23 after 8 weeks should be compared with early PPSV23 followed by a delayed administration of PCV13 1 year later.

Of particular note is whether a booster response will be obtained after PPSV at 8 weeks in the PCV non-responders. In addition to the reduced primary response to PCV, it cannot be ruled out that also the booster response to PPSV23 of serotypes included in PCV13 might be affected in patients with TIDS- and CIDS-induced reduction in their primary PCV response. Another key point is whether there is a remaining hyporesponsiveness to PCV at 1 year after PPSV and whether this response might be associated with increasing age (115). To study the relationship between the number of T cells and antibody responses as well as the relationship between the HLA-DR expression on T cells and antibody responses might be a feasible way to identify non-responders to PCV. It would also be interesting to in a subgroup investigate the relationship between the *in vivo* antibody responses and the *ex vivo* T-cell responses in the model we used in Paper IV.

Until tested in a future study, PCV should probably not be administered early after neurotrauma. Instead, PCV can be postponed a couple of weeks but during this time, when the risk for meningitis is high, the patient has no protection. Awaiting the results of a study demonstrating a reliable response to early PCV13 vaccination, PPSV23 might be considered for early vaccination because of its broader initial coverage and a response that is not inferior to later vaccination. Because PCV13 strains seem to decrease as a consequence of serotype replacement, PPSV23 will result in relevant protection during the most vulnerable period (52, 116, 117). Owing to the large variation in serotypes causing meningitis (118, 119), early vaccination with PPSV23, with its polyserotypic protection against meningitis when the risk is at its peak, might offer an additional advantage over PCV13. For long-term protection, it should probably be followed by PCV. Because of the risk of hyporesponsiveness when vaccines are administered in this sequence, an interval of 1 year is usually recommended based on immunogenicity studies (49). This strategy could be a model for the provision of both early and long-term protection.

Pfizer Inc announced in December 2018 the initiation of a Phase 3 program for its PCV20 candidate for the prevention of invasive disease and pneumonia caused by pneumococcal serotypes in the vaccine in adults aged $\geq$18 years. If successful, a broader long-term protection can be offered to NT patients. Still, determining the optimal timing of the vaccination in neurotrauma patients relative to the trauma remains to be looked into further.

Conclusions

Paper I The results of this study indicate that an impaired response after vaccination with a T-cell-dependent antigen during the first 10 post-traumatic days cannot be excluded.

Paper II The results of this study demonstrate that patients vaccinated with a T-cell-independent pneumococcal polysaccharide vaccine within 10 days after neurotrauma or neurosurgery respond similarly to patients vaccinated after 3 weeks or later.

Paper III There is no correlation between the T-cell-dependent and T-cell-independent vaccine responses in this study. Prediction of the T-cell-dependent vaccine response by grading the trauma or by parameters reflecting the innate immune response is not possible. Because correlations between the responses to the different pneumococcal serotypes are high, it seems reasonable to expect a good response to vaccine serotypes in PPSV23 not analyzed in this study.

Paper IV We noted a significant reduction, although with a wide variation, in the *ex vivo* $CD4^+$ lymphocyte response to pneumococcal T-cell-dependent conjugate vaccine in patients who had suffered from neurotrauma or undergone elective neurosurgery. The defect seems to be general and at the level of specific antigen recognition.

Svensk sammanfattning (summary in Swedish)

Bakgrund

Risken för bakteriell hjärnhinneinflammation ökar efter skallskada med skallbasfraktur (1, 3, 35). Vanligaste agens vid dessa infektioner är *S pneumoniae* och av denna anledning rekommenderas pneumokockvaccination efter skallskada (5). Tidigare har det vanligaste förfarandet i Sverige varit att några veckor till månader efter skadan vaccinera med en dos polysackaridvaccin mot pneumokocker (PPSV23), d v s ett T-cellsoberoende vaccin. I och med utvecklingen av T-cellsberoende konjugatvaccin mot pneumokocker (PCV), finns möjligheten att ge dessa patienter ett långtidsskydd. De svenska rekommendationerna har uppdaterats till att inkludera båda typerna av vaccin, först PCV och 2 månader senare PPSV23, ett förfarande som även rekommenderas i USA (5, 6). Rekommendation om tidpunkt för första vaccinationen i relation till traumat saknas dock. Eftersom risken för hjärnhinneinflammation är allra högst under de första veckorna efter traumat, vore det önskvärt att vaccinera patienterna så tidigt som möjligt (3, 4). Immunsvaret på vaccination given tidigt efter skallskada har dock inte tidigare studerats.

Yttre skador och kirurgi aktiverar det medfödda immunförsvaret och inducerar en systeminflammatorisk reaktion av samma typ som vid sepsis, där pro- och anti-inflammatoriskt svar initieras samtidigt (8). Det anti-inflammatoriska svaret kan leda till traumainducerad immunhämning, här benämnd TIDS, som effekt av bland annat T-cellsdöd och försämrad funktion i befintliga T-celler (9, 11, 12, 58). Ytterligare försämring av T-cellsfunktionen kan vid skallskada orsakas av CNS-skadeinducerad immunhämning (CIDS) (13). Detta tillstånd karaktäriseras av försämrad T-cellsfunktion, medan B-cellernas förmåga att bilda antikroppar tycks påverkas i mindre utsträckning (13, 71). Sammantaget kan skallskada genom mekanismerna TIDS och CIDS resultera i ett försämrat svar på T-cellsberoende vaccin beroende på tidpunkt för vaccinationen.

Syfte och frågeställningar

Avhandlingen består av fyra delarbeten. Det övergripande syftet var att undersöka hur genomgången skallskada påverkar svaret på de vacciner som Folkhälsomyndigheten rekommenderar efter just skallbasfraktur.

I delarbete I var syftet att undersöka antikroppssvaret på ett T-cellsberoende konjugatvaccin hos patienter som vaccinerats inom 10 dagar efter möjligt påslag av TIDS och CIDS orsakat av skallskada eller neurokirurgi. Antikroppssvaret jämfördes med det hos patienter som vaccinerats senare, i så kallad i konvalescensfas.

Delarbete II syftade till att undersöka antikroppssvaret på ett T-cellsoberoende polysackaridvaccin mot pneumokocker hos patienter som vaccinerats inom 10 dagar efter möjligt påslag av TIDS och CIDS orsakat av skallskada eller neurokirurgi. Antikroppssvaret jämfördes med det hos patienter som vaccinerats i konvalescensfas enligt klinisk rutin - när studien genomfördes var polysackaridvaccinet det enda som rekommenderades i Sverige.

I delarbete III undersökte vi sambandet mellan svaren på T-cellsberoende respektive T-cellsoberoende vaccin i delarbete I och II. Om svaren skulle samvariera, talar detta för en mer generell immunhämning där både B- och T-cellsfunktionerna är nedsatt. Det skulle i så fall innebära att individer som inte svarar på det T-cellsberoende vaccinet kanske inte heller svarar på det T-cellsoberoende vaccinet. Om svaren däremot inte skulle samvariera, planerade vi gå vidare och undersöka om man med hjälp av kliniska eller immunologiska parametrar kunde förutsäga svaret på det T-cellsberoende vaccinet. I så fall skulle man på förhand kunna identifiera de patienter som inte förväntas svara på PCV, vilket numera rekommenderas som det första vaccinet, utan som istället skulle få bättre skyddseffekt av PPSV23.

I delarbete IV använde vi en så kallad *ex vivo*-modell för att undersöka det specifika svaret på PCV i blodprover tagna på patienter efter aktivering av det medfödda immunförsvaret genom skallskada eller neurokirurgi. Vi undersökte också om en generell T-cellsfunktionsnedsättning föreligger vid CIDS och TIDS.

Metod

I delarbete I och II inkluderades 85 patienter och vaccinerades vid samma tidpunkt med både ett T-cellsberoende vaccin mot *H influenzae* typ B (Hib) och ett T-cellsoberoende vaccin mot pneumokocker (PPSV). Designen möjliggjorde studier av svaren på dessa två principiellt olika vacciner hos samma individ under exakt samma förhållanden. Patienterna följdes upp med provtagning 3 och 6 veckor efter vaccinationerna. Antikroppsnivåerna mot Hib och 7 olika pneumokockserotyper analyserades.

Delarbete III: Just före vaccinationen av patienterna i delarbete I och II togs blodprover på vilka vi utförde standardiserad endotoxinstimulering *ex vivo* av de perifera blodcellerna. Immuncellerna stimulerades till att producera IL-6, IL-10 och TNF-α. Dessa koncentrationsbestämdes och nivåerna relaterades till antikroppssvaren. Koncentrationsbestämning utfördes även av IL-6, IL-10, pentraxin, fraktalkin och calprotektin utan föregående endotoxinstimulering. Nivåerna relaterades till antikroppssvaren. Uppgifter om CRP och kliniska data för att beräkna Glasgow Coma Scale (GCS), Injury Severity Score (ISS) och New Injury Severity Score (NISS) togs från patientjournalerna. Även dessa parametrar relaterades till antikroppssvaren.

I delarbete IV inkluderades 15 patienter som genomgått skalltrauma, 15 patienter som planerades för neurokirurgi och 15 friska kontroller. De provtogs vid 1-2 tillfällen. FASCIA (flow cytometric assay for specific cell-mediated immune response in activated whole blood) utfördes varvid T-cellerna utsattes för olika generella och specifika stimuli. Därefter utfördes räkning av blastomvandlade T-celler. Uppgifter om CRP och kliniska data för att beräkna GCS och ISS togs från patientjournalerna. Dessa parametrar relaterades till T-cellssvaret.

Resultat och slutsatser

Delarbete I: Signifikant färre patienter svarade på det T-cellsberoende vaccinet givet inom 10 dagar efter skallskada och neurokirurgi jämfört med kontrollgruppen som vaccinerades i konvalescensfas.

Delarbete II: Patienter som vaccinerades med det T-cellsoberoende PPSV23 inom 10 dagar efter skallskada och neurokirurgi svarande likvärdigt med kontrollgruppen som vaccinerades i konvalescensfas.

Delarbete III: Ingen korrelation mellan svaren på T-cellsberoende respektive T-cellsoberoende vaccin kunde påvisas vilket talar för en bevarad B-cellsfunktion efter skallskada och neurokirurgi. Svaret på det T-cellsberoende vaccinet kunde inte förutsägas med hjälp av gradering av skadans svårighetsgrad eller immunologiska parametrar som reflekterar aktiveringen av det medfödda immunförsvaret. Korrelationen mellan antikroppssvaren på de 7 undersökta serotyperna i PPSV23 var hög varför man kan förmoda ett liknande svar även mot de övriga serotyperna i vaccinet.

Delarbete IV: Vi fann en signifikant reduktion av $CD4^+$ T-cellssvar på PCV *ex vivo* hos patienter under första veckan efter skalltrauma eller neurokirurgi. $CD8^+$ T-cellssvar på PCV påverkades inte och inte heller $CD4^+$ T-cellssvaret på generella stimuli. Immundefekten vid CIDS och TIDS tycks således innebära en försämring av $CD4^+$ T-cellers förmåga att reagera på specifika antigen.

Praktisk betydelse

För att säkerställa ett fungerande vaccinationsschema med syfte att skydda patienter mot pneumokockorsakad hjärnhinneinflammation efter skallskada, behövs en randomiserad studie där antikroppssvaret hos patienter som vaccinerats enligt schemat i de svenska riktlinjerna jämförs med patienter som vaccinerats med vaccinerna i omvänd ordning, d v s PPSV23 följt av PCV ett år senare. I väntan på resultaten från en sådan studie föreslås att man, mot bakgrund av resultaten från denna avhandling, avvaktar med PCV minst tre veckor efter skadetillfället. En nackdel är att man då lämnar patienten oskyddad under den period då risken för hjärnhinneinflammation är allra störst. Ett alternativ är att frångå riktlinjerna och istället vaccinera patienterna med PPSV23 direkt efter traumat, vilket i avhandlingen har visat sig ge ett vaccinationssvar likvärdigt med svaret på en senare vaccination, och följa upp med PCV först ett år senare. Intervallet syftar till att undvika så kallad hyporespons vilket kan uppstå om vaccinerna ges i denna ordning med kortare intervall (49). En fördel med detta alternativ är att patienten redan tidigt efter skadetillfället sannolikt får skydd mot hela 23 serotyper varav flera fortfarande cirkulerar i befolkningen. Infektioner orsakade av de serotyper som PCV13 skyddar mot förekommer inte i särskilt stor omfattning längre eftersom PCV ingår i det allmänna barnvaccinationsprogrammet (52, 116, 120).

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Supplementary table

Table 1. Table of antibodies used for flow cytometry

Marker	Fluorescence	Clone	Company
CD3	Alexa fluor 700	UCHT1	BD
CD3	BV650	OKT3	BioLegend
CD4	PB	RPA-T4	BD
CD4	PerCP-Cy5.5	RPA-T4	BioLegend
CD8	BV605	SK1	BD
CD8	APC	RPA-T8	BD
CD19	APC-Cy7	SJ25C1	BD
CD56	PE	B159	BD
CD69	PE-CY7	FN50	BD
HLA-DR	FITC	TU36	BD

Abbreviations: BV: brilliant violet, PB: pacific blue, PerCP: Peridinin chlorophyll protein complex, CY: cyanine, APC: allophycocyanin, PE: phycoerythrin, FITC: fluorescein isothiocyanate.

Companies: BD (BD Biosciences, Franklin Lakes, NJ, USA), BioLegend (San Diego, CA, USA).

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