Role of meningococcal Toll-like receptor agonists in disease and immunity

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Role of meningococcal Toll-like receptor agonists in disease and immunity

Rol van Toll-like receptor agonisten van de meningokok in ziekte en immuniteit

(met een samenvatting in het Nederlands)

Proefschrift

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Chapter 1

General Introduction

Neisseria meningitidis

Biology

Neisseria meningitidis is a Gram-negative bacterium and a major cause of meningitis and sepsis worldwide[1]. It belongs to the bacterial family *Neisseriaceae*, which mainly consist of mammalian commensals such as *N. lactamica*. Another important human pathogen belonging to this group is *N. gonorrhoeae*, the causative agent of gonorrhea. *N. meningitidis* strains are classified into 13 serogroups based on different capsular polysaccharide structures. Strains can be further divided into serotypes and serosubtypes based on differences in the major outer membrane proteins PorB and PorA, respectively[2]. Finally, immunotype is determined by the structure of its lipopolysaccharide (LPS)[3, 4]. Of the 13 serogroups, only 6 (A, B, C, W-135, X, and Y) cause almost all cases of disease[1]. Currently, genome sequences of several *N. meningitidis* strains are available. These strains are serogroup A strain Z2491, serogroup B strain MC58, and serogroup C strain FAM18[5-7]. In addition, whole-genome sequences of three carriage strains were obtained recently[8].

Molecular epidemiology

The traditional typing methods of meningococci described above have some limitations for epidemiological studies of meningococcal disease[9]. Therefore, the multilocus sequence typing (MLST) technique has been developed. In this technique a few housekeeping gene fragments are sequenced. The allelic variations in these genes is thought to reflect the overall mutational rate of the genome. Thus, differences between strains in these genes will provide information about their genetic relatedness. The genetic variations at the different loci are assigned arbitrarily an allele number and the combination of allele numbers over the multiple loci is designated as a sequence type. These sequence types are grouped into clonal complexes based on their similarity. Currently, 37 clonal complexes have been identified in *N. meningitidis*[9]. While it has been become clear that extensive horizontal gene transfer takes place among meningococci, these clonal complexes still manage to persist for long periods. They must therefore represent particular genetic configurations with a strong selective advantage. Almost all meningococcal disease is caused by a particular subset of clonal complexes, the so-called hypervirulent lineages.

Virulence factors

The only known natural habitat of the meningococcus is the human nasopharynx[1]. Therefore, *N. meningitidis* is very well adapted to survive in this environment. Little is known about the mechanisms underlying colonization and infection, but some meningococcal factors are known to play important roles (Fig. 1).

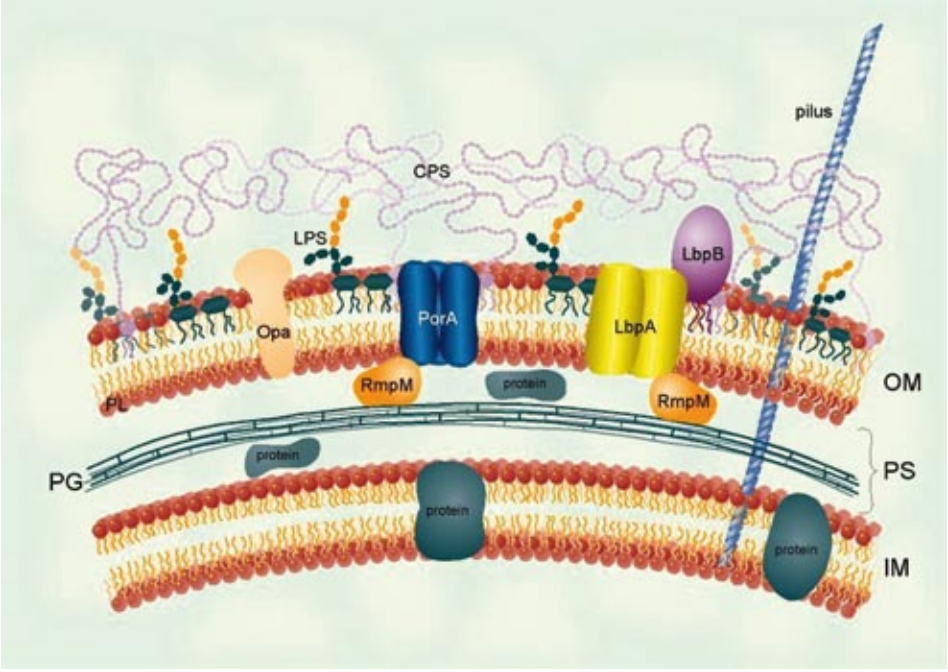

Figure 1. Cross-section of the meningococcal cell envelope. Encapsulated *Neisseria meningitidis* contain an inner membrane (IM) and outer membrane (OM) and is surrounded by capsular polysaccharide (CPS). Lipopolysaccharide (LPS) is anchored in the OM, which also contains several outer membrane proteins such as porin protein A (PorA) and Opacity protein (Opa). Reduction modifiable protein M (RmpM) is located in the periplasmic space (PS), where it forms a bridge between the OM and the peptidoglycan layer (PG)[101].

The membranes of mucosal cells in the human nasopharynx are protected against micro-organisms by the presence of IgA antibodies. *N. meningitidis* may evade this defence by secreting IgA1 protease that destroys these antibodies[10]. Moreover, the human host limits the availability of free iron, which meningococci need for growth. However, *N. meningitidis* has found a solution for this problem by expressing several proteins that can acquire iron from human iron-containing proteins[1]. These meningococcal proteins include HpnA and HpnB (haemoglobin-haptoglobin complex), TbpA and TbpB (transferrin), LbpA and LbpB (lactoferrin), and HmbR (haemoglobin). Capsular polysaccharide protects

the bacterium against phagocytic killing and complement-mediated killing[1]. Type IV pili are thought to be important for the initial attachment of encapsulated *N. meningitidis* to mucosal cells[11]. These type IV pili are composed of several protein subunits forming long filamentous structures that emanate from the bacterial surface and bind to CD46 expressed on human cells. After the initial attachment to the host cells, expression of the capsule and pili is downregulated in order to allow close contact between *N. meningtidis* and the target cell membrane. Proteins required for this intimate adhesion are Opa and Opc proteins[11]. Both can bind at least two molecules: heparin sulphate proteoglycans and sialic acids, but they also have more specific receptors. Opa can also bind CEACAMs, in particular CEACAM1. In addition, Opc binds to vitronectin, fibronectin, and α-actinin. The function of interaction of Opa and Opc with these host structures seems to be adhesion to and invasion of target cells. The meningococcal porins PorA and PorB may also be important for bacterial entry, as it has been shown that porins can translocate spontaneously into plasma membranes and interfere with cell signalling[12]. Finally, the major outer membrane component lipopolysaccharide (LPS) is important in adherence and colonisation[1]. Moreover, it is thought to contribute to the pathogenesis of sepsis and meningitis due to its toxicity.

Another important factor in the adaptation to the human host and evasion of immunity is the genetic diversity of *N. meningitidis*. It can take up foreign DNA from other neisserial strains, or even other species[13]. Moreover, there is extensive antigenic diversity in some genes, due to mutations in non-essential parts of the gene. This is particularly seen in genes under antigenic selection pressures, because their encoded proteins are targeted by the immune system. An example is the *porA* gene, encoding the major immunogenic protein PorA[14]. Finally, several genes can be switched on-and-off by phase-variation, which is associated with short DNA repeats[15]. These sequences are prone to cause slippage of the DNA polymerase during DNA replication, leading to reversible frameshift mutations.

Carriage

Normally, *N. meningitidis* is a commensal of the human upper respiratory tract, where it resides without causing any symptoms (Fig. 2). Around 8-25% of healthy individuals are carrier of the bacterium[1]. Carrier isolates are genetically much more diverse than patient isolates. Only 11 of the 37 clonal complexes presently identified in *N. meningitidis* are disease-associated[9]. Moreover, approximately 40-50% of carrier strains lack capsular polysaccharide, whereas invasive strains almost exclusively express capsule[16, 17]. Risk factors for meningococcal carriage include co-infections of the upper respiratory tract, smoking, very low humidity, and attendance of crowded places[1].

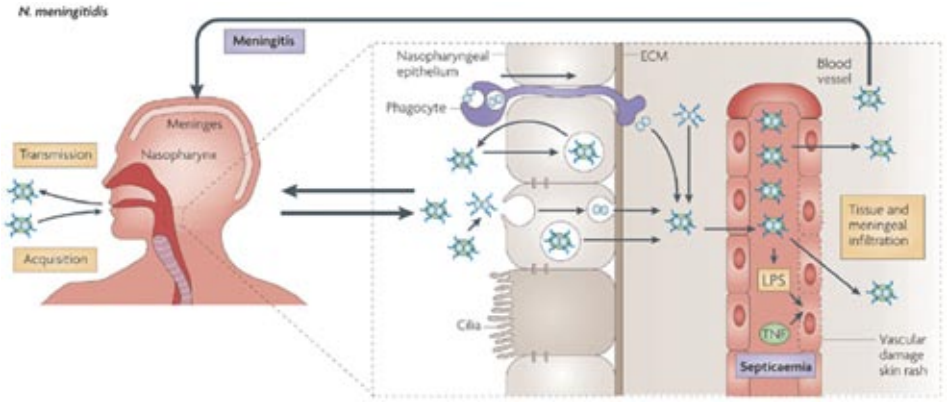

Figure 2. Possible outcomes after encountering *Neisseria meningitidis*. Meningococci enter the human body through the nose or mouth. In most cases individuals will only carry the bacterium asymptomatically in the upper respiratory tract. Occasionally, *N. meningitidis* manages to cross the mucosal layer and enters the bloodstream, which may lead to meningitis and/or sepsis[102].

Disease

Occasionally, *N. meningitidis* manages to cross the mucosal barrier and enters the bloodstream, which may lead to the life-threatening diseases meningitis and sepsis (Fig. 2). Incidence of meningococcal disease is highest in sub-Saharan Africa during epidemics in the dry season[1]. Therefore, this region is often referred to as the "meningitis belt". During these outbreaks up to 2% of the population can be affected. In most cases these epidemics are caused by serogroup A *N. meningitidis*. In endemic situations, incidence of meningococcal disease is about 0.5-5 cases per 100.000 per year. Serogroups B and C are prevalent among patient isolates in developed countries. Serogroup Y disease rates have increased in recent years in the USA and Israel[18]. Incidence of meningococcal disease is highest in young children and there is a second peak in disease rates in adolescents and young adults[19].

Symptoms can be diverse and depend on the focus of infection. However, the majority of patients display haemorrhagical skin lesions, especially on the limbs[1]. Most commonly *N. meningitidis* causes meningitis, after crossing the blood brain barrier, but rapid proliferation of meningococci in the circulation can also lead to sepsis. Meningococcal LPS is thought to play a major role in the pathogenesis of these conditions. It is the most potent inducer of the excessive pro-inflammatory cytokine response, which becomes toxic at such high levels[20, 21]. LPS also activates the coagulation system through upregulation of tissue factor[1]. Excessive stimulation of the coagulation system leads to disseminated

intravascular coagulation (DIC), the most feared complication of meningococcal disease. DIC is clinically characterized by depletion of thrombocytes and coagulation factors, hypotension, and petechial rash. Levels of circulating *N. meningitidis* LPS correlate strongly with morbidity and mortality of meningococcal disease[22, 23]. Mortality rates for meningitis and sepsis are around 10% and 20%, respectively[24]. In addition, approximately 20% of patients who survive remain disabled due to for instance loss of digits or limbs, hearing loss, or mental retardation[25].

It has been known for a long time that levels of antibodies that can activate the complement system to kill *N. meningitidis* correlate strongly with protection against meningococcal disease[26]. Therefore, the current consensus is that a vaccine against *N. meningitidis* should elicit bactericidal antibodies. Apart from absence of bactericidal antibodies, there are also several host factors that increase the risk for meningococcal disease. For example, some individuals with deficiencies in components of the terminal attack sequence of complement (C5-C9) have severe or recurrent infections with Neisseria species[27]. Other complement deficiencies are also associated with meningococcal disease. Specific polymorphisms for the gene encoding mannose binding lectin, an opsonin that mediates complement activation, occur more frequently among children with disease than among controls[28]. Moreover, individuals with deficiencies in properdin or factor D, which are components of the alternative complement pathway, are more susceptible to meningococcal disease[29]. Together these data illustrate the important role of the complement system in protection against *N. meningitidis* (Fig. 3). Other host factors associated with increased risk are polymorphisms in genes coding for the Fcγ receptors CD16 and CD32[30]. Finally, rare mutations in Toll-like receptor (TLR) 4, the receptor for LPS, are found more frequently in patients with meningococcal disease than in healthy controls[31].

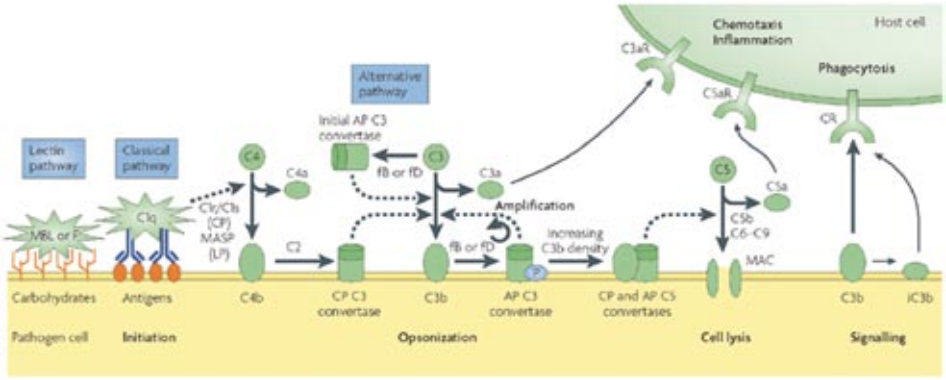

Figure 3. Activation of the complement system. The complement system can be activated in three ways: by antibody complexes (classical pathway (CP)), by binding of mannose-binding lectin (MBL) or ficolin (F) to carbohydrates (lectin pathway (LP)), or by spontaneous and induced C3 hydrolysis (alternative pathway (AP)). All three pathways lead to cleavage of C3 by C3 convertases into its active fragments C3a and C3b. Covalent binding of C3b (opsonization) amplifies the cascade and mediates phagocytosis and adaptive immune responses by binding to complement receptors (CRs). Accumulation of deposited C3b also leads to the assembly of C5 convertases that activate C5 to C5a and C5b. Whereas C5b initiates the formation of the lytic membrane-attack complex (MAC), the anaphylatoxins C3a and C5a induce pro-inflammatory and chemotactic responses by binding to their receptors (C3aR and C5aR). On pathogenic surfaces, properdin (P) induces and stabilizes the alternative pathway C3 convertase, which leads to enhanced complement activity. Abbreviations: fB, factor B; fD, factor D; RCA, regulators of complement activation[103].

Animal models

A good animal model for meningococcal disease would be desirable to study pathogenesis and vaccine efficacy. However, since *N. meningitidis* only infects humans, a perfect animal model does not exist. Many meningoccocal proteins that interact with host structures are highly specific for the human homologue[32]. For example, pili bind to human CD46, Opa proteins interact with human CEACAM1, and the neisserial iron uptake systems are specific for the human iron transport proteins, such as lactoferrin and transferrin. Moreover, even in humans, infection via the natural route progresses only in rare cases to invasive disease. Despite these difficulties some animal models are used frequently. One is the mouse intraperitoneal challenge model. In this model iron dextran or human iron transport proteins are usually administered to the mice to provide a source of iron for the bacteria. Although the model clearly does not represent the natural pathogenesis of the disease, it does model meningococcal sepsis quite well. As in humans, the bacteria can grow to high concentrations in the blood and the mice can become sick or die from the infection. Moreover, active or passive immunization confers protection. A second animal model that is used regularly is intraperitoneal infection of infant rats[32]. The advantage of this model is that an exogenous iron source is not required. In addition, passive protection by human immune sera can be assessed. However, studying protection

by active immunization is not possible. A better animal model might become available in the coming years, for example by using transgenic animals that express human receptors which are important for the virulence of *N. meningitidis*. Indeed it has been demonstrated that mice that express human CD46 are more susceptible to meningococcal disease[33].

Vaccines

Capsular polysaccharide vaccines against serogroup A, C, W-135, and Y are available[1]. These vaccines are effective, but are less immunogenic in children younger than two years. In addition, duration of immunity to the polysaccharide vaccines is restricted to 3-5 years. To overcome these limitations, conjugated capsular polysaccharide vaccines for these serogroups have been developed recently[34]. Unfortunately, a vaccine based on the capsular polysaccharide of serogroup B is not feasible, because it resembles polysialic structures found in human fetal neural tissue. Perhaps for this reason the serogroup B capsule is poorly immunogenic. Even if the immunogenicity can be improved, the use of this structure as a vaccine is not a very attractive approach because of the risk of autoimmunity[35]. Therefore, current strategies to develop a vaccine against serogroup B are mainly focused on meningococcal outer membrane proteins. Outer membrane vesicle (OMV) vaccines have been used successfully to control epidemics of serogroup B disease in Norway, Cuba, Chile, Brazil, and New Zealand[36]. These outbreaks were dominated by one particular strain of defined clonal type. However, an OMV vaccine derived from a single strain is less suitable to prevent disease in an endemic situation where numerous genetically diverse serogroup B meningocci are circulating. The immune response is almost exclusively directed towards a limited number of immunodominant surface proteins like PorA, which are quite variable between strains. As a consequence, there is little cross-protection between the different PorA serosubtypes. Therefore, the Netherlands Vaccine Institute is developing a serogroup B OMV vaccine that contains nine different PorA variants (NonaMen). The expectation is that this vaccine will cover approximately 75% of the serogroup B strains circulating globally[37]. Others have focused on more conserved minor outer membrane proteins for the development of an universal serogroup B vaccine[38].

Immunity

The mammalian immune system can be divided into the innate immune system and the adaptive immune system. The innate immune system consists of mechanisms and cells that provide immediate protection against infection, in a non-specific manner. It is an evolutionary very old system present in all forms of plant and animal life. In addition, an adaptive immune system evolved in vertebrates, which uses randomly generated, clonally expressed, highly specific receptors (T- and B-cell receptors) of seemingly limitless specificity[39, 40]. Adaptive immunity develops relatively slowly, but in contrast to innate immunity it provides immunological memory[41]. This enables the immune system to remember a pathogen after a first encounter and respond much more effectively after a second infection with the same pathogen. Moreover, immunological memory forms the basis of vaccination.

Toll-like receptors

For several decades the innate immune system was ignored by most immunologists. It was thought to consist of a few general and unspecific mechanisms to prevent infection. However, during the last ten years a revolution in immunology took place after the discovery that immune cells express receptors that recognize conserved structures found on broad classes of microbes. These receptors are often referred to as pattern recognition receptors (PRRs). To date, several classes of PRRs have been identified, including Toll-like receptors (TLRs), C-type lectin like receptors, RIG-I like receptors, and Nod-like receptors. The TLR family was the first class to be discovered and is also the best characterized. In 1985, the first Toll gene was found by Christiane Nüsslein-Volhard in the fruit fly *Drosophila melanogaster*[42]. The fly-larva with a mutation in the Toll gene looked peculiar, because the ventral portion of the body was underdeveloped. The comment of Christiane after she saw the larva was “Das war ja toll!”, which is German for ‘That was crazy!” and she decided to name the affected gene Toll. Later in 1996 it was found that besides its role in development, Toll is also important in host defence, as flies with a mutation in this gene were very susceptible to fungal infection[43]. Soon after that, Ruslan Medzhitov and Charles Janeway reported that a mammalian homologue of Toll, a Toll-like receptor, was also involved in activation of the immune system[44]. Finally, Bruce Beutler and colleagues proved that TLR4 is the long-sought receptor for LPS[45].

TLRs can be found in vertebrates and invertebrates, which suggests that TLRs are very ancient and play a pivotal role in host defence. Thirteen TLRs (TLR1-TLR13) have been identified in humans and mice together. However, TLR10 is not expressed in mice and TLR11, TLR12, and TLR13 are not found in humans[46-48]. The extracellular

domains of TLRs consist of leucine-rich repeats (LRRs) and bind to the microbe derived structures[49]. Binding of the ligand causes dimerization of the extracellular domains. Most TLRs form homodimers, but TLR2 forms heterodimers with TLR1 or TLR6. At present numerous TLR ligands have been identified and each TLR recognizes distinct microbial structures (Fig. 4).

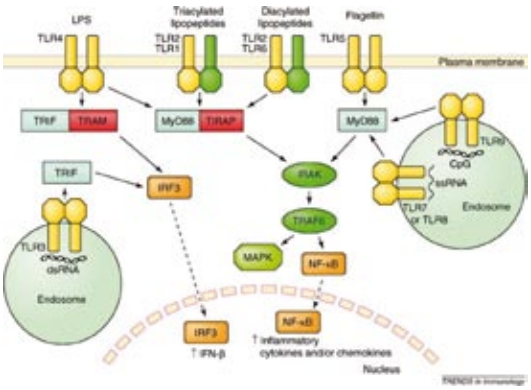

Figure 4. Overview of TLR signalling. All TLRs signal through MyD88, except TLR3. TIRAP (also known as MAL) is a bridging adaptor between MyD88 and TLR4 and TLR2. TLR3 signals through TRIF. The only other TLR that utilizes TRIF is TLR4. The adaptor protein TRAM is only required for TLR4 signalling and forms a bridge between TRIF and TLR4. Dashed arrows indicate translocation into the nucleus. LPS, lipopolysaccharide; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-κB; IRF3, interferon regulatory factor-3[53].

Some TLRs are expressed on the cell surface and generally bind to ligands derived from extracellular microbes. For instance, TLR2/TLR1 recognizes triacylated lipopeptides, TLR2/TLR6 detects diacylated lipopeptides, TLR4 is activated by LPS, and TLR5 senses flagellin. The other TLRs are localized in endolysosomal compartments and recognize nucleic acids mostly derived from intracellular microbes. For example, double-stranded and single stranded RNA produced by certain viruses are recognized by TLR3 and TLR7/8, respectively. Finally, TLR9 senses unmethylated CpG DNA of bacteria and viruses[49]. Signalling is mediated by the cytoplasmic Toll-IL-1 receptor (TIR) domain, which is also found in members of the IL-1 receptor family. After ligand binding, TLR dimerization is believed to trigger the recruitment of specific adaptor proteins to the intracellular TIR domains, which initiates signalling[50]. Four adaptor proteins are utilized by TLRs: MyD88, MAL, TRIF, and TRAM[51]. All TLRs signal through MyD88, except TLR3, which only activates TRIF. In addition, the TRIF pathway is activated by TLR4. MAL is required for signalling by TLR2 and TLR4, serving as a bridge to recruit MyD88.

TRAM is only involved in the TLR4 pathway. It functions as a bridging adaptor for TRIF recruitment. Activation of the MyD88 pathway eventually leads to translocation of specific transcription factors, such as NF-κB, to the nucleus, where they mediate transcription of a large number of genes involved in the immune response[49]. These include pro-inflammatory cytokines such as IL-6, TNF-α, and IL-1β. After stimulation of the TRIF pathway, the transcription factor IRF3 induces production of type I interferons (IFNs). The intracellular TLRs (TLR7-9) also activate type I IFN, but via the MyD88 pathway. Interestingly, recent data suggest that TLR4 first activates MyD88 signalling at the plasma membrane and after internalization activates TRIF signalling from early endosomes[52]. Thus it seems that type I IFN is always induced by a TLR that is located intracellularly.

Dendritic cells

The primary function of TLRs is to recruit and activate innate immune cells to clear the infection immediately. However, TLRs also play an important role in the initiation of adaptive immunity[53, 54]. Dendritic cells (DCs) are considered key players in this process. The main function of DCs is to process antigen material and present it to T cells[55]. Normally DCs in the periphery are sampling the environment looking for threats such as pathogens. In this stage, DCs are in a so called immature state, which means that they are not very efficient in stimulating T-cell immunity. However, when these cells are stimulated by microbial products like TLR ligands they undergo numerous changes referred to as 'maturation' that enables DCs to activate T cells. After TLR activation, DCs will shortly upregulate their endocytic capacity to take up pathogen antigens[56]. Next, these proteins are processed into small peptides and presented on the cell surface by MHC molecules. At the same time activated DCs start to secrete cytokines and chemokines and enhance surface expression of co-stimulatory molecules like CD40, CD80, and CD86. Moreover, DCs upregulate the chemotactic receptor CCR7 to migrate to secondary lymphoid organs[57]. There mature DCs can present pathogen derived epitopes to naïve T cells, which then differentiate into antigen-specific effector cells. Three signals are required to activate naïve T cells, which can all be provided by DCs[55]. Signal 1 is delivered through the binding of the T cell receptor (TCR) to the appropriate peptide-MHC complex. Signal 2 is provided by the attachment of proteins expressed on T cells to the co-stimulatory molecules present on the cell surface of DCs. For example, CD28 on T cells engages CD80 and/or CD86. Signal 3 refers to signals presented by DCs to T cells that shape its differentiation into an effector cell. Which TLR is activated determines what types of cytokines and other factors are produced by the DCs, which in turn dictates

whether the $CD4^+$ T cells differentiate into T helper 1 (Th1), Th2, Th17, or regulatory T cells (Tregs)[58, 59]. In addition, under the influence of certain TLRs DCs can cross-present antigens on MHC I to activate $CD8^+$ T cells. Because TLR ligands can both initiate and direct adaptive immunity, they have great potential as adjuvants.

B cells

B cells are besides T cells an important component of the adaptive immune system. Their principal role is production of antibodies. An antibody consists of two identical light and heavy chains. The genes that encode these domains are found in the variable (V) region and constant (C) region. The heavy-chain V region can be further divided in the V, D, and J segments, which can recombine (VDJ recombination) to form the unique variable domain of the antibody involved in antigen binding[60]. This process creates a large variety of antibodies, each with different specificities. When a B cell binds to antigen with its B cell receptor (BCR), the B cell will proliferate and release antibodies, which are soluble forms of the BCR. In addition, B cells can switch the constant region of the antibody heavy chain. This process is called class switch recombination[60]. During class switching the constant region of the antibody changes, but the variable region remains the same. Thus antigen specificity is not altered. Naïve mature B cells only produce IgM and IgD. After antigen binding, these B cells will proliferate and secrete antibodies of these classes. However, when these B cells also receive help from antigen-specific T cells through CD40-CD40L interaction and certain cytokines, they undergo class switching to produce IgG, IgA, or IgE. Each antibody isotype has different effector functions. Therefore, class switching allows the immune system to fine-tune the antibody response towards a suitable type of reaction for a specific pathogen.

Interestingly, B cells also express certain TLRs[61]. Moreover, it has been demonstrated that direct activation of B cells by TLR ligands is required for the generation of T cell dependent antibody responses[62]. Another study suggests that polyclonal activation of human memory B cells via TLRs is essential for the maintenance of serological memory[63]. It has been proposed that TLRs provide a third signal together with antigen binding to BCR and T cell help to achieve optimal activation of B cells and class switch recombination[63-65]. However, others have reported that activation of TLRs on B cells is not required for the generation of humoral immunity[66, 67]. Therefore, the importance of direct B cell activation by TLR ligands for their adjuvant activity warrants further investigation. In any case it illustrates the important notion that DCs are most likely not the only cell type with an important role in TLR-mediated activation of the adaptive immune system.

Adjuvants

Most of the classical vaccines are live attenuated strains or contain elements of killed microbes[53]. These vaccines are generally very effective and induce immunity which can last for several decades. Their success presumably relates to the intrinsic adjuvant activity of microbial structures that can activate several PRRs such as TLRs. However, now that several new subunit vaccines have been developed that consist of highly pure isolated antigens, it has become clear that such vaccines require adjuvants for generating an effective immune response. Most adjuvants have been empirically identified in experimental models and for many the working mechanisms are still poorly defined. Different classes of adjuvants exist including microbial products, mineral salts, liposomes, microparticles, saponins, and emulsions[68]. Even though many of these compounds have been shown to have adjuvant activity in animal models, they are not suitable for human use because of their toxicity. In fact, aluminium salts (alum) are still the only adjuvants approved for human use in the USA. In addition, the oil-in-water emulsions MF59 and AS03, and the TLR4-agonist monophosphoryl lipid A formulated in alum (AS04) are licensed in Europe[69].

Although alum has been used as an adjuvant for over 70 years, the mechanism of action remained a mystery. However, recent discoveries have shed new light on this puzzle. Several studies independently reported that alum activates the cytoplasmic NOD-like receptor NALP3, which together with ASC and caspase 1 forms a complex called the inflammasome[68]. Eventually NALP3 activation leads to maturation of pro-inflammatory cytokines such as IL-1β, IL-18, and IL-33. Whether NALP3 mediates the adjuvant effect of alum *in vivo* remains controversial. At this point three studies have demonstrated that NALP3 is required by showing that the adjuvant effect of alum is abrogated in NALP3-deficient mice[70-72]. On the other hand, two studies showed that the effect of alum was not impaired in these mice[73, 74]. These differences probably depend on the antigen, the administration route, or the readout for measuring the immune response. Four other particulate adjuvants (chitosan, Quil-A, PLG, and polystyrene microparticles) have been shown to activate NALP3[72, 75]. Furthermore, NALP3 was shown to be required for the enhancement by PLG of cellular immunity but not for the enhancement of humoral immunity[75]. In conclusion, several particulate adjuvants activate NALP3, which contributes to their adjuvant activity. However, they likely enhance immune responses through other mechanisms as well.

Lipopolysaccharide

LPS recognition

That bacteria contain structures that can induce toxicity is already known since the 19th century[76]. Richard Pfeiffer (1858-1945) observed that even heat killed cholera bacteria could kill guinea pigs. His experiments led him to formulate the concept that cholera bacteria had a heat-stable substance that was associated with the insoluble part of the bacterial cell. He named this substance endotoxin. Moreover, Pfeiffer proposed that nearly all groups of bacteria would contain endotoxin. Much later LPS was identified as the structural component of Gram-negative bacteria that had endotoxic activity. More specifically, the lipid A part of LPS was found to be the structure that mediated these effects, as fully synthetic lipid A had similar endotoxic activity[76]. These results suggested that there must be a mammalian receptor for lipid A to mediate its biological activity. This receptor remained elusive for many years until it was demonstrated that two mouse strains (C3H/HeJ and C57BL/10ScCr) already known for their resistance to endotoxin had a missense mutation in the TLR4 gene or complete deletion of the gene, respectively[45].

Now we know that several other proteins are involved in LPS recognition as well. Lipopolysaccharide binding protein (LBP) and CD14 facilitate the transfer of LPS to the co-receptor MD-2[77]. Then the MD-2/LPS complex binds to TLR4. Recently, the crystal structure of this complex has been resolved, showing how the lipid A moiety fits in a hydrophobic cavity formed by both MD-2 and the TLR4 ectodomain[78]. LPS binding induces the formation of a receptor multimer composed of two copies of the TLR4-MD-2-LPS complex arranged symmetrically. Formation of this complex allows recruitment of adaptor proteins to the intracellular domains. This triggers a signaling cascade and subsequent activation of the innate immune system[79]. Among the Toll-like receptors, TLR4 is unique in that it activates both the MyD88 pathway and TRIF pathway, which eventually leads to induction of pro-inflammatory cytokines and type I interferon, respectively.

Relation between LPS structure and its activity

LPS is a major component of the outer-membrane of virtually all Gram-negative bacteria[80]. LPS consists of three distinct domains: lipid A, the core oligosaccharide, and the O-antigen. Lipid A is a phosphorylated glucosamine disaccharide containing several fatty acids that anchor the molecule into the outer membrane. It has an important function in outer membrane stability. Therefore, the structure of lipid A is relatively conserved compared to the other LPS domains. The core oligosaccharide consists of sugars and is attached to the lipid A part. Finally, the O-antigen is a repetitive glycan polymer that is

attached to the core. This is the most variable part of the LPS molecule. However, some bacteria, including *N. meningitidis*, do not contain an O-antigen.

The structure of lipid A determines how well it can form a complex with TLR4 and MD-2. As a consequence the structure of lipid A dictates the biological activity of the LPS molecule. The most important determinant of biological activity is the number of acyl chains that lipid A contains[81]. The number of acyl chains of different bacterial species can vary between 4 and 7. Generally, lipid A with 6 acyl chains has the highest biological activity, lipid A with 7- or 5 acyl chains have intermediate biological activity, and lipid A with 4 acyl chains has low biological activity. In addition, other variations in lipid A structure such as the exact positions of the acyl chains, the length of the acyl chains, and degree of lipid A phosphorylation all have influence on the biological activity[82]. Moreover, there are species-specific differences in recognition of lipid A by TLR4-MD-2[83-86]. In general, lipid A with 4 or 5 acyl chains poorly activates human TLR4-MD-2, but is still relatively well recognized by murine TLR4-MD-2. For example, the intermediate in LPS biosynthesis lipid IVa is an antagonist in the human system, but slightly activates murine or equine TLR4-MD-2[83, 87].

Since TLRs play a pivotal role in host defense one can imagine that pathogens have evolved strategies to subvert TLR recognition, for example by altering the structures which are the targets of these receptors. Although TLR ligands are often portrayed as being conserved, the variation in these molecules between different microbes can indeed be tremendous[88]. This is also the case for lipid A[89]. This molecule varies not only among different bacterial species, but also among different strains or even within a single clone. Probably the best example is the plague bacillus *Yersinia pestis*. In the flea vector at a temperature of 25ºC, this bacterium produces highly active lipid A with 6 acyl chains. However, in mammals at 37ºC, *Y. pestis* produces inactive lipid A with only 4 acyl chains. Interestingly, a mutant that also contains lipid A with 6 acyl chains at 37 ºC was no longer virulent in mice, but showed normal virulence in TLR4-deficient mice[90]. These data strongly suggest that *Y. pestis* alters its lipid A structure to evade TLR4 recognition in mammals. Other species such as *Salmonella* and *Pseudomonas* can also modulate their lipid A structure in response to the host environment [84, 91-95]. Moreover, other human pathogens including *Helicobacter pylori*, *Legionella pneumophila*, and *Francisella spp*. have lipid A moieties that are poorly recognized by human TLR4, which likely contributes to their ability to cause disease in humans[82].

Neisseria meningitidis LPS

In contrast to many enteric bacteria, LPS of *N. meningitidis* lacks repeating O-chains and possesses variable oligosaccharide chains (Fig. 5)[20]. Therefore, some refer to meningococcal LPS as lipo-oligosaccharide. The conserved domain of the oligosaccharide chain is composed of heptose (Hep) and two molecules of unphosphorylated 3-deoxy-D-manno-2-octulosonic acid (KDO) attached to lipid A. Linked to Hep_2-KDO_2-lipid A are variable α and β chain saccharides, which determine the immunotype. *N. meningitidis* LPS can be divided into 12 immunotypes (L1–12) on the basis of specific antibody reactions. Meningococcal lipid A contains 6 acyl chains, the optimal number for TLR4 activation. Indeed, *N. meningiditis* LPS is one the most potent LPS molecules among Gram-negative bacteria. Any alteration of the lipid A structure leads to a decrease in biological activity[96]. The high endotoxicity of this LPS is illustrated by the observation that after meningococcal infection, levels of circulating LPS correlate strongly with morbidity and mortality[22, 23].

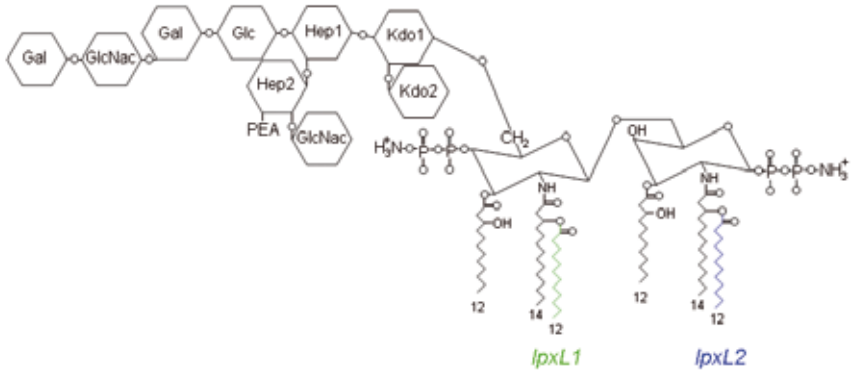

Figure 5. Structure of *Neisseria meningitidis* lipopolysaccharide. The conserved domain of the oligosaccharide chain is composed of heptose (Hep) and two molecules of unphosphorylated 3-deoxy-D-manno-2-octulosonic acid (KDO) attached to lipid A. Linked to Hep_2-KDO_2-lipid A are variable α and β chain saccharides, which determine the immunotype. Meningococcal lipid A contains 6 acyl chains of 12 or 14 carbons in length. The secondary acyl chains are attached to lipid A by LpxL1 and LpxL2.

Modification of LPS structure in vaccine development

An OMV vaccine against *N. meningitidis* serogroup B is currently in development at the Netherlands Vaccine Institute. All OMV vaccines used to date still contain wildtype LPS. On one hand, this is beneficial, as LPS is known for many years for its potent adjuvant activity[76]. On the other hand, the risk of side effects after administration of a molecule with endotoxic properties, makes inclusion of wildtype LPS a less desirable approach. Therefore, our laboratory has made *N. meningitidis* mutants, in which genes involved in the biosynthesis of LPS were inactivated, to obtain modified strains more suitable for vaccine development. Surprisingly, it was possible to inactivate *lpxA*, the gene required for the first step in LPS biosynthesis[97]. Consequently, the *lpxA* mutant has no LPS at all, which at the time was considered an essential component of all Gram-negative bacteria. A strain completely lacking LPS might be an interesting vaccine candidate due to the reduced toxicity. Unfortunately, a vaccine derived from this strain did not induce sufficient immunity in mice, demonstrating that an adjuvant like LPS is required[98]. In addition, we have previously inactivated the *lpxL1* and *lpxL2* genes of *N. meningitidis*, which are needed for addition of the secondary acyl chains to lipid A (Fig. 5)[99]. Therefore,these mutants produce LPS with underacylated lipid A. The *lpxL1* and *lpxL2* mutants showed great reduction in induction of pro-inflammatory cytokines *in vitro*, which is a good measurement for biological activity. Interestingly, *lpxL1* LPS was shown to have comparable adjuvant activity to wildtype LPS in mice[98-100]. Thus it seems that the two properties of an adjuvant 'toxicity' and 'adjuvant activity' can be at least partially separated. Moreover, these data suggest that *lpxL1* LPS is a suitable adjuvant candidate.

Aims and outline of this thesis

The discovery of PRRs in the last decade was the beginning of the comprehension of the working mechanisms of many adjuvants. However, more research is needed to elucidate the pathways that are activated by these adjuvants and how these pathways shape adaptive immunity. This knowledge is needed to pave the way for tailor-made adjuvants that can modulate the immune response in the desired direction. Moreover, it is becoming clear that pathogens adjust the structures that are recognized by PRRs to evade the immune response or modify it to their benefit. The aim of the study described in this thesis was to investigate which TLR ligands are suitable adjuvants for a meningococcal vaccine and to understand their mode of action. In addition, we explored the impact of modulation of meningococcal structures recognized by TLR ligands on the host response after infection with *N. meningitidis*.

In chapter 2, we compared a panel of TLR ligands (TLR1-9) in their ability to improve the immune response towards an LPS-deficient meningococcal outer membrane vaccine in mice. We found that agonists of TLR3, TLR4, TLR7, and TLR9 are suitable adjuvant candidates for a vaccine against *N. meningitidis*.

OMVs derived from wildtype *N. meningitidis* have been used successfully in humans to prevent meningococcal disease. Similarly, whole-cell vaccines against *B. pertussis* have been utilized to diminish cases of whooping cough in the human population. These vaccines contain several TLR ligands, but their contribution to the immunogenicity of the vaccines is not well documented. In chapter 3, we explored the role of genes involved in the recognition of LPS and lipoproteins in the immunogenicity of *N. meningitidis* OMVs and a whole-cell pertussis vaccine in mice. We demonstrated that for both vaccines, LPS signalling is required for optimal adaptive immune responses after immunization. Surprisingly, mice deficient in TLR2 showed normal or even enhanced adaptive immune responses after vaccination with both vaccines.

In chapter 4, we compared a large collection of meningococcal disease isolates for their ability to induce a cytokine response *in vitro*. We found that around 9% of these strains were severely impaired in cytokine induction. Strikingly, all these isolates, except one strain, had lipid A with only 5 acyl chains due to inactivating mutations in the *lpxL1* gene. Moreover, adult meningitis patients infected with *lpxL1* mutants had milder symptoms than patients infected with a wildtype strain, which illustrates the important contribution of LPS to disease severity.

The large majority of people who contract *N. meningitidis* only transiently carry the bacterium in the respiratory tract and never develop disease. Besides meningitis, the bacterium can lead to many other clinical manifestations. Moreover, children and

adolescents have higher risks for becoming infected than adults. Therefore, in chapter 5, we investigated the frequency and impact of *lpxL1* mutations in strains isolated from carriers, patients with all forms of meningococcal disease, and from all different age categories. We show that infections with *lpxL1* mutants are associated with age. Moreover, infections with *lpxL1* mutants gave milder symptoms in the older patients compared to wildtype strain infections, but not in patients younger than 5 years. Unexpectedly, *lpxL1* mutants isolated from carriers were almost exclusively of a single clonal complex, cc23.

The high frequency of *lpxL1* mutants among meningococcal disease isolates suggests there must be an advantage for the meningococcus to inactivate *lpxL1* under certain conditions. In chapter 6, we examined the influence of meningococcal lipid A structure on virulence in a mouse model of meningococcal sepsis. We demonstate that the *lpxL2* mutant is more virulent in mice than the wildtype strain. In contrast, the *lpxL1* mutant was completely avirulent.

Finally, in chapter 7, our findings are summarized and discussed.

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Chapter 2

Agonists of Toll-like receptor 3, 4, 7, and 9 are candidates for use as adjuvants in an outer membrane vaccine against *Neisseria meningitidis*

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Abstract

The bacterium *Neisseria meningitidis* is the causative agent of meningitis and sepsis. A generally effective vaccine against *N. meningitidis* serogroup B is not yet available, but outer membrane vesicle vaccines are in development. These vaccines contain LPS. The inclusion of *N. meningitidis* wild-type LPS in a vaccine is controversial because of its high toxicity. Therefore, the adjuvant activity of a panel of different TLR agonists in combination with LPS-deficient meningococcal outer membrane complexes was compared after immunization of mice. The results demonstrate that TLR3, TLR4, TLR7, and TLR9 agonists enhance immune responses against LPS-deficient outer membrane complexes. Their adjuvant activity was characterized by higher levels of antigen-specific IgG, IgG2a, and IgG2b, a higher IgG2a/IgG1 ratio, lower total IgE levels, and most importantly, higher serum bactericidal antibody titers compared to LPS-deficient outer membrane complexes alone.

Introduction

The Gram-negative bacterium *Neisseria meningitidis* is an exclusively human pathogen that may cause meningitis and sepsis. Capsular polysaccharide vaccines against serogroups A,C,Y, and W-135 are available. However, a vaccine based on the capsular polysaccharide of serogroup B is not feasible, because it is not immunogenic. Therefore, current strategies to develop a vaccine against serogroup B are mainly focused on the use of meningococcal outer membrane proteins. One such strategy is the development of a outer membrane vesicle (OMV) vaccine based on the PorA antigen, which is an important target for bactericidal antibodies[1, 2]. These vaccines contain lipopolysaccharide (LPS), which is a powerful adjuvant, but at the same time is toxic even at low concentrations[3]. Because of possible serious side effects, the use of LPS in vaccines is controversial. Therefore, the genetics of the LPS biosynthesis in *N. meningitidis* was investigated by our group in order to obtain mutants with less toxic LPS for use in vaccine development. Surprisingly, it was possible to inactivate the *lpxA* gene, which is involved in the first step of the LPS biosynthesis, creating a completely LPS-deficient mutant[4]. However, when outer membrane complexes (OMCs) of this mutant were used to immunize mice, the resulting antibody response was too low to be protective[5], demonstrating that an adjuvant like LPS is needed to boost the immune response.

It is now well established that the receptor for LPS is Toll-like receptor 4 (TLR4), which recognizes the lipid component, termed Lipid A. Lipid A is also responsible for the toxic properties of LPS[3]. In particular, the specific acylation pattern of Lipid A determines its biological activity. Thus, one could modify the acylation pattern of *N. meningitidis* Lipid A to obtain less toxic LPS mutants. To this end, the *lpxL1* gene was inactivated in our lab, creating penta-acylated LPS. Importantly, *lpxL1* LPS was much less toxic in *in vitro* studies, but retained adjuvant activity comparable to wildtype *N. meningitidis* LPS in immunization experiments[6, 7].

After the finding of TLR4 as the LPS receptor many more pattern recognition receptors (PPRs) and their corresponding pathogen-associated molecular patterns (PAMPs) have been discovered in recent years[8]. The TLR family is the best studied family of PPRs and recognize a broad spectrum of PAMPs from different classes of microbes. TLRs detect lipoproteins (TLR2), double-stranded RNA (TLR3), LPS (TLR4), flagellin (TLR5), single-stranded viral RNA (TLR7/8), and unmethylated CpG DNA of bacteria and viruses (TLR9). After recognition of their ligand, all TLRs (except TLR3) signal through the adaptor protein MyD88, leading to the activation of NF-kB and consequently induction of many genes involved in immunity[9]. Besides the MyD88-dependent pathway,

TLR3 and TLR4-signalling also occurs through a pathway independent of MyD88, namely via the adaptor protein TRIF. Activation of TRIF ultimately leads to the induction of type I interferon. TLR7/8 and TLR9 are also capable of activating type I interferon, but in a MyD88-dependent manner.

TLRs are expressed on many cell types, but dendritic cells (DCs) are the most widely used to study TLR function. This is because DCs are considered to be the most important professional antigen-presenting cells in infection and vaccination. Normally DCs are in an immature state, but after TLR activation they undergo a process called maturation[10,11]. This process involves phagocytosis, increased surface expression of costimulatory molecules, redistribution of MHC molecules from intracellular compartments to the cell surface, a switch in chemokine receptor expression, and secretion of cytokines and chemokines[12]. After antigen processing and expressing epitopes in a MHC restricted manner, mature DCs can stimulate naïve T-cells to differentiate into effector cells. Depending on the TLR ligand, the immune response may be skewed toward cytotoxic T cell (CTL) responses or particular classes of (Th) T helper responses[13]. Because TLR ligands can initiate adaptive immune responses and in addition can direct the immune response into a certain direction, they have great potential as adjuvants.

In this study the adjuvant activity of a panel of different TLR ligands in combination with LPS-deficient OMCs was compared in mice. We found that among the ligands tested, only those specific for TLR3, TLR4, TLR7, and TLR9 gave a better response to LPS-deficient OMCs. These combinations gave higher antigen-specific total IgG, IgG2a, and IgG2b levels, a higher IgG2a/IgG1 ratio, lower total IgE levels, and higher bactericidal titers compared to LPS-deficient OMCs alone. Together, these data indicate that the adjuvants gave a skewing towards a Th1 response, which is the desired response for a vaccine against *N. meningitidis*. Thus more TLR ligands than wild-type LPS alone can be considered as an adjuvant for an effective vaccine against *N. meningitidis* serogroup B.

Materials and Methods

Animals

Female SPF BALB/c mice were purchased from Harlan Europe (Horst, The Netherlands) and were housed under SPF conditions. Mice were acclimatized for approximately 1 week and were 8-10 weeks old at the start of the experiment. Mice received standard laboratory chow (RMH-GS, Hope Farms, Woerden, The Netherlands) and water ad libitum. Animal experiments were approved by the Institute's Animal Ethics Committee.

Preparation of OMCs

N. meningitidis H44/76, P1.7,16 and LPS-deficient H44/76, P1.7,16 *LpxA* mutant (pLAK33) were grown on GC medium base (Difco laboratories) supplemented with IsoVitaleX (Becton Dickinson) overnight at 37ºC, 5% CO_2 in a humid atmosphere. Next, the bacteria were transferred from plate to Trypticase soy broth liquid medium and grown at 37ºC, 5% CO_2 to late log phase. Then the bacteria were heat inactivated at 56ºC for 1 h. These were used for isolation of OMCs by sarcosyl extraction as described previously[14]. The quantity of protein was determined with the bicinchoninic acid protein assay reagent (Pierce), with bovine serum albumin as standard.

Adjuvants

N. meningitidis L3 LPS, *lpxL1* mutant LPS, *B. pertussis* strain Tohama LPS were isolated with the hot-phenol extraction method as described previously[15]. FSL-1, Pam_3CSK4, Poly(I:C), Monophosphoryl Lipid A (MPL), *B. subtilis* Flagellin, Imiquimod, Loxoribine and Muramyl dipeptide (MDP) were purchased from Invivogen. CpG DNA ODN1826 (5'-tccatgacgttcctgacgtt-3') with phosphorothioate bases was synthesized by Genosys.

Immunizations

Mice were immunized subcutaneously on day 0 and 28 with either 5 μg of H44/76 OMCs, 5 μg of pLAK33 OMCs, or 5 μg of pLAK33 OMCs together with the different adjuvants dissolved in 250 μl PBS. The adjuvants used and their concentrations in weight and molar units are listed in table 1. The Mice which received H44/76 OMCs, pLAK33 OMCs alone, or pLAK33 OMCs in combination with FSL-1, Pam_3CSK4, Poly(I:C), MPL, L3, or LpxL1 were bled on day 42. Mice which received pLAK33 OMCs in combination with Flagellin, *B. pertussis* LPS, Imiquimod, Loxoribine, CpG DNA, or MDP were bled on day 43. Sera were collected and stored at -20ºC.

Table 1

Adjuvant	Concentration per mouse	
	Weight	**Molar units**
FSL-1	10 μg 1 μg	6 nmol 0.6 nmol
Pam_3CSK4	50 μg 5 μg	33.12 nmol 3.31 nmol
Poly(I:C)	100 μg 10 μg	- -
MPL	20 μg 2 μg	13.11 nmol 1.31 nmol
L3	1 μg 0.1 μg	0.26 nmol 0.026 nmol
LpxL1	10 μg 1 μg	2.76 nmol 0.28 nmol
Flagellin	10 μg 1 μg	0.31 nmol 0.031 nmol
LPS *B. pertussis*	10 μg 1 μg	2.47 nmol 0.25 nmol
Imiquimod	100 μg 10 μg	417 nmol 41.7 nmol
Loxoribine	85 μg 8.5 μg	250 nmol 25 nmol
CpG DNA	127 μg 12.7 μg	20 nmol 2 nmol
MDP	100 μg 10 μg	203 nmol 20.3 nmol

Antigen-specific antibodies

Antigen-specific IgG, IgG1, IgG2a, and IgG2b antibodies were determined with enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well microtitre plates (Nunc, Immulon 2) were coated overnight at room temperate with 100 μl/well of H44/76 P1.7,16 OMVs (4 μg/ml PorA) in PBS. Sera were diluted 1:50 and from this three-fold serial dilutions were made in PBS containing 0.1% Tween-80. After extensive washing the plates were incubated with the serial dilutions of the sera (100 μl/well) at 37ºC for 1 h. Again plates were washed and either anti-mouse IgG-HRP, anti-mouse IgG1-HRP, anti-mouse IgG2a-HRP, or anti-mouse IgG2b-HRP (Southern Biotechnology Associates Inc.) diluted 1:5000 in PBS, 0.1% Tween-80, 1% skimmed milk (Protifar, Nutricia, The Netherlands) was added 100 μl/well. Plates were incubated at 37ºC for 1 h. After washing, 100 μl/well peroxidase substrate (3,3',5,5'-tetramethylbenzidine with 0.01% H_2O_2 in 0.11 M sodium acetate buffer pH 5.5) was added. After 20 min, the reaction was stopped by addition

of 100 μl/well of 2 M H_2SO_4. A four-parameter curve fit was made for optical densities values of the serial dilutions, and the antibody titer was calculated in reciprocal dilutions that gave an OD of 0.6. Results are expressed as $\log_{10}$ titers.

Total IgE

Total IgE levels in the sera were measured by ELISA. Flat-bottom 96-well microtitre plates were coated with 100 μl per well of 2 μg/ml rat anti-mouse IgE monoclonal antibody (BD Biosciences) in PBS and incubated overnight at 4ºC. Plates were blocked with 200 μl per well of PBS, 1% skimmed milk at room temperature for 1 h. Next, plates were incubated for 1 h at room temperature with sera of the immunized mice, serum of a naïve mouse, and purified mouse IgE clone C48-2 (BD Biosciences) as standard, all diluted in PBS, 1% skimmed milk, 0.05% Tween-20. Then plates were incubated with 100 μl per well of 2 μg/ml biotinylated anti-mouse IgE (BD Biosciences) in PBS for 1 h at room temperature. Next plates were incubated at room temperature for 30 min with 100 μl per well Streptavidin-Horseradish Peroxidase (Sanquin) diluted 1:5000 in PBS, 1% skimmed milk, 0.05% Tween-20. Peroxidase substrate was used as substrate and the reaction was stopped with 2 M H_2SO_4. Absorbance was determined at 450 nm.

Serum bactericidal assay

Sera of immunized mice were diluted 1:5 in Gey's Balanced Salt Solution (GBSS) + 0.5% BSA and heat inactivated for 30 min at 56ºC. Next, twofold serial dilutions of the sera together with *N. meningitidis* H46/76 P1.7, 16 (10^4 cfu/ml) were incubated in 96-well plates at room temperature for 15 min. After addition of baby rabbit complement (20% of total volume) plates were incubated at 37ºC for 1 h. Bacteria were plated on GC medium base (Difco laboratories) supplemented with IsoVitaleX (Becton Dickinson) and grown overnight at 37ºC, 5% CO_2 in a humid atmosphere. The serum bactericidal titer was determined as the reciprocal serum dilution that gave more than 90% killing of the number of bacteria used. Results are expressed as $\log_{10}$ titers.

Statistics

Before statistical analysis, data from each experiment were $\log_{10}$ converted, which normalized their distribution. In every graph the mean of $\log_{10}$ titers of 8 mice per group including the standard errors of the mean (SEM) are presented, except for IgE where unconverted data are shown. One-way analysis of variance (ANOVA) was performed followed by the post-hoc Dunnett t-test to analyze difference in means between pLAK33 and the other groups (SPSS, Chicago, IL) and were considered significant at p-values of

<0.05. For each experiment the means of at least two groups differed significantly from each other ($p<0.0001$).

Results

Antigen-specific total IgG levels

The ligands used in this study were: FSL-1 (recognized by TLR2/TLR6 heterodimer); Pam_3CSK4 (recognized by TLR2/TLR1 heterodimer); Poly (I:C) (TLR3); different forms of LPS (TLR4); i.e. *N. meningitidis* wildtype L3, mutant penta-acylated *lpxL1* LPS, *Bordetella pertussis* LPS, and monophosphoryl lipid A (MPL); *B. subtilis* flagellin (TLR5); imiquimod and loxoribine (TLR7); CpG DNA (TLR9); and muramyl dipeptide (MDP), which is actually not recognized by a TLR but by NOD2. Each of these adjuvants was used in two concentrations with a factor 10 difference to look at dose-dependency and mixed with 5 μg of LPS-deficient OMCs in PBS. Then BALB/c mice were immunized sc with these formulations, LPS-deficient OMCs alone (pLAK33), or wildtype *N. meningitidis* OMCs (H44/76) as a positive control. Mice were immunized on day 0 and day 28. Forty-two days after immunization, the sera were analyzed by ELISA on IgG antibodies specific for *N. meningitidis* outer membrane proteins (Fig. 1). As expected, pLAK33 induced much lower IgG levels than H44/76, demonstrating the importance of the adjuvant activity of *N. meningitidis* L3 LPS. The addition of the following ligands to pLAK33 gave significant ($p<0.05$) higher IgG levels than pLAK33 alone: MPL (2 μg), L3 (1 μg), LpxL1 (10 μg and 1 μg), LPS *B. pertussis* (10 μg), and Imiquimod (100 μg). Poly(I:C) (100 μg) and CpG DNA (2 nmol) also seemed to enhance IgG levels, but not significantly. There was little difference in response to 10 μg or 1 μg of LpxL1. So apparently, the adjuvant activity of LpxL1 is in a plateau between these two concentrations. The lower concentrations of MPL and CpG DNA gave higher IgG levels than the higher concentrations. For the other ligands there was a dose-dependent effect on IgG levels. As demonstrated in previous studies [6, 7], LpxL1 had a comparable effect on the IgG levels as L3 LPS. However, their positive effect on IgG levels was much lower than that of L3 LPS in H44/76 OMCs, while the concentration of L3 LPS in H44/76 OMCs was lower. This suggests that membrane embedded LPS gives a better response than free LPS.

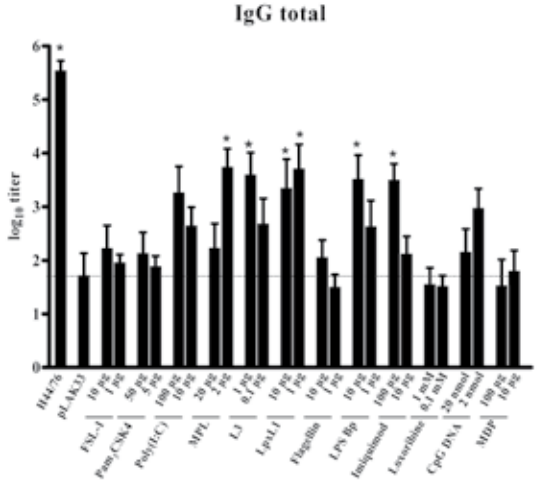

Figure 1. Antigen-specific total IgG levels. Mice were immunized with the following adjuvants: FSL-1 (TLR2/TLR6); Pam_3CSK4 (TLR2/TLR1); Poly (I:C) (TLR3); *N. meningitidis* wildtype L3, LpxL1 LPS, *B. pertussis* LPS, and MPL (TLR4); *B. subtilis* flagellin (TLR5); imiquimod and loxoribine (TLR7); CpG DNA (TLR9); and MDP (NOD2). Sera of immunized mice (8 per group) were analyzed by ELISA for antigen-specific IgG. Data are expressed as mean ± SEM of log_{10} titers. An asterisk indicates that IgG levels of a group were significantly different ($p<0.05$) from pLAK33 immunized mice. The dashed line indicates the level of IgG from pLAK33 immunized mice. Abbreviations: LPS Bp = LPS *B. pertussis*.

Antigen-specific IgG subclasses

Cytokines secreted by T helper cells differentially regulate the production of antibody classes by B-cells. In mice, Th2 cytokines such as IL-4 are involved in isotype switching from IgM and IgD to IgG1 and IgE, while the Th1 cytokines IL-2 and IFN-γ induce class switching to IgG2a and IgG2b[16, 17]. To assess whether the TLR ligands influence the balance between Th1- and Th2-dependent immunoglobulin (Ig) production, sera of the mice were analyzed on the presence of IgG1, IgG2a, and IgG2b antibody subclasses specific for *N. meningitidis* outer membrane proteins (Fig. 2). As for the IgG levels, H44/76 induced more IgG1 than pLAK33 (Fig. 2a). Most ligands did not alter IgG1 levels much. Only LpxL1 (1 μg) induced significant higher levels of IgG1 compared to pLAK33 alone, whereas CpG DNA (20 nmol) gave lower levels of IgG1 (Fig. 2a). The antigen-specific IgG2a and IgG2b levels overall showed the same pattern as total IgG (Fig. 2b,c). Again, Poly(I:C), the LPS variants, Imiquimod, and CpG DNA improved IgG2a and IgG2b levels compared to pLAK33 alone. Moreover, the pattern of dose-dependency was comparable. Thus, LpxL1 was in a plateau, the lower concentrations of MPL and CpG DNA induced more IgG2a and IgG2b than the higher concentrations,

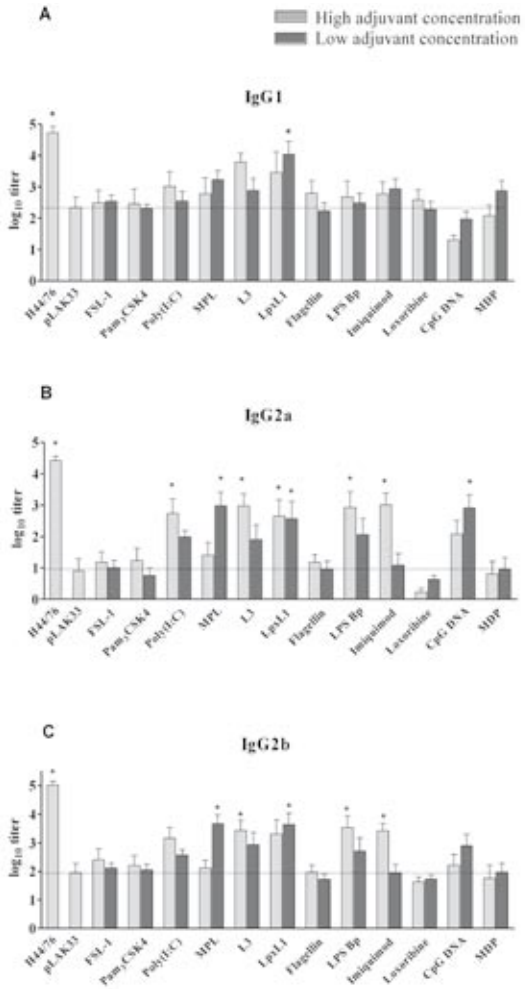

Figure 2. Antigen-specific antibody subclasses. Sera of immunized mice (8 per group) were analyzed by ELISA for the presence of antigen-specific (A) IgG1, (B) IgG2a, and (C) IgG2b. Data are expressed as mean ± SEM of $\log_{10}$ titers. An asterisk indicates that the Ig levels of a group were significantly different ($p<0.05$) from pLAK33 immunized mice. The dashed line indicates the level of Ig from pLAK33 immunized mice. Abbreviations: LPS Bp = LPS *B. pertussis*.

whereas the other ligands showed a dose-dependent effect. As these agonists improved IgG2a and IgG2b levels but not so much IgG1 levels, they gave a skewing towards Th1-dependent Ig production (Fig. 3). This suggests that Poly(I:C), the LPS variants, Imiquimod and CpG DNA promoted a Th1 response, which is in agreement with previous studies that have demonstrated that agonists of TLR3, TLR4, TLR7, and TLR9 can induce Th1-polarization[18-21].

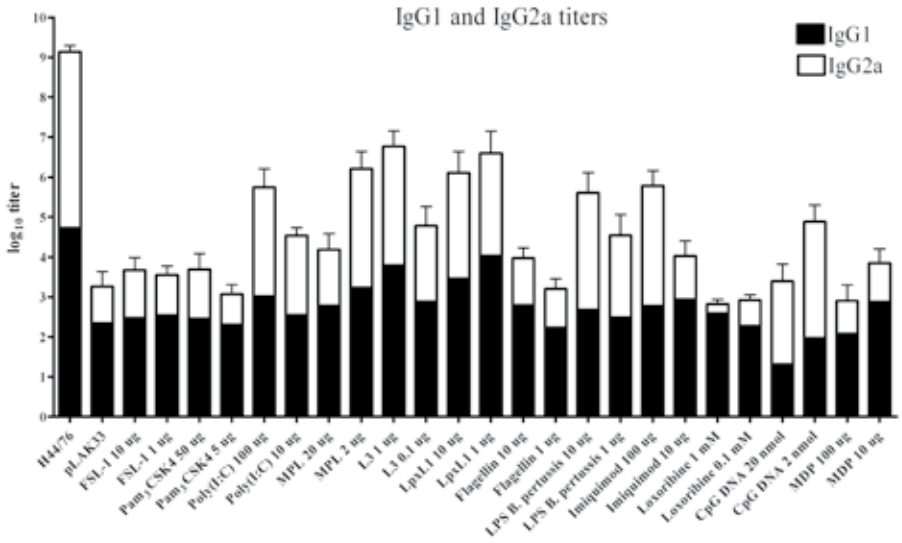

Figure 3. Comparative log titers of IgG1 and IgG2a. Sera of immunized mice (8 per group) were analyzed by ELISA for the presence of antigen-specific IgG1 and IgG2a. Data are expressed as means of $\log_{10}$ titers of IgG1 plus means of $\log_{10}$ titers of IgG2a.

Total IgE levels

To further establish Th2-dependent Ig production, the total levels of IgE antibodies were determined in the sera of the immunized mice (Fig. 4). Antigen-specific IgE antibodies were not detected (data not shown). Compared to a naïve mouse, the IgE levels in the sera of mice after immunization with H44/76 OMCs barely increased. However, after immunization with LPS-deficient OMCs the IgE levels were much higher than that of a naïve mouse. This suggests that in the absence of LPS the *N. meningitidis* vaccine skews the balance more towards a Th2 response. The combination of FSL-1 and pLAK33 induced even higher levels of total IgE levels in the sera, which indicates that FSL-1 might be a Th2 inducing ligand. In that respect it is interesting to note that other studies have also found that TLR2 ligands can induce Th2 responses[22-24]. In contrast, pLAK33 in combination with both doses of Poly(I:C), MPL, L3, LpxL1, CpG DNA or the highest dose of *B. pertussis* LPS strongly reduced the IgE levels in the sera compared to pLAK33 alone. The reduction in total IgE was also dose-dependent for most of these ligands. These data suggest that these ligands reduced pLAK33 induced Th2-dependent Ig production and together with the observation that most of these ligands also promoted higher IgG2a/IgG1 ratios, these data further support that these ligands induce Th1-polarization.

Exception on this correlation between higher IgG2a/IgG1 ratios and lower total IgE levels is Imiquimod, which induced a higher IgG2a/IgG1 ratio but not lower IgE levels.

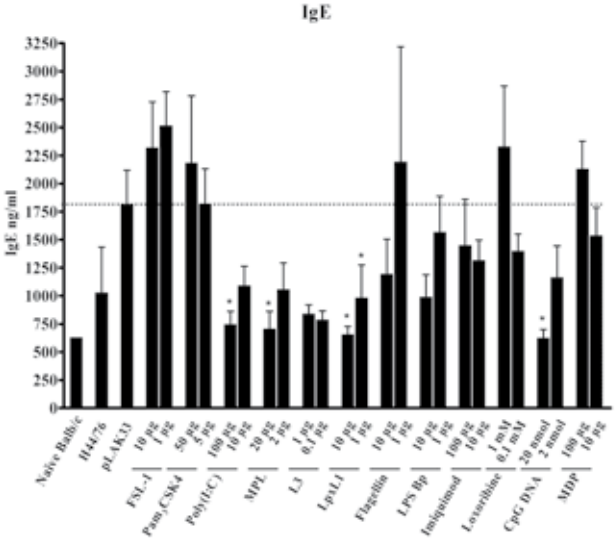

Figure 4. Total IgE levels. Amounts of IgE antibodies were determined in sera of immunized mice (8 per group, except CpG DNA 2 nmol, which were 7 mice) with ELISA. Data are expressed as mean ± SEM of ng/ml IgE. An asterisk indicates that the IgE levels of a group were significantly different ($p<0.05$) from pLAK33 immunized mice. The dashed line indicates the level of IgE from pLAK33 immunized mice. Abbreviations: LPS Bp = LPS *B. pertussis*.

Serum bactericidal antibodies

We have shown that TLR ligands elevated the production of certain antibody subclasses. However, that does not automatically imply that it contributed to a better protection against *N. meningitidis* serogroup B infection. Previously, it has been demonstrated that the presence of serum antibodies that mediate complement-dependent killing of *N. meningitidis* strongly correlate with protection against the bacterium[25]. Therefore, sera of the immunized mice were analyzed on the presence of bactericidal antibodies (Fig. 5). The sera of mice immunized with H44/76 showed the highest bactericidal activity. In contrast, LPS-deficient pLAK33 OMCs hardly induced bactericidal antibodies. However, the addition to pLAK33 of Poly(I:C), the LPS variants, Imiquimod, and CpG DNA clearly led to sera with enhanced bactericidal activity. Moreover, the pattern of dose-dependency was the same as for antigen-specific IgG and IgG2a. Again both concentrations of LpxL1 induced similar levels of bactericidal activity, the lower

concentrations of MPL and CpG DNA induced more bactericidal activity than the higher concentrations, and the other ligands had a dose-dependent effect.

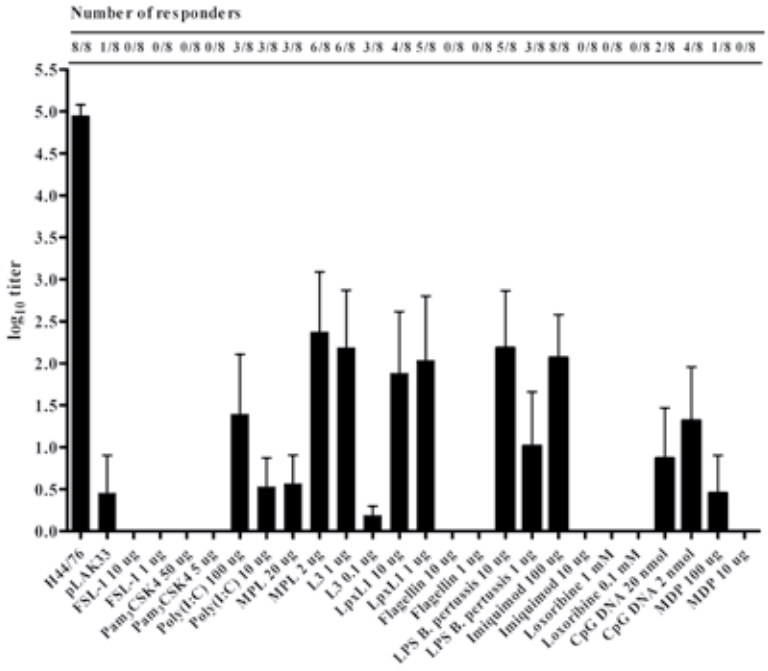

Figure 5. Serum bactericidal antibodies. Serial dilutions of sera of immunized mice (8 mice per group) were incubated with *N. meningitidis* and rabbit complement. The highest dilution that killed ≥ 90% of bacteria was determined. The number of responders is indicated in the graph. A responder was defined as a mouse whose serum killed ≥ 90% at at least 1:2 dilution. Data are expressed as mean ± SEM of $\log_{10}$ titers.

We also assessed the correlation coefficient between bactericidal activity and the distribution of different antibody types in individual mice (Table 2). For most ligands, the IgG2a and IgG2b levels correlated much more strongly than IgG1 with bactericidal activity. Indeed, IgG2a and IgG2b but not IgG1 isotypes are known for their ability to activate complement[26].

Table 2. Correlation between serum bactericidal activity and antibody isotypes

	IgG	IgG1	IgG2a	IgG2b	IgE
H44/76	0.69	-0.06	0.79	0.76	-0.48
pLAK33	0.74	0.49	0.76	0.77	-0.23
FSL-1 10 μg	-	-	-	-	-
FSL-1 1 μg	-	-	-	-	-
Pam_3CSK4 50 μg	-	-	-	-	-
Pam_3CSK4 5 μg	-	-	-	-	-
Poly(I:C) 100 μg	0.85	0.86	0.86	0.75	-0.41
Poly(I:C) 10 μg	0.84	0.72	0.81	0.74	0.07
MPL 20 μg	0.83	0.78	0.75	0.88	0.16
MPL 2 μg	0.82	-0.49	0.91	0.82	-0.26
L3 1 μg	0.89	0.61	0.92	0.82	-0.28
L3 0.1 μg	0.48	0.12	0.52	0.57	0.00
LpxL1 10 μg	0.70	0.40	0.81	0.78	0.60
LpxL1 1 μg	0.76	0.33	0.85	0.79	-0.46
Flagellin 10 μg	-	-	-	-	-
Flagellin 1 μg	-	-	-	-	-
LPS B. pertussis 10 μg	0.92	0.76	0.96	0.91	-0.58
LPS B. pertussis 1 μg	0.86	0.16	0.88	0.89	0.27
Imiquimod 100 μg	0.87	0.13	0.86	0.77	0.33
Imiquimod 10 μg	-	-	-	-	-
Loxoribine 1 mM	-	-	-	-	-
Loxoribine 0.1 mM	-	-	-	-	-
CpG DNA 20 nmol	0.88	0.01	0.90	0.79	0.36
CpG DNA 2 nmol	0.83	0.42	0.74	0.79	-0.63
MDP 100 μg	0.93	0.76	0.91	0.98	-0.52
MDP 10 μg	-	-	-	--	-

Data shown are correlation coefficients, which were determined by comparing the SBA titers of individual mice with their titers of IgG, IgG1, IgG2a, IgG2b, and IgE. A dash indicates that a correlation coefficient could not be calculated due to the absence of serum bactericidal activity. Correlation coefficients were calculated with the formula:

$$\text{Correl}(X,Y) = \frac{\Sigma(x - \tilde{x})\,(y - \tilde{y})}{\sqrt{(x - \tilde{x})^2\,\Sigma(y - \tilde{y})^2}}$$

Discussion

To test their adjuvant activity, the different TLR agonists were mixed with LPS-deficient OMCs prior to immunization. To what extent adjuvant and antigen associate in larger complexes was not determined. Only in the case of wild type H44/76 OMCs one can assume that the adjuvant (LPS) is co-incorporated into the antigen complex. The LPS content in OMCs is between five and ten percent, which is similar to the LPS content in OMVs[27]. In all our assays these wild-type OMCs showed superior responses compared to the other formulations. This difference is most striking when wild-type OMCs are compared with the combination of LPS-deficient OMCs and *N. meningitidis* wild-type LPS, because these two formulations are practically the same except the presentation form of the LPS. Apparently, an adjuvant is much more effective if it is somehow physically linked to the antigen. For CpG DNA it has indeed been demonstrated that its adjuvant effect was strongly enhanced when CpG DNA and OVA were emulsified in incomplete Freund's adjuvant (IFA), cross-linked by biotin-avidin bridges, or co-encapsulated in liposomes, which suggests that close proximity of CpG DNA to the antigen is beneficial[28]. Recent studies have provided a possible mechanistic explanation for these observations. Blander et al. have demonstrated that antigens phagocytosed by DCs depend on TLR ligands within the phagocytosed cargo for efficient presentation[29]. Similarly, Spörri et al. showed that direct TLR signaling on DCs resulted in activated DCs capable of inducing effector T-cells, whereas indirect activation by inflammatory mediators derived from TLR ligand stimulation on other cells did not result in DCs with these capabilities[30]. Thus, if a DC takes up antigen together with TLR ligands, an effective immune response against the antigen will be mounted, but not if the DC encounters antigen without TLR ligands in close proximity.

Each adjuvant was tested at two concentrations with a factor 10 difference. As expected, most adjuvants that enhanced antibody responses showed a dose-dependent effect. However, the lower concentrations of CpG DNA and MPL augmented antibody titers more than the higher concentrations. Previously, it also has been demonstrated that 100 μg of RC-529, a synthetic lipid A mimetic that resembles MPL, gave lower responses to Hepatitis B surface antigen than 25 μg of RC-529[31]. These findings suggest that too much stimulation of the immune system may lead to lower antibody production.

Interestingly, most adjuvants did not alter the level of antigen-specific IgG1 induced by LPS-deficient OMCs alone. The only formulations that induced significant higher IgG1 titers were wild-type OMCs and pLAK33 in combination with 1 μg of LpxL1. Thus only some forms of LPS could augment IgG1 levels. Indeed, it has been found previously that LPS can promote both Th1- and Th2-dependent Ig production[32, 33]. Others have

demonstrated that TLR ligands, even the ones that induce a Th1 response, can augment antigen-specific IgG1 levels[20, 34]. However, in these studies TLR ligands were combined with a soluble protein. These proteins do not activate the immune system themselves and hence do not modulate the Th1/Th2 balance. In contrast, LPS-deficient OMCs contain endogenous adjuvants that appear to induce a Th2 response, demonstrated by the low IgG2a/IgG1 ratio and high IgE levels. This is in agreement with studies that demonstrated that the LPS-deficient mutant mainly activates TLR2[35-37] and other reports that showed that TLR2 ligands induce a Th2 response[22-24]. It seems reasonable that the level of Th2-dependent IgG1 induced by LPS-deficient OMCs can not be augmented by Th1 polarizing adjuvants. Similar results were found by Vasilakos et al[38]. They examined the effects of Th1 adjuvants R-848 and CpG DNA on Ab production induced in the presence of the Th2 adjuvant Alum. It was found that IgG2a but not IgG1 levels induced by Alum-OVA were enhanced by R-848 and CpG DNA.

One formulation, pLAK33 in combination with 20 nmol of CpG DNA, induced lower levels of IgG1. Possibly, this is a unique feature of CpG DNA as it has been demonstrated that CpG directly inhibits IgG1 and IgE switching in purified B-cells, while promoting the production of IgG2a[39, 40]. Previously, it was assumed that CpG influenced antibody class-switching only by its ability to induce Th1-skewing cytokines, like IL-12, IL-18, interferon-α and IFN-γ.

Most importantly, agonists of TLR3, TLR4, TLR7, and TLR9 increased the bactericidal activity of sera of the immunized mice. The increase of bactericidal antibodies strongly correlated with an increase in IgG2a and IgG2b antibodies. This demonstrates that these agonists can enhance the functionality of an *N. meningitidis* outer membrane based vaccine. As bactericidal activity depends on class-switching to Th1-dependent antibodies, these results were not unexpected, because these agonists are known to induce Th1-polarization[18-21]. What remains an interesting question however is why TLR3, TLR4, TLR7, and TLR9 agonists showed adjuvant activity whereas TLR2, TLR5, and NOD2 agonists did not seem to have much influence. A remarkable distinction between these two groups is their capability to induce type I IFN. Activation of TLR3, TLR4, TLR7, or TLR9 in contrast to activation of TLR2, TLR5, or NOD2 is reported to induce production of type I IFN[41]. Possibly, TLR-induced type I IFN drives Th1 development and is responsible for the adjuvant effect of TLR ligands. Previously, Le Bon et al. demonstrated that the adjuvant effect of Poly(I:C) and complete Freund's Adjuvant was greatly diminished in mice lacking the receptor for type I IFN[42]. Moreover, purified murine type I IFN had comparable adjuvant activity. These results demonstrate the importance of type I IFN in adjuvant activity for at least some adjuvants. In another study, it was found that Poly(I:

C)- and LPS-induced upregulation of costimulatory molecules on antigen-presenting cells was entirely dependent on type I IFN receptor signaling[43]. Finally, production of IL-12p70 by DCs depends on type I IFN[44]. DC-derived IL-12p70, the bioactive form of IL-12, stimulates IFN-γ production in naïve Th cells and, therefore, strongly promotes Th1 responses[45].

There are several possible explanations for the apparent lack of adjuvant activity of the TLR2, TLR5, and NOD2 agonists in our model. Therefore, we can not conclude from these data that activation of these receptors does not lead to adjuvant activity. In fact, agonists of these receptors were reported to enhance immune responses in previous studies[46-53]. The NOD2 agonist MDP is the minimum active component of complete Freund's adjuvant , which has long been considered the "gold standard" for adjuvant activity in mice[51]. In that formulation however, MDP is dissolved in a water-in-oil emulsion and others have also found that MDP alone barely improves responses[52, 53]. Possibly, the depot effect of an emulsion is particularly important for MDP, which might relate to the cytoplasmic localization of the NOD2 receptor. Regarding TLR2, the LPS-deficient mutant itself is reported to mainly activate TLR2[35-37]. For example, it has been demonstrated that PorB, which is a major component of Neisserial OMCs, activates DCs and B cells via a TLR2-dependent mechanism[54, 55]. Moreover, it has been shown that a conjugate vaccine consisting of the capsular polysaccharide of *Haemophilus influenzae* type b linked to a complex of meningococcal outer membrane proteins (OMPC) needed TLR2 engagement by OMPC for optimal immunogenicity[47]. Together these results suggest that the remaining responses in mice after immunization with LPS-deficient OMCs depend at least in part on TLR2 activation. Therefore, inclusion of additional TLR2 ligands might have limited influence on the response to LPS-deficient OMCs. Finally, as flagellin is reported to promote a Th2-type response[49, 50], this effect might be concealed by the endogenous TLR2 ligands of the LPS-deficient OMCs that also seem to induce a Th2-like response.

Recently, Gavin et al. reported that the adjuvants alum, FCA, Freund's incomplete adjuvant, and Ribi adjuvant mount robust antibody responses in mice completely deficient in TLR-signaling[56]. These findings might have implications for vaccine design. TLR-induced responses can have side-effects, which could be avoided by excluding TLR ligands from vaccines altogether. However, as these studies were done with an artificial antigen, it remains to be established whether existing vaccines or vaccines in development are also effective independent of TLRs. At this point it can not be ruled out that during natural infection TLRs do initiate adaptive immune responses and that vaccines should resemble a natural infection as close as possible to induce effective responses and long

lasting memory. In that respect it is interesting to note that yellow fever vaccine 17D activates DCs via multiple TLRs generating polyvalent immune responses, which might be the reason why this vaccine is one of the most effective available[57]. Moreover, our data show that immunization with bacteria derived OMCs enhance antibody responses mainly by LPS of the bacterium itself. Therefore, it seems likely that *N. meningitidis* also initiates adaptive immunity via LPS-dependent mechanisms during natural infection.

In summary, we have demonstrated that TLR3, TLR4, TLR7, and TLR9 agonists are suitable adjuvant candidates for an outer membrane-based *N. meningitidis* sergroup B vaccine. Wild-type LPS from *N. meningitidis* is very toxic[58]. In fact, LPS levels in the blood of patients, who suffer from septicaemia caused by meningococci, correlate with severity of the disease. Therefore, inclusion of this compound in a vaccine seems undesirable, especially when alternatives are available. With the LPS-deficient mutant at our disposal, we have the opportunity to combine neisserial outer membrane proteins with less reactogenic adjuvants. Here we show that wild-type *N. meningitidis* LPS can be replaced by other adjuvants. Some of these adjuvants, like Imiquimod, CpG DNA and in particular MPL, have already been tested extensively in humans and were found to be safe[28, 31, 59].

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Chapter 3

Differential effect of TLR2 and TLR4 activation on the adaptive immune response after immunization with a *Neisseria meningitidis* outer membrane vesicle vaccine and a *Bordetella pertussis* whole cell vaccine

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Manuscript in preparation

Abstract

Neisseria meningitidis and *Bordetella pertussis* are Gram-negative bacterial pathogens that can cause serious diseases in humans. *N. meningitidis* outer membrane vesicle (OMV) vaccines and whole cell pertussis vaccines have been successfully used in humans to control infections with these pathogens. The mechanisms behind their effectiveness are poorly defined. Here we investigated the role of Toll-like receptor (TLR) 2 and TLR4 in the induction of immune responses in mice after immunization with these vaccines. Innate and adaptive immune responses were compared between wild type mice and mice deficient in TLR2, TLR4, or TRIF. Unexpectedly, immune responses were not lower in TLR2-/- mice but tended to be even higher after immunization. In contrast, TRIF-deficient and TLR4-deficient mice showed impaired immunity after immunization. The whole cell pertussis vaccine induced lower levels of all IgG subclasses in these mice. The adaptive immune response after *N. meningitidis* OMV vaccine immunization was clearly shifted towards Th2 in TRIF-deficient mice. Moreover, although antibody responses were not greatly reduced, T cell responses were much lower in TLR4-deficient mice immunized with *N. meningitidis* OMVs. Together our data demonstrate that TLR4 activation contributes to the immunogenicity of the *N. meningitidis* OMV vaccine and the whole cell pertussis vaccine, but that TLR2 activation is not required.

Introduction

The innate immune system senses microbes through a number of receptors present on innate immune cells that can recognize a wide variety of microbial structures[1]. This group of receptors is often referred to as pattern recognition receptors (PRRs). There are several classes of PRRs, including Toll-like receptors (TLRs), C-type lectin like receptors, RIG-I like receptors, and Nod-like receptors. The TLR family is the best characterized class to date. In humans, 10 different TLRs have been described and each TLR recognizes distinct microbial structures[2]. For example, lipopolysaccharide (LPS), a major component of the outer membrane of virtually all Gram-negative bacteria, activates TLR4, lipoproteins and several other structures activate TLR2, unmethylated CpG DNA of bacteria and certain viruses activate TLR9, and viral dsRNA is recognized by TLR3[2]. TLRs become activated by dimerization of the ectodomains after ligand binding. Dimerization leads to recruitment of adaptor proteins to the intracellular TLR domains. This initiates a signalling cascade, which eventually leads to the induction of many genes involved in the innate immune response[3]. Four adaptor proteins mediate TLR signalling: MyD88, TRIF, MAL, and TRAM[4]. All TLRs signal through MyD88, except TLR3, which signals solely through TRIF. Moreover, TLR4 is the only TLR which utilizes both MyD88 and TRIF[5]. Activation of these proteins eventually leads to induction of pro-inflammatory cytokines and type I interferon, respectively. Activation of TLR7/8 and TLR9 also leads to the induction of type I interferon, but in a MyD88-dependent manner[1].

The primary function of TLRs is to detect pathogens and activate innate immune cells to clear the infection immediately. However, TLRs also play an important role in the initiation of adaptive immune responses[6, 7]. Dendritic cells (DCs) are thought to play a central role in linking innate and adaptive immunity after TLR triggering, because of their superior capacity to stimulate T cells[8]. Which TLR is activated determines what types of cytokines and other factors are produced by the DCs, which in turn dictates whether the $CD4^+$ T cells differentiate into Th1, Th2, Th17, or Treg[1, 9]. Because TLR ligands can both initiate and direct adaptive immunity, they have great potential as adjuvants. However, the claim that TLR activation plays an important role in the induction of an adaptive immune response after vaccination has been challenged recently[10].

Many of the currently licensed vaccines are live attenuated strains or contain elements of killed microbes[6]. These vaccines likely contain structures that are recognized by TLRs and contribute to the immunogenicity. This has indeed been demonstrated for a number of vaccines[6, 11-14], but for the majority of vaccines this information is still lacking. It is important to identify the pathways induced by these successful vaccines for the rational design of new vaccines and/or adjuvants. Moreover, the human population is

genetically very diverse and some individuals might have deficiencies in the pathways that are induced by the vaccine, which could explain why some individuals respond poorly after vaccination[15]. We decided to study the role of TLRs in the immunogenicity of two LPS-containing vaccines against the Gram-negative bacterial pathogens *Neisseria meningitidis* and *Bordetella pertussis*.

N. meningitidis is a leading cause of meningitis and sepsis worldwide[16]. The bacterium can be divided into several serogroups based on its capsule. For most serogroups (A, C, Y, and W-135) capsular polysaccharide vaccines are available, but not for serogroup B, because its capsular polysaccharide is not immunogenic. An attractive alternative for serogroup B is an outer membrane vesicle (OMV) vaccine[17]. OMV vaccines have been shown to be effective in controlling epidemics in Cuba, Norway, and New Zealand, where one particular clone of *N. meningitidis* serogroup B was causing high rates of meningococcal disease. *B. pertussis* is the causative agent of whooping cough in humans. To prevent this disease, whole cell pertussis vaccines have been used for many decades in developed countries and are still used today in developing countries. However, due to adverse effects the whole cell vaccine has now been replaced in developed countries with a safer subunit vaccine consisting of a few *B. pertussis* antigens[18, 19]. The *N. meningitidis* OMV vaccine and whole cell pertussis vaccine both contain LPS and lipoproteins, which activate TLR4 and TLR2 respectively[20-24]. Ligands of these TLRs have been shown to have adjuvant activity in numerous studies in mice[12, 25-28]. Interestingly, the major outer membrane proteins in both vaccines are porins, which have been shown to be immunogenic[29-31]. Moreover, porins of several Gram-negative bacteria, including the PorB porin of *N. meningitidis*, have been shown to have adjuvant activity and to activate TLR2[32-38].

Here we investigated the role of TLR2 and TLR4 in the induction of immune responses in mice after immunization with a *N. meningitidis* OMV vaccine and a whole cell pertussis vaccine. Innate cytokine induction, T cell responses, and antibody production were compared between wild type mice and mice deficient in either TLR2, TLR4, or TRIF. Surprisingly, TLR2-/- mice were not compromised in any of the responses after immunization. In contrast, TRIF-deficient and TLR4-deficient mice showed impaired immunity after immunization. We conclude that TLR4 activation contributes to the immunogenicity of the *N. meningitidis* OMV vaccine and the whole cell pertussis vaccine, but that TLR2 activation is not required.

Materials and Methods

Animals

Specific-pathogen-free C57BL/6J, C3H/HeJ (TLR4-deficient), and C3H/HeOuJ (TLR4-proficient) mice were purchased from Charles River Laboratories. TLR2-/- mice[39] and TRIF-deficient mice[40], both from a C57BL/6 background, were kindly provided by respectively Dr. Shizuo Akira (Osaka University, Japan) and Dr. Bruce Beutler (Scripps Research Institute, La Jolla, CA) via Dr. Tom van der Poll (Academic Medical Center, Amsterdam, the Netherlands). For experiments, female mice were used, aged 10-14 weeks. Animal experiments were approved by the Institute's Animal Ethics Committee.

Vaccine preparation

The OMV vaccine was obtained from the PorB/RmpM-negative *N. meningitidis* strain TR52 [P1.5-1,2-2][41] by extraction of bacteria with 0.5% deoxycholate in 0.1 M Tris-HCl-10 mM EDTA (pH 8.6) and were purified by differential centrifugation[42]. Protein and LPS content were determined as described previously[43]. OMVs were stored at 4ºC and prior to immunization diluted to 20 μg/ml in PBS. For preparation of the whole cell pertussis vaccine, *B. pertussis* strains 134 and 509 were grown in defined synthetic medium[44]. After 18-22 hours of culture, bacteria were heat inactivated for 10 min at 56ºC in the presence of 16 mM formaldehyde. Next cells were centrifuged for 10 min at 16,100 x g and resuspended in PBS to 200 international opacity units (IOU)/ml. The suspensions were stored at 4ºC. Prior to immunization, both strains were mixed 1:1 and diluted to a final concentration of 16 IOU/ml in PBS. Tetanus toxoid was produced by the QC department at the NVI and stored at 4ºC. Before immunization, tetanus toxoid was diluted to 4 Lf units/ml in PBS.

Immunizations

Mice were immunized subcutaneously on days 0 and 21 with either 250 μl PBS, 1 Lf units tetanus toxoid in 250 μl PBS, or 5 μg of *N. meningitidis* P1.5-1,2-2 OMVs in 250 μl PBS. Whole cell pertussis vaccine (4 IOU in 250 μl PBS) was administered subcutaneously on days 0 and 28. Groups that received PBS or tetanus toxoid contained 3 mice per group and groups that received OMVs or whole cell pertussis vaccine contained 6 animals per group. A blood sample of all mice was taken 2 and 4 hours after immunization. Sera were collected and stored at -20ºC. From the mice that received PBS, tetanus toxoid, or *N. meningitidis* P1.5-1,2-2 OMVs blood and spleen were taken on day 28. From the mice that received whole cell pertussis vaccine blood and spleen were taken on day 42. Sera were collected and stored at -20 ºC. Single-cell suspensions of spleen cells were produced

by mechanical dissociation of organs through 70-μm-pore-size nylon filters. Spleens cells were used either fresh or after freezing and storage at –135°C.

Antigen-specific antibodies

Antigen-specific IgG, IgG1, IgG2a/c, IgG2b, and IgG3 antibodies were determined by enzyme-linked immunosorbent assay (ELISA) as described previously[26] with slight modifications. Briefly, flat-bottom 96-well microtiter plates (Immulon 2; Nunc) were coated overnight at room temperature with 100 μl/well 4 Lf units/ml tetanus toxoid in PBS, 5 μg/ml *N. meningitidis* P1.5-1,2-2 OMVs (4 μg/ml PorA) in PBS, or 2 IOU/ml of *B. pertussis* strain 509 (inactivated as described above) in PBS. Plates were incubated with serial dilutions of the sera of the immunized mice (100 μl/well) at 37°C for 1 h. Antibody isotypes were detected with anti-mouse antibodies conjugated to horseradish peroxidase (Southern Biotechnology Associates, Inc.). Next, peroxidase substrate was added to the wells and the reaction was stopped by the addition of 2 M H_2SO_4. A four-parameter curve fit was made for optical density values of the serial dilutions, and the antibody titer was calculated in reciprocal dilutions that gave 50% of the maximum absorbance. The results are expressed as $\log_{10}$ titers.

Total IgE

Total IgE levels in the sera were measured by ELISA as described previously[26]. Briefly, flat-bottom 96-well microtiter plates were coated with rat anti-mouse IgE monoclonal antibody (BD Biosciences) and incubated overnight at 4°C. Next, the plates were incubated for 1 h at room temperature with the sera of the immunized mice and purified mouse IgE clone C48-2 (BD Biosciences) as a standard. For detection of IgE, biotinylated anti-mouse IgE (BD Biosciences) was used followed by streptavidin-HRP (Sanquin). Peroxidase substrate was used as a substrate, and the reaction was stopped with 2 M H_2SO_4. The absorbance was determined at 450 nm.

Serum bactericidal assay

The sera of immunized mice were diluted 1:5 in Gey's balanced salt solution plus 0.5% bovine serum albumin and then heat inactivated for 30 min at 56°C. Next, twofold serial dilutions of the sera, together with *N. meningitidis* P1.5-1,2-2 (10^4 CFU/ml), were incubated in 96-well plates at room temperature for 15 min. After the addition of baby rabbit complement (20% of total volume) plates were incubated at 37°C for 1 h. Bacteria were plated on GC medium base (Difco Laboratories) supplemented with IsoVitaleX (Becton Dickinson) and grown overnight at 37°C in 5% CO_2 in a humid atmosphere. The

serum bactericidal titer was determined as the reciprocal serum dilution that gave more than 90% killing of the number of bacteria used. The results are expressed as $\log_{10}$ titers.

Peptide synthesis

Overlapping 18-mer peptides covering the entire *B. pertussis* P.69 Prn protein or *N. meningitidis* P1.5-1,2-2 PorA protein were prepared by solid-phase synthesis using N^{α}-(9-fluorenyl)methoxycarbonyl (FMOC)-protected amino acids and a Syro II simultaneous multiple-peptide synthesizer (MultiSyntech GmbH, Witten, Germany). The purity and identity of the synthesized peptides were assessed by reverse-phase high-performance liquid chromatography and mass spectometry.

Spleen cell proliferation

Spleen cells from immunized mice were cultured at 1.5 x 10^5 in 150 μl IMDM (Gibco BRL) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 300 μg/ml L-glutamine (Gibco BRL), 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL), and 50 μM beta-mercaptoethanol in 96-well round-bottom plates (Greiner). Spleen cells were co-cultured with either medium, 1 Lf unit/ml tetanus toxoid, or 1 μM of P.69 Prn or P1.5-1,2-2 PorA peptides. On day 4, 100 μl of supernatant was removed and stored at -20ºC. Next, 0.5 μCi (18.5 kBq) [^{3}H]thymidine (Amersham) was added to the wells, and cells were cultured for another 18 h. Cells were harvested, and [^{3}H]thymidine incorporation was determined as counts per minute using a Wallac 1205 Betaplate liquid scintillation counter. Results are expressed as stimulation indices from triplicate wells, calculated as (counts per minute of cultures in the presence of antigen)/(counts per minute of cultures in the presence of medium only).

Luminex

A 6-plex Bio-Plex assay (Bio-Rad) containing beads for mouse IL-1β, IL-6, IL-10, IL-12p70, RANTES, and TNF-α was used to measure levels of these cytokines in the sera of the mice taken 2 and 4 hours after immunization. A 7-plex Bio-Plex assay (Bio-Rad) containing beads for mouse IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ was used to determine amounts of these cytokines in the supernatants of spleen cells restimulated with antigen or peptide. Cytokine concentrations were determined with a Bio-Plex system (Bio-Rad).

Statistics

One-way analysis of variance (ANOVA) was performed, followed by the post-hoc Dunnett *t* test to analyze differences in means between the experimental groups of C57BL/6 mice and TLR2-/-, and TRIF-deficient mice (GraphPad Prism 4). The unpaired two-tailed Students' t test was used to analyze differences in means between the experimental groups of C3H/HeJ and C3H/HeOuJ mice. Variances were compared with the F-test. In case of significant different variances ($p<0.05$), the Welch's correction was included (GraphPad Prism 4). In case the SD of one of the means was 0, the One-sample T test was used instead (SPSS Statistics 17.0). P values of <0.05 were considered significant.

Results

TRIF-deficient and TLR4-deficient mice have reduced innate cytokines levels after immunization

As a *N. meningitidis* OMV vaccine we chose OMVs derived from serosubtype P1.5-1,2-2. This serosubtype is one of the prevalent variants in The Netherlands. Moreover, serosubtype P1.5-1,2-2 is among the more immunogenic serosubtypes in humans and mice[43, 45, 46]. Finally, we also included tetanus toxoid as a control vaccine. As tetanus toxoid is not known to activate any TLR, it should induce a similar response in all the different mice. In humans these vaccines are administered with alum as the adjuvant[17-19]. However, we did not include alum to study more specifically the intrinsic adjuvant activity of the vaccines.

The role of TLR2 and TRIF in vaccine-induced responses after immunization was examined by immunizing wild type C57BL/6 mice, TLR2-/- mice, and TRIF-deficient mice, which all have a C57BL/6 background[39, 40]. To investigate the role of TLR4, responses were compared between TLR4-deficient C3H/HeJ mice and wild type C3H/HeOuJ mice[47]. All mouse strains were immunized subcutaneously with either PBS, tetanus toxoid, *N. meningitidis* OMVs, or whole cell pertussis vaccine. After two and four hours a blood sample was taken from all mice to analyze serum cytokine levels. Concentrations of IL-1β, IL-6, IL-10, IL-12p70, RANTES, and TNF-α were measured with luminex. As expected, tetanus toxoid did not induce cytokine production in any of the mice (data not shown). *N. meningitidis* OMVs only induced slightly higher levels of IL-6 and RANTES compared to PBS injected mice (Fig. 1A and data not shown). IL-6 levels after 2 hours were significantly lower in TRIF-deficient mice and TLR4-deficient mice and tended to be higher in TLR2-/- mice compared to wild type mice. RANTES was equally induced in all mouse strains. Whole cell pertussis vaccine induced higher levels of IL-6, IL-12p70, RANTES, and IL-10 (Fig. 1B and data not shown). Again TRIF deficient mice and C3H/HeJ mice were clearly compromised in cytokine induction compared to wild type mice. TLR2-/- mice were not impaired in cytokine production, IL-6 even tended to be higher. These results suggest that serum cytokine production *in vivo* after immunization with *N. meningitidis* OMVs or whole cell pertussis vaccine depends mainly on LPS signalling and not on TLR2 activation.

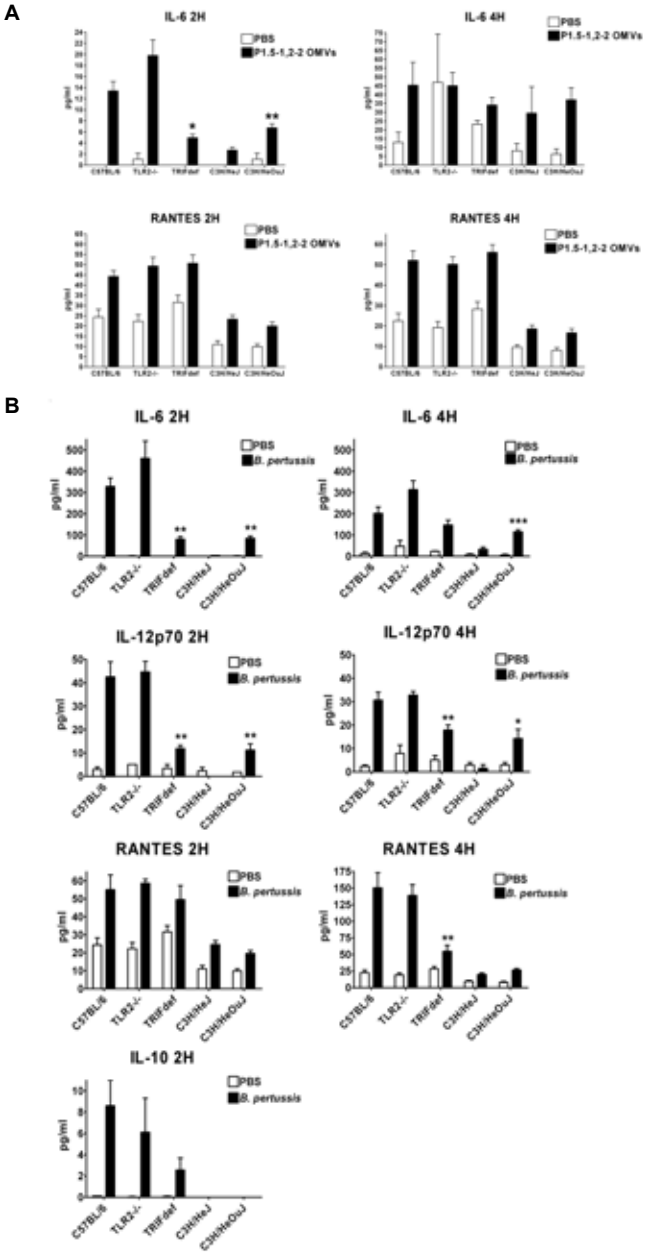

Figure 1. Serum cytokine levels shortly after vaccination. Two and four hours after immunization blood samples were taken from all mice and cytokine levels in the sera were analyzed with Luminex. Results for *N. meningitidis* OMVs are shown in panel A, results for whole cell pertussis vaccine are shown in panel B. There were 3 mice per group for the animals that received PBS and 6 mice per group for *N. meningitidis* OMVs and whole cell pertussis vaccine. The data are expressed as means, error bars indicate S.E.M. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Comparison of antigen-specific antibody levels

The amount of antibodies was determined in all sera. It has been demonstrated previously that class switching to IgG2a/c, IgG2b, and IgG3 depends on a Th1 response and class switching to IgG1 and IgE depends on a Th2 response[48-50]. Therefore, to analyze antibody production and the type of immune response that was induced, antigen-specific total IgG, antigen-specific IgG subclasses, and total IgE were determined in the serum. Tetanus toxoid immunization led to a comparable antibody response in the C57BL/6, TRL2-/-, and TRIF-deficient mice (Fig. 2A). Antigen-specific IgG3 was not detected, probably because IgG3 antibodies mainly target carbohydrates[51, 52]. As expected, total IgE levels were higher in mice immunized with tetanus toxoid than mice immunized with PBS, which indicates that tetanus toxoid induced a Th2 response. Levels of especially total IgE, but also IgG2c and IgG2b tended to be higher in TLR2-/- mice, but not significantly. Unexpectedly, levels of IgG, IgG1, and IgG2b were significantly lower in the C3H/HeOuJ mice compared to the C3H/HeJ mice (Fig. 2B). However, overall these results show that antibody production after tetanus toxoid immunization is quite comparable between the mice.

As expected, *N. meningitidis* OMVs induced a more Th1-dependent antibody profile than tetanus toxoid in wild type C57BL/6 mice, reflected by high levels of IgG2b and IgG2c, almost no IgG1, and no increase in total IgE compared to PBS injected mice (Fig. 3A). However, IgG3 levels were also very low, although IgG3 switching is reported to be induced by Th1 responses[49]. Strikingly, TLR2-/- mice immunized with *N. meningitidis* OMVs were not impaired at all in the antibody response, IgG1 and IgG3 levels even tended to be higher. On the other hand, *N. meningitidis* OMVs induced significant lower amounts of IgG in TRIF-deficient mice, which was mainly due to lower levels of IgG2b and IgG2c (Fig. 3A). In contrast, IgG1 and IgG3 levels to OMVs were significantly higher in TRIF-deficient mice compared to wild type mice. These results suggest that the anti-OMV response was more polarized towards Th2 in the TRIF-deficient mice. Surprisingly, TLR4-deficient C3H/HeJ mice did not have significantly lower antibody levels to OMVs than wild type C3H/HeOuJ mice. However, IgG, IgG2a, IgG2b, and IgG3 tended to be lower in the C3H/HeJ mice and IgG1 tended to be higher. Therefore, *N. meningitidis* OMVs seem to skew the response more towards Th2-dependent antibody production in absence of the LPS signal in C3H/HeJ mice, which is in agreement with one of our previous studies[26].

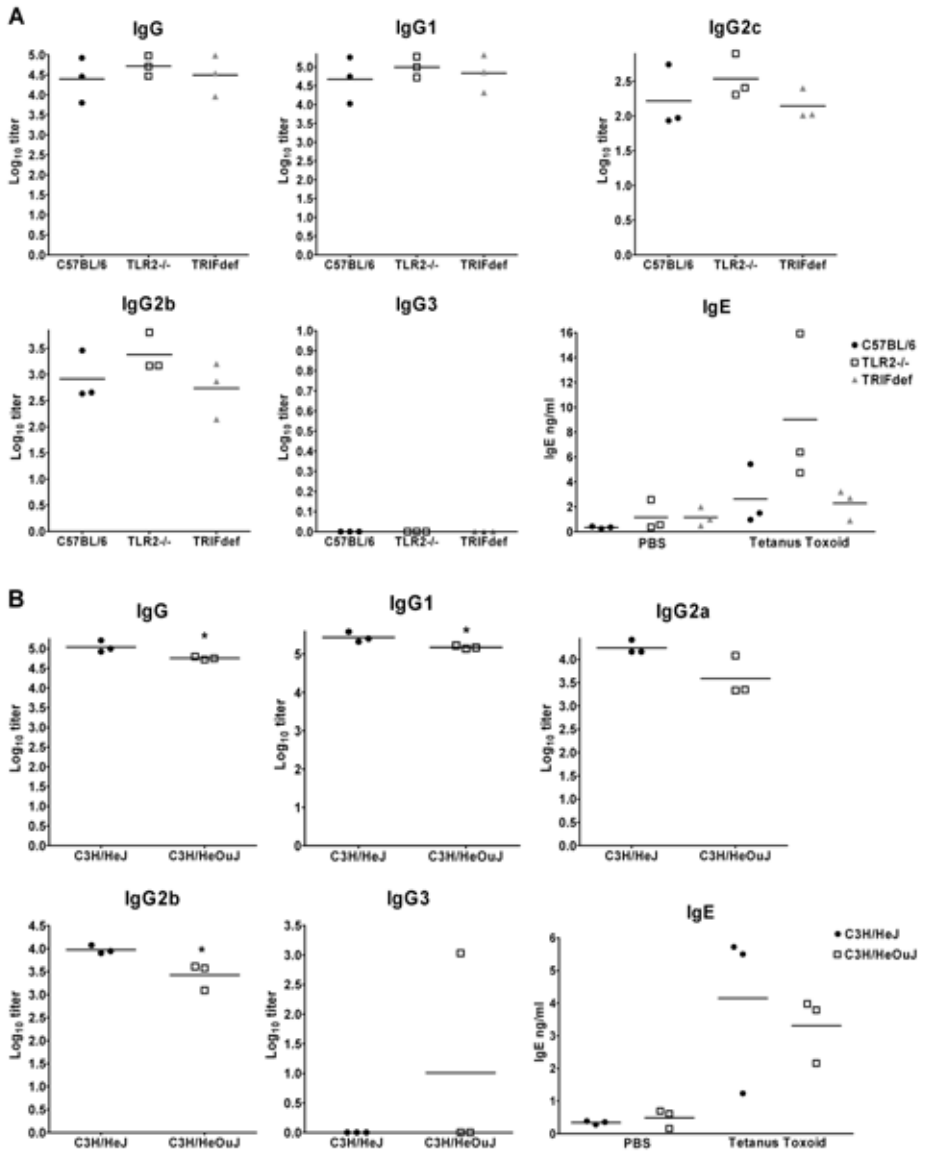

Figure 2. Antibody titers of mice after immunization with tetanus toxoid. Mice were immunized with tetanus toxoid and antigen-specific titers of IgG, IgG1, IgG2a/c, IgG2b, and IgG3 in sera were determined with ELISA. Also total IgE in sera was measured with ELISA. Results for C57BL/6, TLR2-/-, and TRIF-deficient (TRIFdef) are shown in panel A, results for C3H/HeJ and C3H/HeOuJ mice are shown in panel B. Data are expressed as means of $\log_{10}$ titers for 3 mice per group. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

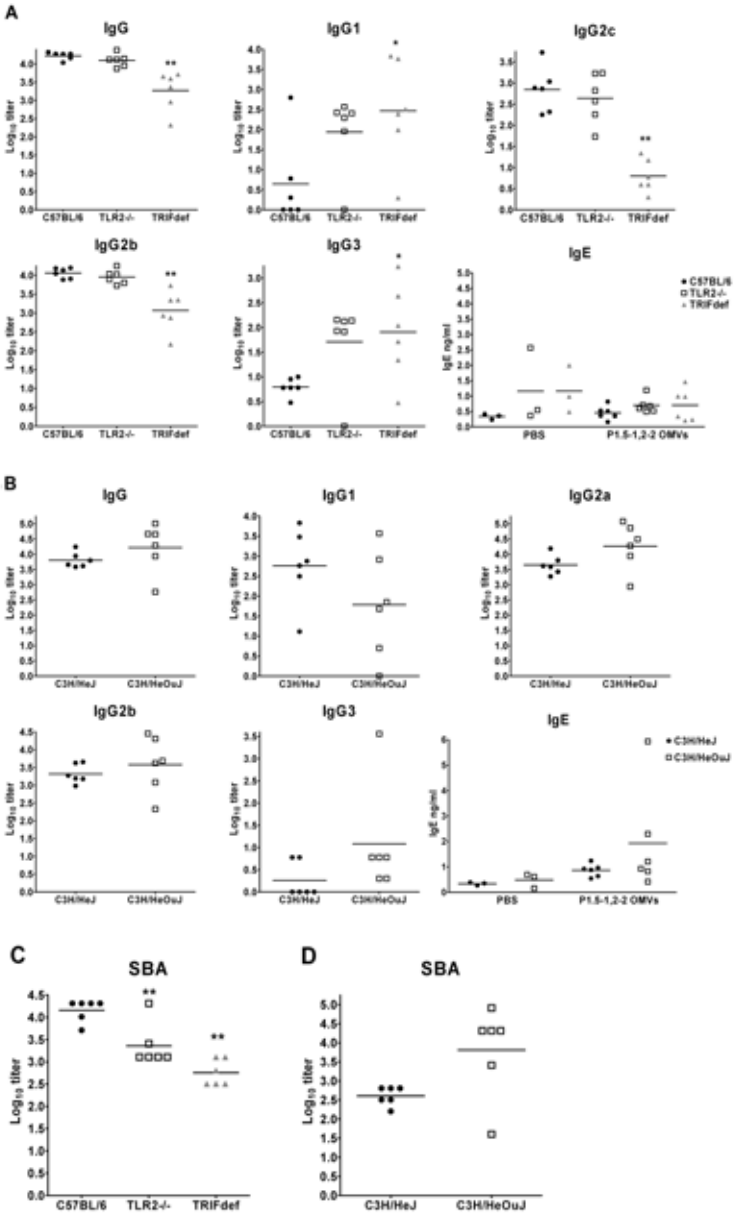

Figure 3. Antibody titers of mice after immunization with *N. meningitidis* P1.5-1,2-2 OMVs. Mice were immunized with *N. meningitidis* P1.5-1,2-2 OMVs and antigen-specific titers of IgG, IgG1, IgG2a/c, IgG2b, and IgG3 in sera were determined with ELISA. Also total IgE in sera was measured with ELISA. Results for C57BL/6, TLR2-/-, and TRIF-deficient (TRIFdef) are shown in panel A, results for C3H/HeJ and C3H/HeOuJ mice are shown in panel B. Levels of serum bactericidal antibodies after immunization with *N. meningitidis* P1.5-1,2-2 OMVs are shown in panel C and D. Data are expressed as means of $\log_{10}$ titers for 6 mice per group. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

We showed that the different mouse strains differed in antibody production after *N. meningitidis* OMV immunization. However, levels of antibodies alone gives no information about their functionality. It has been demonstrated previously that individuals with complement deficiencies are extremely susceptible to infections with Neisseria species[53]. Moreover, it has been shown that in humans the presence of serum antibodies that mediate complement-dependent killing of *N. meningitidis* strongly correlate with protection against the bacterium[54]. Therefore, bactericidal antibodies are currently considered to be the most important correlate of protection for meningococcal disease. Furthermore, IgG2a/c, IgG2b, and IgG3, but not IgG1, isotypes can activate complement[55]. We tested the bactericidal activity of the sera of all mice immunized with *N. meningitidis* OMVs. Consistent with the lower IgG2b and IgG2c levels, TRIF-deficient mice also had significantly lower levels of bactericidal antibodies compared to wild type mice (Fig. 3C). However, also TLR2-/- mice had significantly lower amounts of bactericidal antibodies, although induction of IgG2b and IgG2c was not impaired. This could result from the higher IgG1 levels and therefore reduced IgG1 to IgG2b/c ratios in these mice, as the TLR2-/- mouse with no IgG1 titer had the highest level of bactericidal antibodies. Finally, C3H/HeJ mice tended to have less bactericidal antibodies than C3H/HeOuJ mice (Fig. 3D). Together these results suggest that LPS signalling also contributes to the generation of bactericidal antibodies after immunization with *N. meningitidis* OMVs. But also TLR2 activation might contribute to the generation of antibodies that can activate complement, although it does not seem to be required for antibody generation in general.

The differences between the different mouse strains in antibody levels were quite similar for the mice that received whole cell pertussis vaccine and the mice that received *N. meningitidis* OMVs. Again TLR2-/- mice did not show any defects in the induction of antibodies (Fig. 4A). Moreover, IgG3 and IgE levels tended to be higher in TLR2-/- mice compared to wild type mice. TRIF-deficient mice had significant lower amounts of IgG, which was mainly due to lower levels of isotypes IgG2b and IgG2c, as with *N. meningitidis* OMVs. However, in contrast to *N. meningitidis* OMVs, whole cell pertussis vaccine did not induce higher levels of IgG1 and IgG3 in TRIF-deficient mice. C3H/HeJ mice had lower amounts of all the antibodies compared to C3H/HeOuJ mice, except IgE (Fig. 4B). This difference was significant for IgG, IgG2a, but also for IgG1. Together these results suggest that LPS in the whole cell pertussis vaccine does not skew the response as much towards Th1-dependent antibody production as in *N. meningitidis* OMVs. Finally, the whole cell pertussis vaccine also induced the production of total IgE, in contrast to *N. meningitidis* OMVs. This suggests that the whole cell pertussis vaccine induced more Th2 responses than *N. meningitidis* OMVs. In summary, our results demonstrate that LPS signalling contributes to the antibody response after immunization with *N. meningitidis* OMVs and whole cell pertussis vaccine. However, activation of TLR2 is not required.

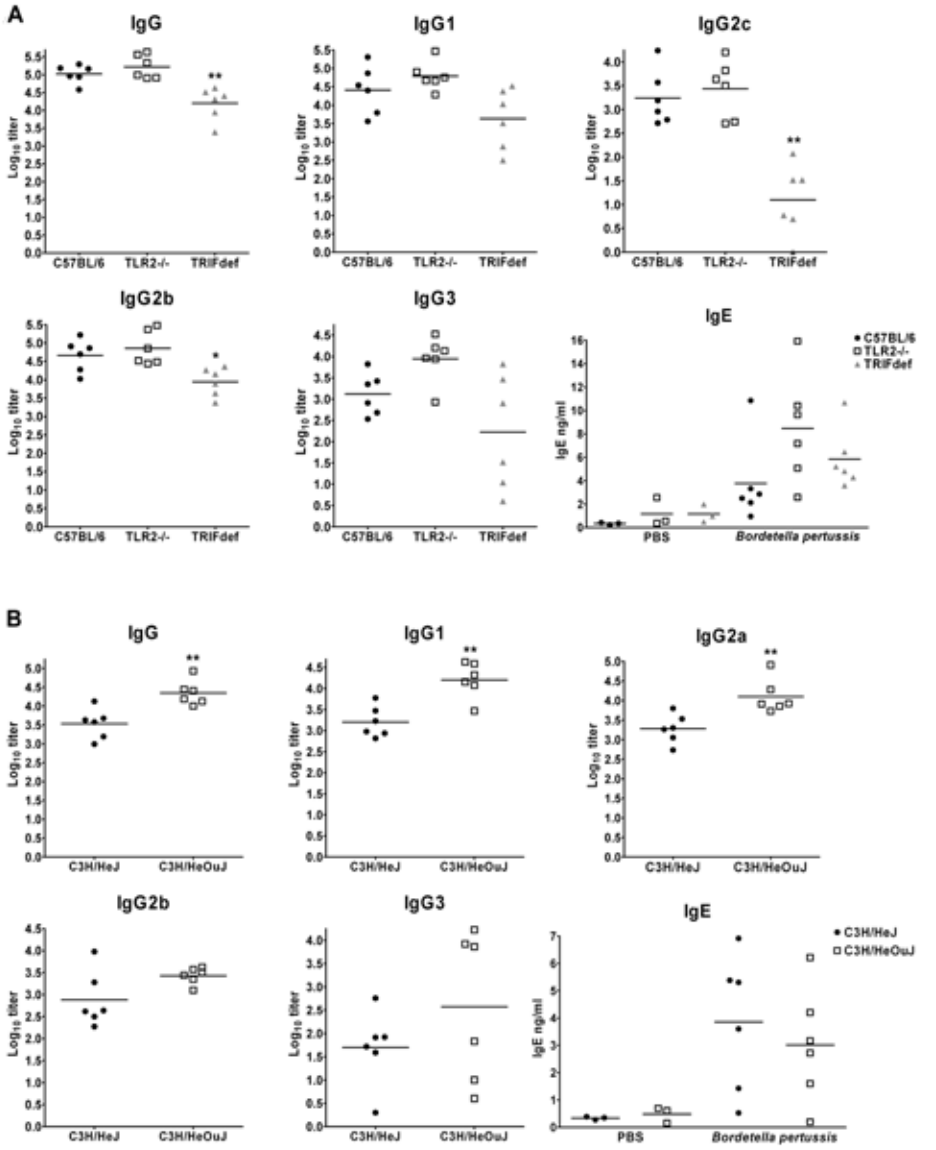

Figure 4. Antibody titers of mice after immunization with whole cell pertussis vaccine. Mice were immunized with whole cell pertussis vaccine (*Bordetella pertussis*) and antigen-specific titers of IgG, IgG1, IgG2a/c, IgG2b, and IgG3 in sera were determined with ELISA. Also total IgE in sera was measured with ELISA. Results for C57BL/6, TLR2-/-, and TRIF-deficient (TRIFdef) mice are shown in panel A, results for C3H/HeJ and C3H/HeOuJ mice are shown in panel B. Data are expressed as means of $\log_{10}$ titers for 6 mice per group. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Spleen cells of the different mouse strains proliferate equally after in vitro antigen restimulation

Also the spleen was taken from the mice immunized as described above. Spleen cells were restimulated with antigen or peptides for 4 days and proliferation of the cells was assessed by measuring [^{3}H]thymidine incorporation. Spleen cells of tetanus toxoid immunized mice or PBS injected mice were restimulated with tetanus toxoid. No significant differences in proliferation of spleen cells of tetanus immunized mice were found among the different mouse strains (Fig. 5A). However, proliferation of TRIF-deficient cells tended to be higher than proliferation of wild type C57BL/6 cells. Moreover, proliferation of C3H/HeJ cells tended to be higher than proliferation of C3H/HeOuJ cells.

Spleen cells of mice with a C57BL/6 background and immunized with either *N. meningitidis* OMVs or PBS were restimulated with two PorA peptides, which were previously identified as two P1.5-1,2-2 PorA epitopes recognized by C57BL/6 mice (M. Poelen and C. van Els, unpublished data). Spleen cells from *N. meningitidis* OMV immunized mice clearly proliferated in response to the two peptides, suggesting that CD4$^+$ T cells specific for those epitopes were generated after immunization (Fig. 5B). Interestingly, there was no difference between the three mouse strains in spleen cell proliferation. Which epitopes of P1.5-1,2-2 PorA were recognized by C3H/HeJ and C3H/HeOuJ mice was not known to us. Therefore, spleen cells of these mice immunized with either PBS or *N. meningitidis* OMVs were restimulated with different peptides pools of P1.5-1,2-2 PorA (data not shown). We found that one peptide pool induced the highest proliferation of spleen cells derived from *N. meningitidis* OMV immunized mice (Fig. 5B). There was also no difference in spleen cell proliferation between C3H/HeJ and C3H/HeOuJ mice. Together these results suggest that activation of TLR2 or TLR4 has no influence on CD4$^+$ T cell proliferation after immunization with *N. meningitidis* OMVs.

We previously demonstrated that *B. pertussis* P.69 pertactin (P.69 Prn) is recognized by CD4$^+$ T cells of mice immunized with whole cell pertussis vaccine or *B. pertussis* infection[56]. Moreover, two P.69 Prn epitopes were identified in C57BL/6 mice (R. Stenger, M. Poelen, and C. van Els, unpublished observations). Therefore, spleen cells of mice with a C57BL/6 background immunized with either whole cell pertussis vaccine or PBS were restimulated with two peptides representing these two epitopes (Fig. 5C). Unfortunately, the splenocytes of whole cell pertussis vaccine immunized mice proliferated poorly. Cells from TLR2-/- mice gave a slightly better response. As with PorA, epitopes of P.69 Prn were not known in C3H/HeJ and C3H/HeOuJ mice. Therefore, spleen cells from C3H/HeJ and C3H/HeOuJ mice immunized with either whole cell pertussis vaccine or PBS were restimulated with different peptide pools of P.69 Prn. However, none of the peptide

pools induced spleen cell proliferation of the whole cell pertussis vaccine immunized mice (data not shown). Thus it seems that immunization with the whole cell pertussis vaccine generated little P.69 Prn-specific $CD4^+$ cells in the spleen.

LPS signalling and TLR2 activation influence quality of T cell responses

Naïve $CD4^+$ T cells can differentiate into Th1, Th2, Th17, or Treg cells. Th1 cells produce IFN-γ, Th2 cells produce IL-4, IL-5, and IL-13, Th17 cell produce IL-17, and most Treg cell subsets produce IL-10[57, 58]. These cytokines were measured in the supernatant of the spleen cells stimulated with antigen or peptides to identify the type of T cell responses that were induced. For the mice with a C57BL/6 background, stimulation of spleen cells with tetanus toxoid induced more production of IL-4, IL-5, IL-10, and IL-13 by cells of mice immunized with tetanus toxoid than the same mice immunized with PBS (Fig. 6A). Levels of IL-5 and IL-13 were especially high, suggesting that tetanus toxoid induced mainly a Th2 response. There were no significant differences between the C57BL/6, TLR2-/-, and TRIF-deficient mice, but IL-4 and IL-13 tended to be higher for the TLR2-/- mice. Tetanus toxoid restimulation induced production of all cytokines tested by the spleen cells of the tetanus toxoid immunized C3H/HeJ and C3H/HeOuJ mice (Fig. 6B). Apparently, tenanus toxoid immunization leads to a more mixed Th response in these mice compared to C57BL/6 mice. Moreover, levels of all cytokines, except IL-17, tended to be higher in the supernatants of C3H/HeJ cells. This is in good agreement with the higher tetanus toxoid specific antibodies that we observed in the serum of these mice (Fig. 2B).

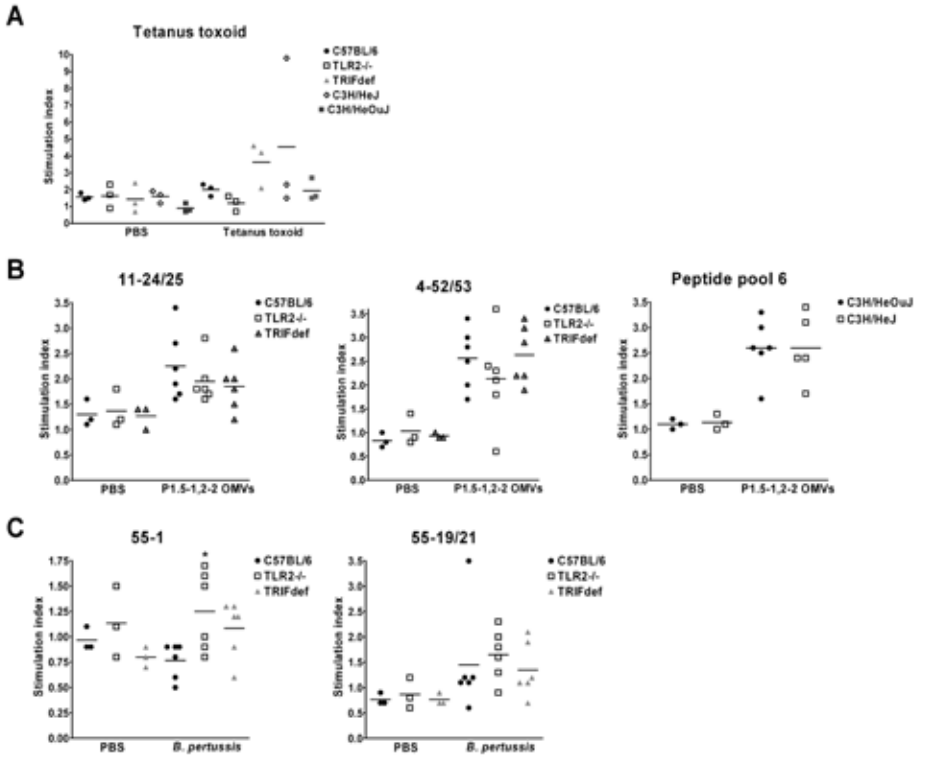

Figure 5. Proliferation of spleen cells after restimulation with antigen or peptide. Spleens were taken from the mice after immunizations and spleen cells were incubated for 4 days with medium, antigen, or peptides, after which [^{3}H]thymidine incorporation was determined. Spleen cells of C57Bl/6, TLR2-/-, TRIF-deficient (TRIFdef), C3H/HeJ, and C3H/HeOuJ mice immunized with PBS (3 mice per group) or tetanus toxoid (3 mice per group) were restimulated with 1 Lf/ml tetanus toxoid (A). Spleen cells of C57Bl/6, TLR2-/-, and TRIF-deficient mice immunized with PBS (3 mice per group) or *N. meningitidis* P1.5-1,2-2 OMVs (6 mice per group) were restimulated with 1 μM of peptides 11-24/25 and 4-52/53. Spleen cells of C3H/HeJ and C3H/HeOuJ mice immunized with PBS (3 mice per group) or *N. meningitidis* P1.5-1,2-2 OMVs (6 mice per group) were restimulated with peptide pool 6 (1 μM of each peptide, B). Spleen cells of C57BL/6, TLR2-/-, and TRIF-deficient mice immunized with PBS (3 mice per group) or whole cell pertussis vaccine (*B.pertussis*, 6 mice per group) were restimulated with 1 μM of peptides 55-1 and 55-19/21 (C). Data are expressed as means of stimulation indices. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

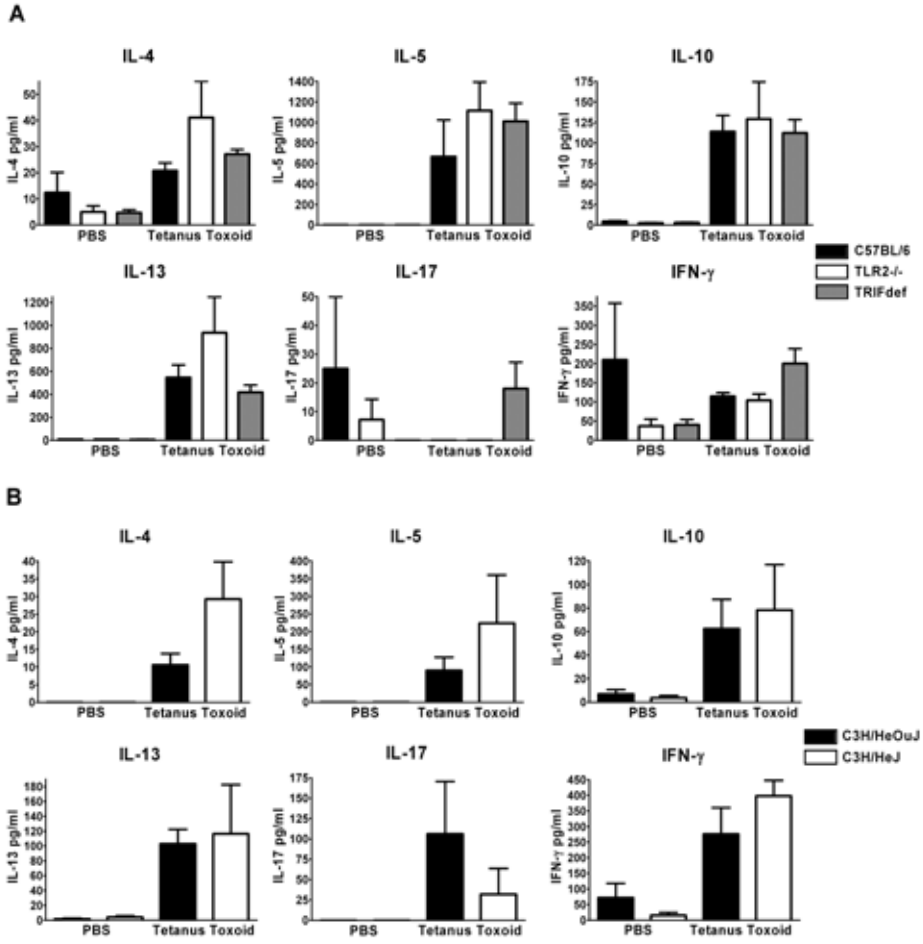

Figure 6. Cytokines produced by spleen cells after restimulation with tetanus toxoid. Spleen cells from mice immunized with PBS or tetanus toxoid were incubated for 4 days with 1 Lf/ml tetanus toxoid, after which IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ were determined in the supernatant with Luminex.. Results for C57BL/6, TLR2-/-, and TRIF-deficient mice are shown in panel A. Results for C3H/HeJ and C3H/HeOuJ mice are shown in panel B. Data are expressed as means of three mice per group, error bar indicates S.E.M. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Spleen cells of mice with a C57BL/6 background immunized with either *N. meningitidis* OMVs or PBS were restimulated with two P1.5-1,2-2 PorA peptides. Cytokines were analyzed in the supernatant for both peptides. Cells from TLR2-/- mice immunized with *N. meningitidis* OMVs tended to produce more IL-5, IL-10, IL-13, and IFN-γ after stimulation with peptide 11-24/25 (Fig. 7A). The other peptide, 4-

52/53, generally induced higher production of cytokines by spleen cells of all the mice immunized with *N. meningitidis* OMVs (Fig. 7B). Moreover, peptide 4-52/53 induced spleen cells of TRIF-deficient mice to produce significant higher levels of IL-4, IL-5, and IL-13 and tended to produce less IFN-γ compared to spleen cells of wild type C57BL/6 mice. Clearly, the response was skewed towards a Th2 response in the TRIF-deficient mice, consistent with what we noticed at the antibody level (Fig. 3A). Spleen cells of TLR2-/- mice also produced significant higher levels of IL-13 than wild type spleen cells (Fig. 2B). Furthermore, IL-5 and IL-17 tended to be higher. Spleen cells of C3H/HeJ and C3H/HeOuJ mice were restimulated with different peptide pools. Since peptide pool 6 induced the highest proliferation, cytokine production by cells stimulated with this pool was measured (Fig. 7C). Remarkably, spleen cells from C3H/HeJ mice immunized with *N. meningitidis* OMVs did not produce any of the cytokines analyzed, although the cells clearly proliferated as much as the C3H/HeOuJ cells (Fig. 5B). In contrast, C3H/HeOuJ cells produced all cytokines, except IL-4. Overall these results show that after immunization of wild type mice with *N. meningitidis* OMVs, antigen-specific T cells mainly produce IFN-γ. This suggests that *N. meningitidis* OMVs mainly induce a Th1 response in wild type mice. We also demonstrate that some antigen-specific T cells produce IL-17. Furthermore, our results suggest that TLR4 activation is required for the generation of cytokine producing T cells and that TRIF signalling contributes to a Th1 response. Finally, TLR2 activation is not required for the generation of cytokine producing T cells, but in contrast seems somewhat inhibitory.

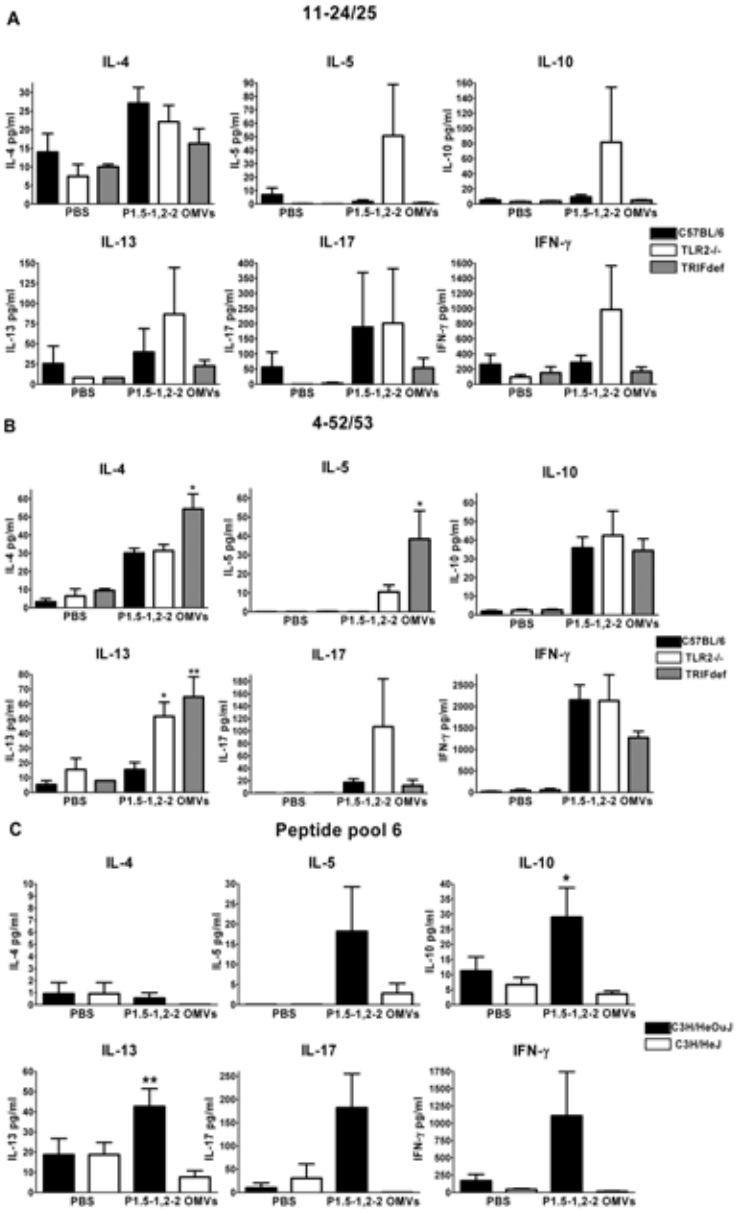

Figure 7. Cytokines produced by spleen cells after restimulation with P1.5-1,2-2 PorA peptides. Spleen cells from C57BL/6, TLR2-/-, or TRIF-deficient mice immunized with PBS or *N. meningitidis* P1.5-1,2-2 OMVs were incubated for 4 days with peptide 11-24/25 (A) or peptide 4-52/53 (B). Spleen cells from C3H/HeJ or C3H/HeOuJ mice immunized with PBS or *N. meningitidis* P1.5-1,2-2 OMVs were incubated for 4 days with peptide pool 6 (C). Cytokines IL-4, IL-5, IL-10, IL-13, IL-17, and IFN-γ were determined in the supernatant with Luminex.. Data are expressed as means of three mice per group (PBS), or six mice per group (*N. meningitidis* OMVs), error bar indicates S.E.M. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Spleen cells of C57BL/6, TLR2-/-, and TRIF-deficient mice immunized with whole cell pertussis vaccine were restimulated with two P.69 Prn peptides. Because the cells did not proliferate much, we did not expect very high levels of cytokines. Therefore, supernatants of cells stimulated with the two peptides were pooled and cytokine production was analyzed. Only IL-17 was specifically induced in spleen cells of whole cell pertussis vaccine immunized mice compared to PBS immunized mice (Fig. 8 and data not shown).

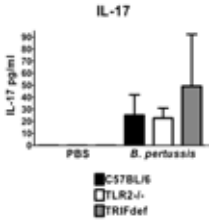

Figure 8. IL-17 production by spleen cells after restimulation with P.69 Prn peptides. Spleen cells of C57BL/6, TLR2-/-, and TRIF-deficient mice immunized with PBS or whole cell pertussis vaccine (*B. pertussis*) were incubated with peptides 55-1 and 55-19/21. Supernatants of cells stimulated with the two different peptides were pooled and IL-17 was measured with Luminex. Data are expressed as means of three mice per group (PBS), or six mice per group (whole cell pertussis vaccine), error bar indicates S.E.M. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Indeed it has been demonstrated previously that whole cell pertussis vaccine induces a Th17 response[56, 59, 60]. As we did not observe proliferation after peptide restimulation of spleen cells from C3H/HeJ and C3H/HeOuJ mice after immunization with whole cell pertussis vaccine, cytokine production of these cells was not measured.

Discussion

In the present study, we demonstrate that TLR4 signalling by LPS contributes to generation of adaptive immune responses after immunization with *N. meningitidis* OMVs or whole cell pertussis vaccine, while TLR2 activation was not required. Instead, TLR2-/- mice overall showed higher rather than lower responses compared to C57BL/6 mice after immunization with the *N. meningitidis* and *B. pertussis* vaccines. For example, IL-6 serum levels just after vaccination tended to be higher in the TLR2-/- mice. At the antibody level, IgG1 and IgG3 tended to be higher in *N. meningitidis* OMVs immunized TLR2-/- mice, whereas IgG3 and IgE tended to be higher in TLR2-/- mice immunized with whole cell pertussis vaccine. Finally, spleen cells of TLR2-/- mice immunized with *N. meningitidis* OMVs restimulated with P1.5-1,2-2 PorA peptides produced higher levels of several cytokines, including significantly higher levels of IL-13. Together these results indicate that TLR2 activation inhibited immune responses, especially Th2 responses, after vaccination. Similarly, TLR2-deficient mice were shown to have greatly enhanced Th1 and Tc1 responses after vaccination with yellow fever vaccine YF-17D[14]. Moreover, it has been demonstrated recently that activation of TLR2 by the yeast cell wall derivative zymosan induces a Treg response[61, 62]. On the other hand, TLR2 ligands have been shown to have adjuvant activity in several studies[11, 12, 28, 63]. In addition to induction of a Treg response, TLR2 activation has also been shown to induce a Th1 reponse[1, 64], or Th2 response 65-67. How TLR2 activation can lead to all these different responses is currently not clear, but it might depend for example on which ligand is used, whether the ligand is recognized by TLR2/TLR1 or TLR2/TLR6, or which cell type is targeted. In any case, our results show that TLR2 activation is not required for induction of adaptive immunity when TLR4 is also activated. However, even TLR4-deficient mice still develop adaptive immune responses after vaccination. This residual response might be due to TLR2 activation. To address this issue, also mice deficient in both receptors should be tested. Moreover, the vaccines might contain ligands of other TLRs or PRRs, which also contribute to the immunogenicity.

TRIF-deficient mice clearly showed altered immune responses after immunization with *N. meningitidis* OMVs and whole cell pertussis vaccine. Antigen- specific IgG2b and IgG2c levels were significantly reduced for both vaccines, which resulted in less bactericidal antibodies in the case of *N. meningitidis* OMVs. In addition, spleen cells of TRIF-deficient mice immunized with *N. meningitidis* OMVs restimulated with PorA peptides produced significantly higher amounts of IL-4, IL-5, and IL-13, while IFN-γ production was reduced. Together these data show that activation of the TRIF pathway leads to a Th1 response after immunization with *N. meningitidis* OMVs or whole cell

pertussis vaccine. Therefore, targeting of the TRIF pathway is important, because for both vaccines optimal responses after vaccination should include Th1-responses. Induction of the TRIF pathway eventually leads to the induction of type I interferon, which is not induced by the MyD88 pathway downstream of TLR2 or TLR4[4, 40, 68]. We have shown previously that ligands of TLR3, TLR4, TLR7, and TLR9 enhance the immunogenicity of a *N. meningitidis* outer membrane vaccine by promoting Th1-dependent antibody production, whereas ligands of other TLRs did not show adjuvant activity[26]. Importantly, activation of TLR3, TLR4, TLR7, and TLR9 leads to the induction of type I interferon, but activation of the other TLRs does not[69]. Possibly, TLR-induced type I interferon drives Th1 development and contributes to the adjuvant effect of TLR ligands. Indeed, the adjuvant effect of poly IC by inducing $CD4^+$ Th1 immunity and humoral immunity depends on type I interferon[70, 71]. Interestingly, the vaccine adjuvant Monophosphoryl lipid A, a low-toxicity LPS derivative, has been shown to preferentially activate the TRIF pathway[72].

We also show that TLR4-deficient mice have impaired immune responses after immunization with *N. meningitidis* OMVs and whole cell pertussis vaccine. After whole cell pertussis immunization of the TLR4-deficient C3H/HeJ mice, IL-6 and IL-12p70 were almost not detectable in the serum. Moreover, IgG, IgG1, and IgG2a levels were significantly lower in these mice. In contrast, others have found that antibody levels are not decreased in TLR4-deficient mice after immunization with whole cell pertussis vaccine[59, 60]. However, in these studies alum was added to the vaccine as an adjuvant, which mainly enhances humoral immunity. Presumably, the adjuvant effect of LPS is more redundant in the presence of another potent adjuvant like alum. The fact that we did not include alum could also explain why spleen cells did not respond greatly after restimulation with P.69 Prn peptides, because in the study where these P.69 Prn epitopes were identified, whole cell pertussis vaccine was also mixed with alum prior to immunization[56].

Surprisingly, TLR4-deficient C3H/HeJ mice did not have significantly decreased OMV-directed antibody titers compared to wild type C3H/HeOuJ mice. By contrast, we previously found that LPS-deficient *N. meningitidis* outer membrane complexes (OMCs) induced more than 1000-fold lower antibody titers in TLR4-proficient mice than wild type OMCs[26]. This suggests that LPS improves the immune response not only by activating TLR4, but also by other mechanisms. Possibly, LPS could target the vaccine to antigen presenting cells by binding to other receptors, for example CD14 or CR3. In contrast to antibody levels, cytokine production after restimulation with PorA peptides was severely impaired in spleen cells from C3H/HeJ mice. Similarly, it has been demonstrated that whole cell pertussis vaccine immunization led to comparable antibody titers in TLR4-deficient

and wild type mice, but IFN-γ and IL-17 production of T cells after antigen restimulation was much lower for TLR4-deficient mice[60]. These results suggest that TLR4 activation is more important for the induction of effector T cells than for antibody production. Remarkably, the C3H/HeJ spleen cells restimulated with PorA peptides proliferated as much as spleen cells from the wild type C3H/HeOuJ mice. This suggests that in C3H/HeJ mice, PorA-specific $CD4^+$ T cells were generated without effector function. Interestingly, it has been shown that DCs that had a mature phenotype but did not produce IL-12 and other pro-inflammatory cytokines promoted expansion of CD4+ T cell populations lacking effector function[73]. Possibly, *N. meningitidis* OMV vaccination induced that type of DCs in C3H/HeJ mice. After whole cell pertussis vaccine immunization, serum levels of IL-6 and IL-12p70 were very low in C3H/HeJ mice. In addition, it has been demonstrated that serum IL-12p70 production in vivo after administration of a TLR9 ligand depended entirely on DCs[74]. Unfortunately, in our experiments *N. meningitidis* OMVs were not potent enough to enhance serum IL-12p70 levels even in wild type animals, but in a recent report where a 10-fold higher dose of a comparable preparation of native *N. meningitidis* OMVs was used, IL-12p70 was elevated in serum of wild type mice but not in TLR4-deficient mice. Serum levels of MCP-1, TNF-α, IL-6, and IL-10 were also much lower in the TLR4-deficient mice[75].

Deficiencies in innate immunity genes in mice might have unanticipated effects on the immune system. Immune cells might respond differently to commensal bacteria in the gut for example. Indeed it has been demonstrated recently that mice deficient in TLR signalling spontaneously produce high levels of antibodies against their commensal bacteria to maintain host-commensal mutualism[76]. To check whether the deficiencies of the mice used in our study might have unexpected effects on the immune response after vaccination, we immunized them with tetanus toxoid, which is not known to activate TLRs. We did not find huge differences between the different mouse strains in the response to tetanus toxoid. However, we found that TLR4-deficient C3H/HeJ mice had slightly higher antibody titers than wild type C3H/HeOuJ mice. Consistently, T cell cytokine production was also higher in the C3H/HeJ mice. These results indicate that C3H/HeJ mice might respond differently to vaccination even in the absence of LPS. Also TLR2-/- mice tended to have higher levels of especially IgE, IL-4, IL-13 after tetanus toxoid immunization compared to wild type mice, which suggests that a stronger Th2 response was induced in the TLR2-/- mice. Interestingly, it has been demonstrated that TLR2-/- mice have a lower number of Treg cells than wild type controls, presumably because TLR2 ligands can activate Treg cells directly to expand[77, 78]. Therefore, induction of effector T cells might be less inhibited in TLR2-/- mice after vaccination.

Identification of the pathways that are activated by successful vaccines is important for the rational design of new vaccines and/or adjuvants. Moreover, most vaccines do not confer protection in 100% of individuals. These low responders might have deficiencies in the pathways induced by the vaccine. Additionally, environmental factors can influence the expression of PRRs. For example, TLR2 and CD14 expression was found to be significantly higher in farmers' children than non-farmers' children[79]. Moreover, individuals at the extremes of age often respond poorly to vaccination and have also been reported to have impaired responses after TLR stimulation[80, 81]. Little is known about the influence of PRR functionality on the immunogenicity of vaccines in humans. However, it has been demonstrated that individuals with very low antibody titers after vaccination with *Borrelia burgdorferi* OspA had lower cell-surface expression of TLR1[11]. OspA stimulation, which activates TLR2/TLR1, induced less TNF-α and IL-6 in macrophages of these low responders. Moreover, a polymorphism in TLR4 was associated with lower antibody titers in children after whole cell pertussis vaccine immunization[82]. Also polymorphisms in other genes in the TLR4 signaling pathway were associated with antibody titers in children after vaccination with this vaccine[83]. These data support a role for TLR4 in the immunogenicity of the whole cell pertussis vaccine also in humans.

In conclusion, our results show that two successful LPS-containing vaccines against the Gram-negative bacterial pathogens *N. meningitidis* and *B. pertussis* require TLR4 signaling for optimal immunogenicity and induction of Th1 responses in mice. On the other hand, TLR2 activation was not required. These findings confirm that TLR4 and the TRIF pathway are attractive targets for adjuvants in vaccines.

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Chapter 4

Naturally occurring lipid A mutants in *Neisseria meningitidis* from patients with invasive meningococcal disease are associated with reduced coagulopathy

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Abstract

Neisseria meningitidis is a major cause of bacterial meningitis and sepsis worldwide. Lipopolysaccharide (LPS), a major component of the Gram-negative bacterial outer membrane, is sensed by mammalian cells through Toll-like receptor 4 (TLR4), resulting in activation of proinflammatory cytokine pathways. TLR4 recognizes the lipid A moiety of the LPS molecule, and the chemical composition of the lipid A determines how well it is recognized by TLR4. *N. meningitidis* has been reported to produce lipid A with six acyl chains, the optimal number for TLR4 recognition. Indeed, meningococcal sepsis is generally seen as the prototypical endotoxin-mediated disease. In the present study, we screened meningococcal disease isolates from 464 patients for their ability to induce cytokine production *in vitro*. We found that around 9% of them were dramatically less potent than wildtype strains. Analysis of the lipid A of several of the low-activity strains by mass spectrometry revealed they were penta-acylated, suggesting a mutation in the *lpxL1* or *lpxL2* genes required for addition of secondary acyl chains. Sequencing of these genes showed that all the low activity strains had mutations that inactivated the *lpxL1* gene. In order to see whether *lpxL1* mutants might give a different clinical picture, we investigated the clinical correlate of these mutations in a prospective nationwide observational cohort study of adults with meningococcal meningitis. Patients infected with an *lpxL1* mutant presented significantly less frequently with rash and had higher thrombocyte counts, consistent with reduced cytokine induction and less activation of tissue-factor mediated coagulopathy. In conclusion, here we report for the first time that a surprisingly large fraction of meningococcal clinical isolates have LPS with underacylated lipid A due to mutations in the *lpxL1* gene. The resulting low-activity LPS may have an important role in virulence by aiding the bacteria to evade the innate immune system. Our results provide the first example of a specific mutation in *N. meningitidis* that can be correlated with the clinical course of meningococcal disease.

Author Summary

Neisseria meningitidis is a Gram-negative bacterium that can cause the life-threatening diseases meningitis and sepsis. Most of the symptoms seen in these diseases are the result of excessive stimulation of the immune system by lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria. The structure of the membrane-anchoring part of LPS, named lipid A, determines how well it is recognized by TLR4, the receptor which triggers this response. *N. meningitidis* has been shown previously to make a highly active form of LPS with six fatty acyl chains in its lipid A moiety. We now show that a surprisingly large fraction of meningococcal patient isolates have a mutation in one of their lipid A biosynthesis genes which makes their LPS underacylated and which strongly decreases their capability to activate the immune system. Presumably, selection of these mutants is promoted because the reduced activation aids the bacteria to escape elimination within the human host. We further demonstrate that meningitis patients infected with these LPS mutants have less severe symptoms than patients infected with wildtype strains. This is the first instance where a specific bacterial mutation can be correlated with the clinical course of meningococcal disease.

Introduction

Neisseria meningitidis is a major cause of bacterial meningitis and sepsis worldwide[1]. While it is a frequent commensal of the human upper respiratory tract, in some individuals the bacterium spreads to the bloodstream causing meningitis and/or sepsis, serious conditions with high morbidity and mortality. As in all Gram-negative bacteria, lipopolysaccharide (LPS) is a major component of the outer membrane of *N. meningitidis*. It is now well established that LPS is sensed by mammalian cells through Toll-like receptor 4 (TLR4), in combination with coreceptors MD-2 and CD14[2]. Activation of this complex leads to recruitment of the adapters MyD88, Mal, TRIF, and TRAM to the cytoplasmic domain of TLR4[3]. These adapters initiate signal transduction pathways that lead to induction of innate immunity. These pathways are classified in a so called "MyD88-dependent" pathway involving MyD88 and Mal, and a "MyD88-independent" pathway involving TRIF and TRAM. Hallmarks of MyD88-dependent and MyD88-independent signaling are induction of pro-inflammatory cytokines and type I IFN respectively. While the response to LPS can be beneficial to the host by containing a beginning infection, it can also be detrimental when excessive stimulation occurs through growth of large numbers of bacteria in the bloodstream as happens during sepsis[2, 4, 5].

TLR4 recognizes the lipid A moiety of the LPS molecule[2]. The chemical composition of the lipid A determines how well it is recognized by TLR4 and consequently it determines the biological activity of the LPS. *N. meningitidis* has been reported to produce lipid A with six acyl chains, the optimal number for TLR4 recognition[6]. Indeed purified LPS of this bacterium is highly active and plasma concentrations of LPS in patients with meningococcal disease correlate strongly with mortality risk[7]. LPS is also important in the activation of the coagulation system through upregulation of tissue factor. Excessive activation of the coagulation system can lead to disseminated intravascular coagulation (DIC), the most feared complication of invasive meningococcal disease[1]. DIC is clinically characterized by hypotension, petechial rash, and depletion of thrombocytes and coagulation factors.

Uniquely among Gram-negative bacteria, *N. meningitidis* can grow without LPS, as was shown by us when we constructed a mutant with an inactivated *lpxA* gene, required for the first step in LPS biosynthesis[8]. In addition, we have previously shown that insertional inactivation of the *lpxL1* or *lpxL2* genes required for addition of secondary acyl chains leads to reduced biological activity of meningococcal LPS[9, 10]. The possibility that such mutations might also occur naturally was suggested to us by a report showing that the group Y strain HF13 was defective in signaling through the MyD88-independent pathway and TLR4[11].

Here we report that strain HF13 has penta-acylated lipid A due to a mutation in its *lpxL1* gene. Screening of a selection of clinical isolates revealed *lpxL1* mutations in approximately 13% of meningococcal disease isolates of all major serogroups and clonal complexes. Several different kinds of mutations were found. We also found evidence for on-and-off switching of *lpxL1 in vivo* in humans. Importantly, patients with meningococcal meningitis that were infected with an *lpxL1* mutant strain had less severe systemic inflammation and reduced coagulopathy.

Materials and Methods

Ethics statement

This observational study with anonymous patient data was carried out in accordance with the Dutch privacy legislation. Written informed consent to use data made anonymous was obtained from the patient (if possible) or from the patient's legal representative.

N. meningitidis strains

Strain HF13 was a kind gift from M. Kilian. The constructed *lpxA* and *lpxL1* mutants were generated in the H44/76 strain as previously described[8, 9]. All other strains were selected from the collection of the Netherlands Reference Laboratory for Bacterial Meningitis. Details about year of isolation, serogroup, genotype and anatomical site of isolation are presented in Table S1. Meningococci were cultured in GC broth or on GC plates (Difco laboratories) supplemented with 1% (vol/vol) Vitox (Oxoid) at 37ºC in humified atmosphere of 5% CO_2[12]. Bacteria were suspended in PBS and the A_{620} was determined. The bacteria were heat inactivated at 56ºC for 30 min. Serogrouping were performed as described elsewhere[13]. MLST was performed as described by Maiden et al[14].

Lipid A structure

Bacteria were grown as described above and suspended in isobutyric acid-ammonium hydroxide 1M (5:3, v/v). Lipid A was extracted as described previously[15] with slight modifications. The lipid A structure was analyzed by nanoelectrospray tandem mass spectrometry (MS/MS) on a Finnigan LCQ in the negative (MS) or positive (MS/MS) ion mode[16].

Sequencing

DNA was extracted from boiled cultures of *N. meningitidis*. Sequencing of *lpxL1* was carried out using primers 344-2 and 670-1 (Table S2) and BigDyeTerminator chemistry (Applied Biosystems) according to the instructions of the manufacturer. The primers used to obtain sequences upstream and downstream of *lpxL1* are presented in Table S2. Sequence traces were obtained with ABI Big-dyes and an ABI 3730 sequencer.

Cell lines and PBMCs

PBMC from HLA-oligotyped donors after leukapheresis were isolated by centrifugation of buffy coat cells on Ficoll-Hypaque (Pfizer) and were used after cryopreservation. For experiments and/or maintenance, the human monocyte cell line Mono-mac-6 (MM6), the mouse macrophage cell line J774A.1, and PBMCs were suspended in IMDM (Gibco

BRL) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 300 µg/ml l-glutamine (Gibco BRL), and 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL). For experiments and maintenance of HEK-293 cells stably transfected with human TLR4A, MD-2, and CD14 (Invivogen), DMEM (Gibco BRL) was used, supplemented with 10% FCS, 10 μg/ml blasticidin (Invivogen), and 50 μg/ml Hygromycin B (Invivogen).

ELISA

Depending on the experiment either J774A.1, MM6, PBMCs, or HEK-293 hTLR4/MD-2/CD14 cells were used. Different plates and quantities of cells were used: 1.10^6 cells in 1 ml medium per well in 12-well plates, 9.10^4-5.10^5 cells in 250-1000 μl medium per well in 24-well plates, and 1.10^5-3.10^5 cells in 200-300 μl medium per well in 96-well plates. Cells were stimulated with bacteria and incubated o/n at 37 °C in a humidified atmosphere containing 5% CO_2. Cytokine concentrations in the culture supernatants were quantified with ELISA. Mouse IP-10 was determined with mouse IP-10 ELISA kit (R&D systems) and human IL-6, TNF-α, IL-1β, and IL-8 with PeliPairTM reagent sets (Sanquin).

Meningitis cohort study

The Dutch Meningitis Cohort Study included 258 patients with meningococcal meningitis; from 254 patients the bacterial strain was stored in the Netherlands Reference Laboratory for Bacterial Meningitis[17]. Inclusion and exclusion criteria have been described extensively elsewhere[13]. In summary, eligible patients were older than 16 years, had bacterial meningitis confirmed by culture of cerebrospinal fluid (CSF), and were listed in the database of the Netherlands Reference Laboratory for Bacterial Meningitis from October 1998 to April 2002. This laboratory receives CSF isolates from about 85% of all patients with bacterial meningitis in the Netherlands. The treating physician was contacted, and informed consent was obtained from all participating patients or their legally authorized representatives. This observational study with anonymous patient data was carried out in accordance with the Dutch privacy legislation. Patients underwent a neurologic examination at discharge, and outcome was graded with the Glasgow Outcome Scale. This measurement scale is well validated with scores varying from 1 (indicating death) to 5 (good recovery). A favourable outcome was defined as a score of 5, and an unfavourable outcome as a score of 1-4. Focal neurologic deficits were defined as focal cerebral deficits (aphasia, monoparesis, or hemiparesis) or cranial nerve palsies. Serogrouping, MLST, and susceptibility testing of meningococcal isolates were performed by the Netherlands Reference Laboratory for Bacterial Meningitis.

Statistics

The Mann-Whitney U test was used to identify differences between groups in continuous variables, and dichotomous variables were compared by the chi-square or Fisher exact test. All statistical tests were 2-tailed, and a p value less than 0.05 was regarded as significant.

List of accession numbers/ID numbers for genes mentioned in the text

Please see Table S3 for accession numbers.

Results

Strain HF13 is a natural lpxL1 mutant

Mogensen et al. demonstrated that the serogroup Y strain HF13 is defective in TLR4 activation and initiation of MyD88-independent signaling[11]. Reduced biological activity of meningococcal LPS is associated with altered lipid A structure[9, 10]. Therefore, the lipid A structure of strain HF13 was assessed by mass spectrometry (Fig. 1A). The spectrum shows major peaks that correspond with lipid A with only five acyl chains. One of the two secondary C_{12} acyl chains is absent, but the spectrum is not conclusive on which one, since the C_{12} acyl chains have the same mass. This result implies that in strain HF13 either *lpxL1* or *lpxL2* is inactive, as we previously found that the addition of the secondary C_{12} acyl chains to lipid A requires active *lpxL1* and *lpxL2*[9]. Sequence analyses of both genes showed a normal *lpxL2* sequence, but the *lpxL1* sequence contained one adenosine deletion in a poly adenosine tract, leading to a frameshift and a premature stop of the translated protein (Fig. 2, Table 1).

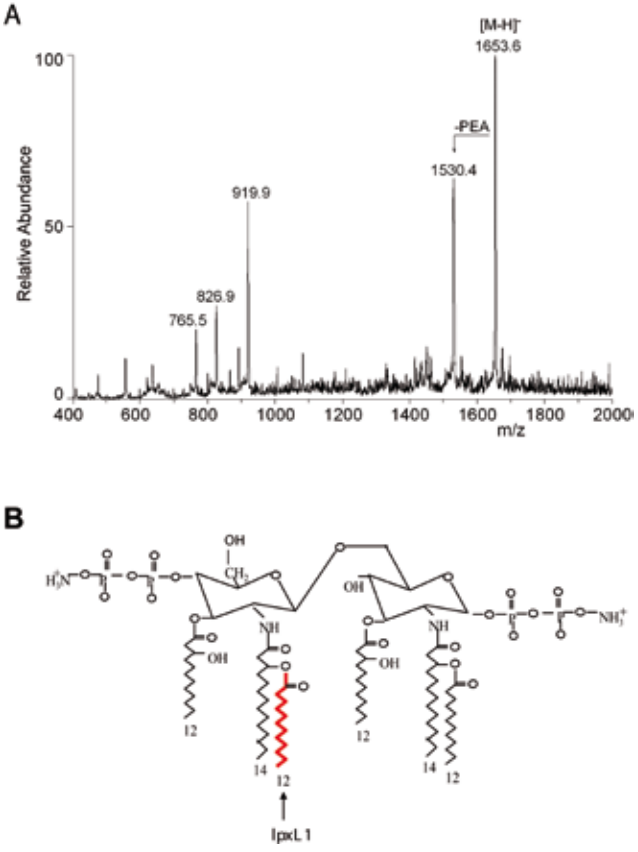

Figure 1. Strain HF13 is an *lpxL1* mutant. (A) Mass spectrum of HF13 lipid A. The highest peak (1653.6) corresponds to penta-acylated lipid A with two phosphate groups and one phosphoethanolamine (PEA), the second peak (1530.4) corresponds to penta-acytlated lipid A with two phosphate groups without PEA. (B) Depiction of *N. meningitidis* wildtype lipid A. The acyl chain that is added by LpxL1 is indicated.

Figure 2. Presentation of all *lpxL1* mutations**.** The *lpxL1* gene sequence of strain MC58 is shown including the different types of mutations and their position in the gene found among isolates from patients. Each type of mutation is indicated with a different color. An '_' indicates a nucleotide that is deleted in the mutant strain, pink indicates an insertion element (type I and II mutations), yellow indicates a deletion of a guanine in a stretch of five guanines (type III mutation), green indicates a deletion of an adenosine in a stretch of five adenosines (type IV mutation), light blue indicates a deletion of an adenosine in a stretch of seven adenosines (type V mutation), dark blue indicates an insertion of an adenosine in a stretch of seven adenosines (type V mutation) and the sequences that are highlighted in red or gray are deleted in the mutant strain (type VI and VII mutations respectively).

The inactivated *lpxL1* gene in strain HF13 results in a penta-acylated lipid A lacking the secondary acyl chain at the 2'-position in lipid A, while *N. meningitidis* typically has a hexa-acylated lipid A (Fig. 1B). These results provide an explanation for the inability of strain HF13 to activate TLR4 and to initiate MyD88-independent signaling.

Mutations in lpxL1 are present in several serogroups and clonal complexes

To evaluate the distribution of *lpxL1* mutations among meningococcal isolates from patients, we initially screened a panel of 56 serogroup Y meningococcal isolates for their capacity to induce the MyD88-independent cytokine IP-10 in the mouse macrophage cell line J774A.1 (Fig. S1). As controls, strain H44/76 and HF13 were included. Of 56 serogroup Y isolates, eight strains induced like HF13 little or no IP-10. Sequence analyses of *lpxL1* of these isolates revealed that they all had mutations in *lpxL1*, resulting in an inactive gene. Five strains had one adenosine deletion in a poly A tract just like strain HF13 (type V mutation, Fig. 2, Table 1), two strains had a deletion of ten nucleotides (type VI mutation), and one strain had an insertion of the insertion element IS1301 (Type I mutation).

Table 1. List of all *lpxL1* mutant strains.

Strain no.	Isolated from	serogroup	ST	Clonal complex	Type of mutation in *lpxL1*[a]
lpxL1 mutants among 56 serogroup Y isolates					
HF13	Nd[b]	Y	nd	nd	V
2011169	blood	Y	23	23	V
2040760	blood	Y	23	23	V
970455	blood	Y	nd	nd	V
2050913	joint puncture	Y	2786	23	V
971523	CSF	Y	nd	nd	V
971886	CSF	Y	nd	nd	VI
982195	blood	Y	nd	nd	VI
2040608	CSF	Y	nd	nd	I
lpxL1 mutants among 114 isolates representing major serogroups and clonal complexes					
2000569	blood	X	750	750	V
2011833	blood	C	3553	269	V
2041268	blood	B	4926	35	V
2050093	blood	B	461	461	V
	blood	C	337	41/44	V
2051372	blood	B	461	461	V
2010151	blood	C	461	461	IV
2021270	CSF	C	461	461	III
2041396	CSF	B	4930	18	III
2050806	CSF	B	213	213	III
2071416	blood	Y	23	23	VI
2020799[c]	CSF	B	35	35	conserved amino acid change
2050392	CSF	B	213	213	VII
lpxL1 mutants among multiple isolates from a single patient					
Patient 94176					
941761 I	CSF	C	nd	nd	conserved amino acid change
941761 III	Troat swab	C	nd	nd	wildtype
Patient 9707010					
9707101	CSF	C	nd	nd	wildtype
970710 III	nose swab	C	nd	nd	IV
Patient 971859					
9718591	CSF	C	nd	nd	IV
971859 III	Throat swab	C	nd	nd	wildtype
lpxL1 mutants among isolates from 254 patients in the prospective cohort study					
2012202	CSF	B	41	41/44	V
2020434	CSF	C	11	11	V
991174	CSF	C	11	11	V
990576	CSF	B	571	41/44	V
991382	CSF	B	191	41/44	V
2011833	CSF	C	3553	269	V
991344	CSF	B	42	41/44	III
2000607	CSF	B	40	41/44	III
2000311	CSF	B	461	461	III
991093	CSF	B	5451	32	III
2020622	CSF	B	5458	41/44	IV
990344	CSF	B	5449	41/44	IV
2010640	CSF	B	1474	41/44	conserved amino acid change
2011334	CSF	C	11	11	II
2011764	CSF	B	303	41/44	conserved amino acid change
992008	CSF	B	146	41/44	Not detected

[a]Type of mutations found in *lpxL1*. Colors in parentheses correspond to colors shown in figure 2. Type I mutation: insertion of IS1301 (pink), type II mutation: insertion of IS1655 (pink), type III mutation: deletion of a guanine in a stretch of five guanines (yellow), type IV mutation: deletion of an adenosine in a stretch of five adenosines (green), type V mutation: deletion or insertion of an adenosine in a stretch of seven adenosines (light and dark blue), type VI mutation: deletion of ten nucleotides (red), type VII mutation: deletion of C-terminal part of the *lpxL1* gene (gray). For all strains with conserved amino acid changes, the inactivation of *lpxL1* has been confirmed with analysis of the lipid A by mass spectrometry. [b]Nd: not determined. [c]Strain 2020799 was part of both the panel of 114 isolates representing all major serogroups and clonal complexes and the panel of 254 isolates from patients in the prospective cohort study.

These results prompted us to investigate the distribution of *lpxL1* mutations among meningococci of the major serogroups and clonal complexes. Previously, we have shown that at higher dilutions an *lpxL1* mutant induces less pro-inflammatory cytokines than wildtype *N. meningitidis*[9, 10]. To identify meningococcal isolates with mutations in *lpxL1*, isolates were tested on their capacity to induce IL-6 in the human monocytic cell line Mono Mac 6 (MM6). Of 114 isolates, representing all major serogroups and clonal complexes, 13 were found to induce low amounts of IL-6 (Fig. S2). Sequence analyses of *lpxL1* showed that 12 isolates had a mutation in *lpxL1*, rendering the gene inactive (Fig. 2, Table 1). Of these strains, 10 had an insertion or deletion in a polyadenosine or polyguanosine tract (type III, IV and V mutations); six of these had the same mutation as found in the majority of mutant serogroup Y strains. One strain had a type VI mutation, like in the two aforementioned serogroup Y strains. One strain had a deletion of the C-terminal part of the gene (type VII mutation). The remaining strain (2020799) had apparently no mutation in *lpxL1* that would lead to its inactivation. However, closer examination of its putative amino acid sequence showed that one amino acid was altered at a position conserved in all known *lpxL1* homologues. Therefore, the LpxL1 protein of this strain is probably nonfunctional. Indeed, we confirmed that strain 2020799 had penta-acylated lipid A by mass spectrometry (data not shown). As a control, also *lpxL1* of 34 strains that induced a normal level of IL-6 was sequenced. As expected, these strains had no mutations in *lpxL1* (data not shown). Together, seven unique *lpxL1* mutations were found among this panel of different serogroups and different clonal complexes, indicating that inactivation of *lpxL1* must have occurred multiple times independently. The results show that *lpxL1* mutations are not associated with serogroup or clonal complexes and occur also among the serogroup B and C strains, which are prevalent among isolates from patients with meningococcal disease in Europe.

Screening of lpxL1 mutations in a panel of multiple isolates per patient

Most of the identified *lpxL1* mutations were in nucleotide repeats of adenosines and guanosines, the type III, IV and V mutations (Fig. 2, Table 1). These sequences are prone to cause slippage of the DNA polymerase during DNA replication, leading to reversible frameshift mutations. This slipped-strand mispairing is the most common mechanism of translational phase variation, the process of random and reversible on-and-off switching of a gene. Phase variation creates a phenotypically diverse population, allowing the bacterium to adapt to different microenvironments within the human host. To investigate whether *N. meningitidis* can switch *lpxL1* on-and-off we screened a panel of strains obtained from different anatomical locations within individual patients: isolates from the

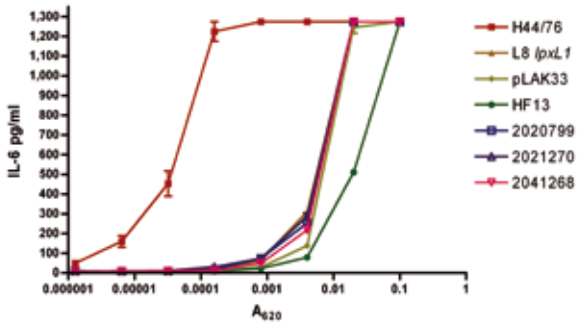

Figure 3. Comparison of IL-6 induction in MM-6 cells between wildtype strains and lpxL1 mutants. MM-6 cells were stimulated for 18 h with titrations of indicated strains and IL-6 in supernatant was quantified with ELISA. H44/76 is a wildtype strain, L8 lpxL1 is a constructed lpxL1 mutant, pLAK33 is an LPS-deficient mutant, and all other strains are spontaneous lpxL1 mutants. Results of one representative experiment of three independent experiments are shown. Error bars indicate S.E.M. of triplicates.

blood and/or cerebrospinal fluid (CSF) as well as from the throat and/or nose of 40 patients were used. The MM6 cell line was stimulated with these strains and IL-6 production was measured with ELISA. Three strains induced low levels of IL-6 compared to wildtype *N. meningitidis* (Fig. S3). These isolates were from three different patients. Two strains were isolated from the cerebrospinal fluid and one strain was isolated from the throat. The other isolates of these patients induced normal levels of IL-6. The *lpxL1* genes of all isolates of these three patients were sequenced and found to be mutated in the isolates that induced low IL-6, but not in the isolates that induced normal IL-6 (Fig. 2, Table 1). Two strains had a type IV mutation, which potentially is reversible. The third strain had a point mutation leading to substitution of a conserved amino acid. These results suggest that in the host the expression status of *lpxL1* of meningococci is subject to phase variation.

lpxL1 mutants induce less pro-inflammatory cytokines in a TLR4-dependent manner

The identified *lpxL1* mutations occurred in strains of widely varying genetic background, and it is therefore conceivable that other factors besides altered LPS contribute to their reduced cytokine induction. To investigate this, titrations of four of the spontaneous *lpxL1* mutants were compared in their capacity to induce cytokines in MM6 cells with titrations of our previously constructed *lpxL1* knockout mutant and its parent strain H44/76, as well as the completely LPS-deficient strain pLAK33 (Fig. 3). Clearly, the LPS-deficient strain pLAK33 is much less potent in inducing IL-6 than the wildtype strain H44/76. IL-6 induction by the constructed *lpxL1* mutant is similar to that by pLAK33 and the four *lpxL1* mutants isolated from patients.

To demonstrate that the *lpxL1* mutants induced less cytokines than wildtype strains because their LPS is less well recognized by the LPS receptor complex, titrations of a similar panel of strains was used to stimulate HEK293 cells transfected with human TLR4,

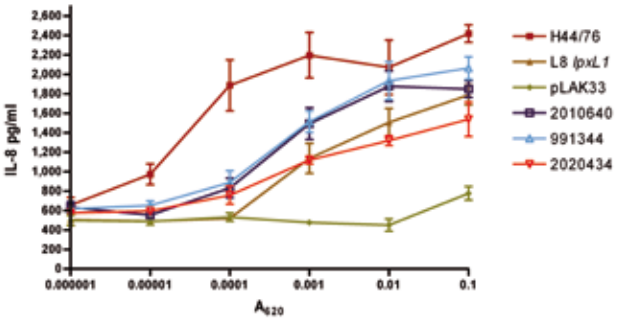

Figure 4. Comparison between wildtype strains and lpxL1 mutants of IL-8 induction in HEK293 cells transfected with human TLR4. HEK293 cells transfected with human TLR4, CD14, and MD-2 were stimulated for 18 h with titrations of indicated strains and IL-8 in supernatant was quantified with ELISA. H44/76 is a wildtype strain, L8 *lpxL1* is a constructed *lpxL1* mutant, pLAK33 is an LPS-deficient mutant, and all other strains are spontaneous *lpxL1* mutants. Results of one representative experiment of three independent experiments are shown. Error bars indicate S.E.M. of triplicates.

MD-2, and CD14. Activation of the receptor complex was assessed by measuring IL-8 production (Fig. 4). Wildtype strain H44/76 was much more efficient in TLR4 activation than the mutants. All *lpxL1* mutants, either constructed or isolated from patients, showed a similar decrease in IL-8 induction, while the LPS-deficient pLAK33 cells were even less active. Together, these results demonstrate that the *lpxL1* mutants activate human TLR4 less efficiently, and this is the sole reason for their reduced biological activity.

We have shown that *lpxL1* mutants induce less cytokines in human and murine cell lines. However, these *in vitro* models do not necessarily represent the situation *in vivo* and do not take into account the genetic diversity of the human population. To mimic a systemic meningococcal infection more closely, also human peripheral blood mononuclear cells (PBMCs) of several donors were stimulated with titrations of a selection of *N. meningitidis* strains. After stimulation, concentrations of IL-6, TNF-α, and IL-1β were determined in the supernatant (Fig. 5). These pro-inflammatory cytokines are known to mediate the toxic effects of LPS[2]. In all donors, wildtype strain H44/76 induced much more IL-6, TNF-α, and IL-1β than the mutants. Overall, the constructed and spontaneous *lpxL1* mutants showed a similar reduction in cytokine induction.

Meningitis patients infected with lpxL1 mutant meningococci have reduced inflammation and coagulopathy

We next explored whether infection with *lpxL1*-mutant meningococcal strains was associated with a particular clinical phenotype. The meningococcal isolates from 254 patients from a prospective nationwide observational cohort study of 696 adults with community-acquired bacterial meningitis in the Netherlands (period, 1998-2002)[13, 17] were

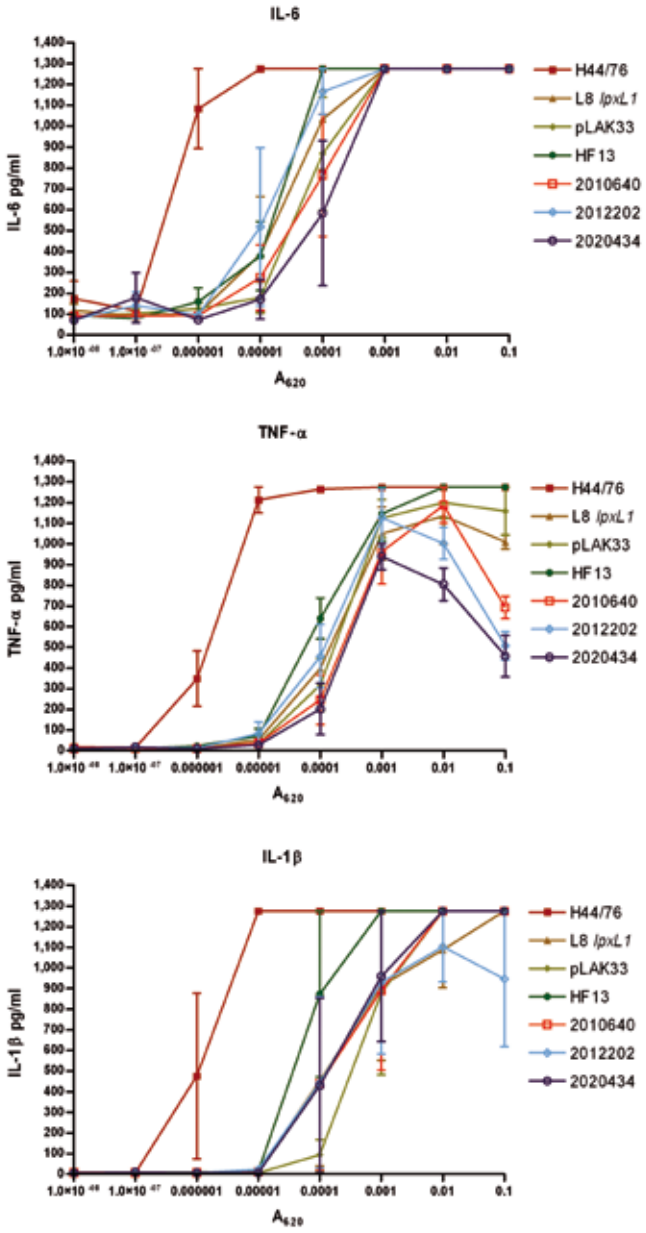

Figure 5. Comparison between wildtype strains and lpxL1 mutants in pro-inflammatory cytokine induction in PBMCs. PBMCs from three different donors were stimulated with titrations of the indicated strains and IL-6, TNF-α, and IL-1β were quantified in the supernatant 18 h after stimulation. H44/76 is a wildtype strain, L8 *lpxL1* is a constructed *lpxL1* mutant, pLAK33 is an LPS-deficient mutant, and all other strains are spontaneous *lpxL1* mutants. Results of one representative experiment of two independent experiments are shown. Error bars indicate S.E.M. of triplicates.

Table 2. Clinical features of 254 adults with meningococcal meningitis due to lpxL1 mutant and wildtype strains.

Characteristic	Wildtype (N=237)	*lpxL1* mutant[e] (N=17)	P-value[f]
Admission			
Age in yr - median (IQR[a])	31 (19-51)	21 (19-30)	0.053
Fever (temperature > 38.0 ºC) – no. (%)	87/231 (38)	2/16 (13)	0.057
Neck stiffness – no. (%)	205/234 (88)	17/17 (100)	0.23
Median score on GCS[b] (IQR)	13 (10-15)	12 (11-15)	0.97
Rash	157/236 (67)	5/16 (31)	0.006
Hypotension (Systolic BP[c] <90mmHg) – no. (%)	23/204 (11)	0/16 (0)	0.39
Focal neurological deficits – no. (%)	51/237 (22)	2/17 (12)	0.54
Laboratory investigations			
Cerebrospinal fluid white cell count – median per mm^3 (IQR)	5205 (1466-12605)	5376 (3063-11416)	0.89
Positive blood culture – no. (%)	117/207 (49)	11/16 (69)	0.44
Platelet count – median 10^9/L (IQR)	162 (123-211)	215 (169-270)	0.005
Serum creatinine – median μmol/L (IQR)	95 (77-128)	84 (69-99)	0.051
Clinical course			
Septic shock – no. (%)[d]	30/237 (13)	0/17 (0)	0.23
Neurologic complication – no. (%)	98/237 (41)	5/17 (29)	0.45
Outcome			
Death – no. (%)	18/237 (8)	1/17 (6)	1.00
Focal neurological deficits – no. (%)	25/218 (11)	3/16 (19)	0.42
Unfavourable outcome – no. (%)	27/237 (11)	3/17 (18)	0.43

[a]IQR denotes interquartile range, [b]GCS Glasgow Coma Scale, [c]BP blood pressure. [d]Septic shock was defined as systolic blood pressure < 90 mmHg with positive blood culture. Systolic blood pressure was measured on admission in 243 patients, GCS in 253 patients, CSF white cell count in 238 patients, and serum platelet count in 241 patients. [e]In 16 strains a mutation in *lpxL1* was found, but not in strain 992008. [f]The Mann-Whitney U test was used to identify differences between groups in continuous variables, and dichotomous variables were compared by the chi-square or Fisher exact test.

analyzed for their ability to induce IL-6. Of the 254 isolates, 172 (68%) were of serogroup B, 78 (31%) of serogroup C, 3 (1%) of serogroup Y, and one (<1%) of serogroup W135. Multilocus sequence typing showed 91 unique sequence types. The most prevalent clonal complexes were cc41/44 (41%), cc11 (24%), and cc32 (16%) [17].

MM6 cells were stimulated with these strains and IL-6 induction was assessed (Fig. S4). The isolates of 17 patients (7%) showed a decreased IL-6 induction and sequencing revealed mutations in *lpxL1* in all but one (Fig. 2, Table 1). Twelve isolates had a type III, IV or V mutation. Three strains had a point mutation leading to substitution of an essential amino acid, and one strain had an IS1655 insertion. In one strain (992008) we were unable to identify a mutation in *lpxL1* that could lead to gene inactivation or inactive gene product. Further analyses with mass spectrometry to determine the mass of its lipid A and silver staining of a Tricine-SDS-PAGE gel to analyze the size and quantity of its LPS, demonstrated that LPS was not detectable in this strain (results not shown). The responsible mutation remains to be identified. There were no overall differences in *lpxL1*

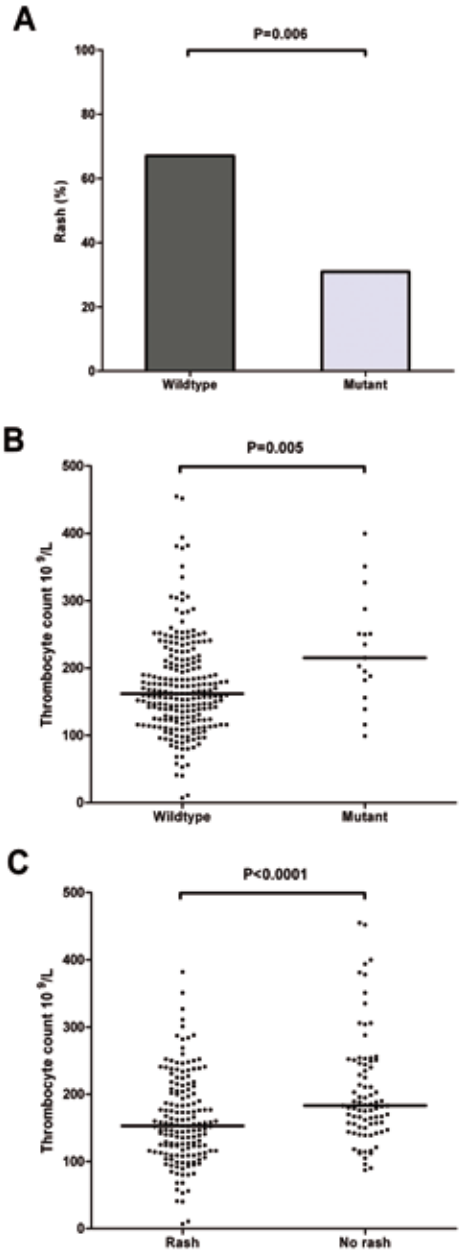

Figure 6. Clinical correlate of lpxL1 mutations in meningococcal meningitis. (A) Frequency of rash in patients presenting with meningitis infected by *lpxL1* wildtype and mutant strains. (B,C) Platelet counts on admission for *lpxL1* wildtype and mutant strains (B) and patients presenting with and without rash (C). Horizontal bars reflect medians. The Mann-Whitney U test was used to identify differences between groups in continuous variables, and dichotomous variables were compared by the chi-square or Fisher exact test.

mutation frequency between serogroups (P=0.85) and clonal complexes (P=0.56).

Next, we correlated results of the mutation analysis with clinical data (Table 2)[13, 17]. Patients infected with *lpxL1* mutant strains tended to be younger (P=0.053) and to present less frequently with fever (P=0.057). None of the patients infected with an *lpxL1* mutant strain presented with hypotension and these patients had correspondingly lower levels of serum creatinine. They were less likely to present with rash compared with those infected with wildtype meningococci (5/16 [31%] vs. 157/236 (67%); P=0.006; Fig. 6) and had higher platelet counts (P=0.005). Rash was strongly related with lower platelet counts (P<0.0001). To investigate the possibility that the clinical differences found between the two patient groups were confounded by the different ages of the patient groups, a multivariate analysis adjusting for age was performed. The difference in platelet count (P=0.003) and rash (P=0.004) remained statistically significant after adjusting for age. Subgroup analysis of clonal complex 41/44 showed similar results. The differences in platelet count (P=0.007), rash (P=0.006), and age (P=0.053) between patients infected by mutant and wildtype strains were also present in the subgroup of clonal complex 41/44.

None of the patients infected with *lpxL1* mutant strains developed septic shock during clinical course, while 13% of the wildtype-infected patients did. One patient infected with an *lpxL1* mutant strain died of respiratory failure after multiple seizures. By contrast, sepsis was the leading cause of death among patients infected with wildtype meningococci (14 of 16 fatalities, 88%).

Thus, the *lpxL1* mutation occurs frequently among meningococci causing meningitis. Patients infected by mutant strains have a clinical phenotype consistent with less systemic inflammation and reduced activation of the coagulant system.

Discussion

Overall, we screened meningococcal isolates of 464 different patients and identified 40 strains with an *lpxL1* mutation. An additional low-activity strain had no *lpxL1* mutation but appeared to be LPS-deficient; the responsible mutation is currently under investigation. Thus, 8.6% of patients were infected with an *lpxL1* mutant, which is surprisingly common. There are several lines of evidence making it very likely that *lpxL1* mutants arise spontaneously in the host instead of introduction of an *lpxL1* mutation into one or several clones and subsequent spreading among the meningococcal population. Firstly, *lpxL1* mutations exist in isolates of many serogroups and clonal complexes. Secondly, we identified 12 unique mutations in *lpxL1*. Thirdly, most *lpxL1* mutations (71%) are due to frameshifts in homopolymeric nucleotide tracts, making phase variation likely. Finally, we found evidence for switching from wildtype to *lpxL1* mutant *in vivo* in patients for which multiple isolates were available.

A picture emerges of *N. meningitidis* modulating its lipid A structure under selective pressure. Under some conditions, hexa-acyl lipid A has to be beneficial to compensate for enhanced recognition by the innate immune system. Lipid A with six acyl chains can protect bacteria from the antibacterial molecules in mucosal secretions, consistent with the observation that many bacteria inhabiting the respiratory tract and gut still produce hexa-acyl LPS[18]. Chronic inflammation of these environments due to LPS stimulation is probably prevented because epithelial cells express low levels of either TLR4, MD-2, or CD14 at the mucosal surface. On the other hand, the submucosal spaces are normally sterile and the defense cells present there, such as macrophages, dendritic cells, and neutrophils, express all the components of the LPS receptor complex and can therefore respond potently after an encounter with a Gram-negative bacterium[18]. Perhaps for this reason most species of Gram-negative bacteria with hexa-acyl lipid A that inhabit the mucosal surfaces rarely become invasive. On the other hand, many Gram-negative pathogens that cause systemic infection do not produce hexa-acyl lipid A. Most of these bacteria have other habitats than the mucosa and enter the body via nonmucosal routes[6]. A good example is the plague bacillus *Yersinia pestis*. At mammalian body temperature *Y. pestis* normally produces tetra-acyl LPS that is poorly recognized by TLR4. Interestingly, a modified strain that produced hexa-acyl LPS at 37ºC was no longer virulent in wildtype mice but fully virulent in TLR4-deficient mice, demonstrating the importance of evasion of TLR4 activation for this bacterium[19]. *N. meningitidis* seems to be one of the exceptions to the general rule that Gram-negative bacteria with hexa-acyl lipid A do not cause systemic disease. However, our observation that a proportion of clinical isolates have penta-acylated LPS suggests that evasion of TLR4 activation might aid the

bacterium to circumvent host defences after crossing the nasopharyngeal epithelium. The hypothesis that TLR4 plays an important role in the prevention of meningococcal disease corroborates with the finding that subjects with rare TLR4 mutations have an increased risk for developing the disease[20]. If the assumption is correct that hexa-acyl LPS gives the bacterium an advantage on mucosal surfaces and that non hexa-acyl LPS is better for bacteria in submucosal spaces, one would expect that the frequency of *lpxL1* mutants is lower in meningococcal isolates from the respiratory tract compared to meningococcal isolates from the cerebrospinal fluid or blood.

Mogensen et al. showed that strain HF13 is specifically defective in activation of the MyD88-independent pathway, but not in inducing the MyD88-dependent pathway[11]. However, we demonstrate that strain HF13 and other *lpxL1* mutants are also defective in inducing the MyD88-dependent cytokines IL-6, TNF-α, and IL-1β. Our experiments indicate that *lpxL1* mutants or purified *lpxL1* LPS compared to wildtype controls are not specifically deficient in inducing the MyD88-dependent vs. independent pathway. This apparent discrepancy might be explained by the dose of bacteria used. If cells are stimulated with a high dose of bacteria the difference between *lpxL1* mutant and wildtype is only detectable for the MyD88-independent pathway. This is because LPS is the only bacterial component capable of inducing the MyD88-independent pathway, while many other bacterial components can induce the MyD88-dependent pathway (e.g. TLR2 ligands). When cells are stimulated with lower doses of bacteria the difference in induction of the MyD88-dependent pathway becomes apparent, because LPS is by far the most active component of the bacterium and the other non-TLR4 ligands that can activate the MyD88-dependent pathway are diluted too far to be still active.

The relatively high frequency of phase variation raises the question whether the *lpxL1* mutations might have arisen *in vitro* after isolation from the patient. Previously, we have performed extensive research on the phase variation of *porA* in *N. meningitidis*. In this gene, homopolymeric nucleotide tracts are found in the promoter (polyguanidine) and in the coding region (polyadenine). The frequencies by which these sequences vary in length are 10^{-3} [12, 21]. Others showed phase variation of capsule expression caused by insertion of IS1301 in the siaA gene with a frequency of phase variation of 9×10^{-4} [22, 23]. *In vitro* selection of porA phase variants and siaA phase variants have not been reported. Meningococcal isolates received by the Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) are low passages (up to 2 passages). We sequenced the *lpxL1* gene of 20 individual colonies of a culture of a mutant isolate (971859 I) and of 25 individual colonies of a culture of isolate 971859 III and found in each instance the same sequence, i.e. 20 mutant sequences and 25 wildtype sequences, respectively. Therefore, we estimate

the frequency of phase switching to be less than 2.2 x 10^{-2}. In addition, we sequenced *lpxL1* of DNA extracted from a swap taken from 4 different quadrants of another culture plate of isolate 971859 III. All 4 *lpxL1* sequences were homogeneous and identical. Thus we are confident that the discovered *lpxL1* mutations are not caused by *in vitro* phase variation.

Infection with *lpxL1*-mutant meningococcal strains is associated with a particular clinical phenotype, which consisted of less systemic inflammation and reduced activation of the coagulant system, reflected in less fever, higher serum platelet counts, and lower numbers with rash. Moreover, our *in vitro* data have shown that *lpxL1* mutants induce much less pro-inflammatory cytokines than wildtype strains. The coagulation system is activated through upregulation of tissue factor[1]. It has been demonstrated that LPS upregulates tissue factor on monocytes and endothelial cells[24-26]. Furthermore, in particular the pro-inflammatory cytokine IL-6 appears to mediate *in vivo* expression of tissue factor[27, 28]. Finally, IL-1β and TNF-α inhibit anticoagulant pathways by downregulating thrombomodulin at the endothelial surface and by increasing plasminogen activator inhibitor type-1 (PAI-1)[29, 30]. Thus, our finding that patients infected with an *lpxL1* mutant show less activation of the coagulation system is consistent with our results that show that *lpxL1* LPS is less potent and that *lpxL1* mutants induce less pro-inflammatory cytokines. Remarkably, the *lpxL1* mutants induced the same degree of CSF leukocytosis as wildtype strains. There are several explanations for "normal" CSF white cell counts in patients infected by mutant strains. Patients in the cohort all had positive CSF cultures; almost all had clinical signs of meningitis and CSF leukocytosis. Likely, leukocytosis is not only mediated by lipid A, but also by other microbial constituents.

It should be noted that not all groups of patients were included in our analysis of clinical patient data. The study only included adults with meningitis. Patients younger than 16 years or patients with sepsis only were not included. Therefore, our results are potentially biased by excluding these patient groups. Patients with meningitis often have a less severe form of the disease, as reflected by the overall low mortality of 8% in our study. However, patients with sepsis have very serious symptoms resulting from high concentrations of bacteria in the circulation. Mortality rates in these patients can be as high as 50%. Also, patients younger than 16 years are an import group, because rates for meningococcal disease are highest for young children[1]. It would be interesting to see whether *lpxL1* mutants also exist in these patients groups, and if so, if these patients have a different clinical course compared to patients infected with a wildtype strain. These additional data are needed to fully understand the impact of *lpxL1* mutations on meningococcal disease.

Meningococcal sepsis is generally seen as the prototypical endotoxin-mediated disease. Here we report for the first time that meningococcal lipid A mutants which are defective in TLR4 activation occur naturally. Their frequency is unexpectedly high, suggesting an important role in virulence for the resulting low-activity LPS. Our results suggest that in most cases this mutation has occurred through phase variation, and may give the bacteria an advantage because they are less well sensed by the innate immune system. Patients infected with these mutant strains endure milder symptoms with less systemic inflammation and reduced activation of the coagulant system, showing that our findings are clinically relevant. Importantly, these results with *lpxL1* also provide the first example of a specific bacterial mutation which can be associated with the clinical course of meningococcal disease. More generally, it shows how there can be an underestimated heterogeneity in the TLR4-activating capacity of pathogenic bacteria.

Acknowledgements

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Supporting information

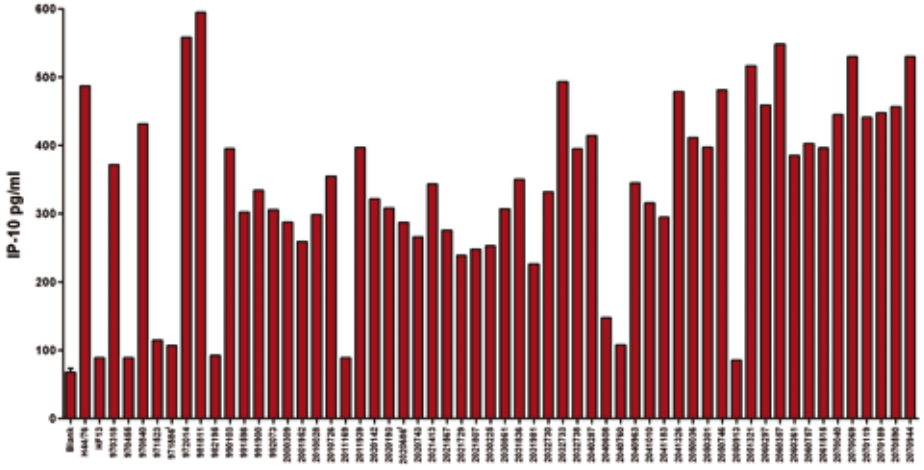

Figure S1. Screening of N. meningitidis group Y clinical isolates on cytokine induction. J774A.1 cells were stimulated for 3 h with a panel of group Y strains (0.1 OD) and IP-10 in the supernatant was determined with ELISA.

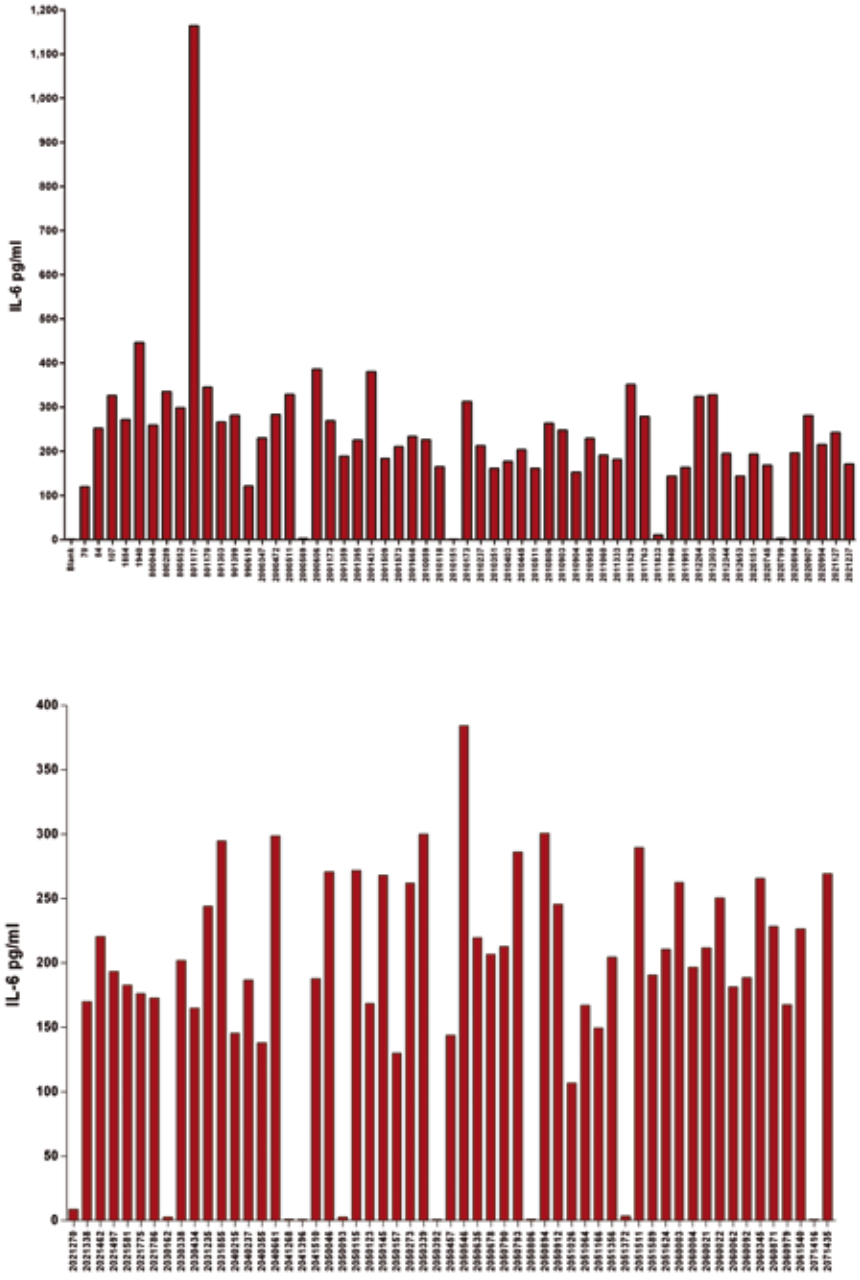

Figure S2. Screening of panel of N. meningitidis clinical isolates representing all major serogroups and clonal complexes. MM6 cells were stimulated for 18 h with a selection of clinical isolates (0.001 OD) representing all serogroups and clonal complexes. IL-6 was determined in the supernatant with ELISA.

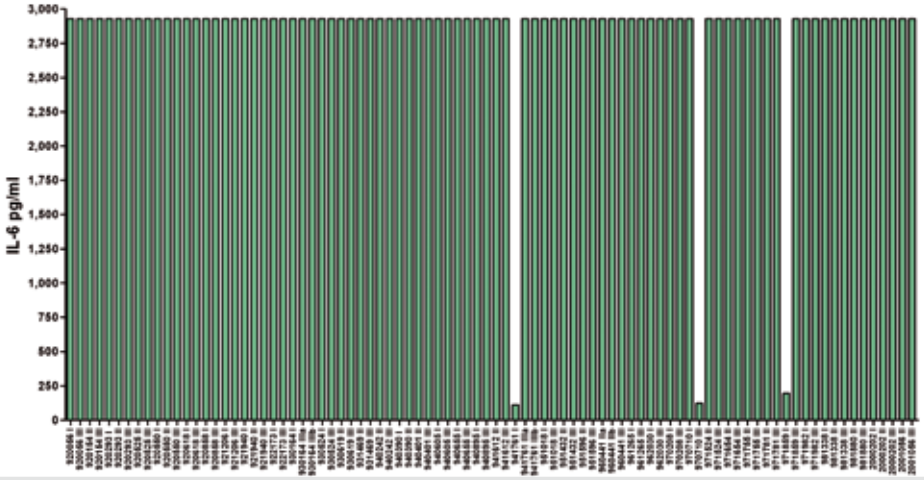

Figure S3 Sceening of panel of multiple isolates per patient. MM6 cells were stimulated for 18 h with a panel of clinical isolates (0.001 OD), of which multiple isolates were obtained from a single patient. IL-6 was determined with ELISA.

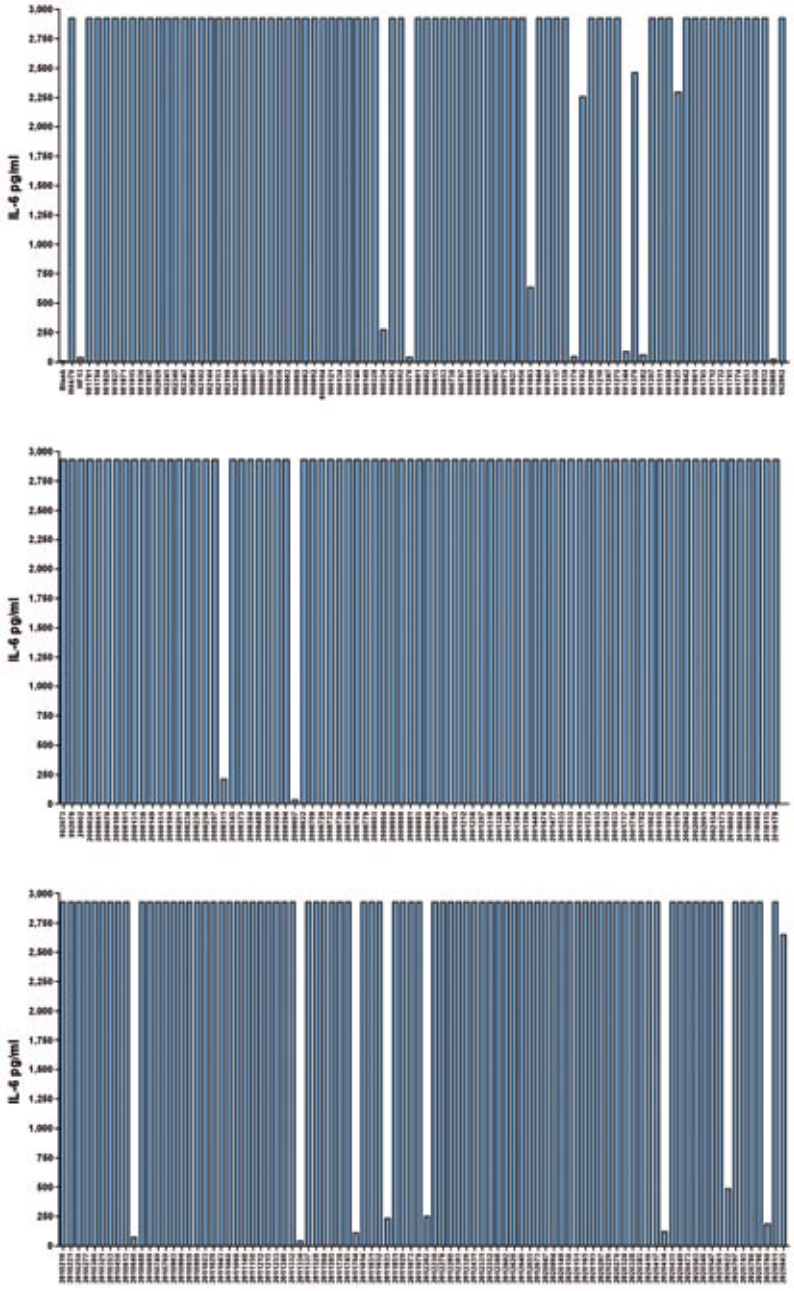

Figure S4 Screening of clinical isolates of patients included in the Dutch meningitis cohort study. MM6 cells were stimulated for 18 h with 254 isolates from patients with meningitis (0.001 OD). IL-6 was determined with ELISA.

Table S1

Serogroup Y isolates					
Year of isolation	Number	serogroup	ST	clonal complex	anatomical site
1997	970318	Y	nd	nd	CSF
1997	970455	Y	nd	nd	blood
1997	970840	Y	nd	nd	CSF
1997	971523	Y	nd	nd	CSF
1997	971886	Y	nd	nd	CSF
1997	972014	Y	nd	nd	blood
1998	981811	Y	nd	nd	blood
1998	982195	Y	nd	nd	blood
1999	990103	Y	nd	nd	blood
1999	991598	Y	nd	nd	CSF
1999	991900	Y	nd	nd	blood
1999	992073	Y	nd	nd	CSF
2000	2000309	Y	nd	nd	CSF
2000	2001952	Y	nd	nd	blood
2001	2010028	Y	nd	nd	blood
2001	2010726	Y	167	ST-167 complex	CSF
2001	2011169	Y	23	ST-23 complex/Cluster A3	blood
2001	2011939	Y	nd	nd	blood
2002	2020142	Y	23	ST-23 complex/Cluster A3	blood
2002	2020193	Y	3544	ST-174 complex	CSF
2002	2020686	Y	nd	nd	CSF
2002	2020743	Y	nd	nd	sputa
2002	2021413	Y	nd	nd	blood
2002	2021567	Y	1627	ST-167 complex	CSF
2002	2021729	Y	nd	nd	blood
2002	2021807	Y	23	ST-23 complex/Cluster A3	blood
2003	2030225	Y	nd	nd	blood
2003	2030861	Y	nd	nd	unknown
2003	2031836	Y	nd	nd	blood
2003	2031981	Y	nd	nd	CSF
2003	2032730	Y	nd	nd	CSF
2003	2032733	Y	nd	nd	blood
2003	2032738	Y	nd	nd	blood
2004	2040287	Y	1466	ST-174 complex	sputa
2004	2040608	Y	nd	nd	CSF
2004	2040760	Y	23	ST-23 complex/Cluster A3	blood
2004	2040963	Y	nd	nd	blood
2004	2041010	Y	4921	-	blood
2004	2041183	Y	4924	ST-103 complex	blood
2005	2050036	Y	103	ST-103 complex	CSF
2005	2050301	Y	1466	ST-174 complex	blood
2005	2050746	Y	4938	ST-167 complex	blood
2005	2050913	Y	2786	ST-23 complex/Cluster A3	blood
2005	2050915	Y	2786	ST-23 complex/Cluster A3	juncture
2005	2051321	Y	4921	ST-174 complex	blood
2006	2060297	Y	5461	ST-103 complex	blood
2006	2060357	Y	nd	nd	unknown
2006	2060361	Y	1466	ST-174 complex	blood
2006	2060757	Y	3544	ST-174 complex	blood
2006	2061515	Y	167	ST-167 complex	CSF
2007	2070040	Y	6286	ST-174 complex	blood
2007	2070069	Y	167	ST-167 complex	CSF
2007	2070119	Y	nd	nd	blood
2007	2070189	Y	1466	ST-174 complex	CSF
2007	2070590	Y	5436	-	blood
2007	2070944	Y	894	ST-23 complex/Cluster A3	blood
Isolates representing major serogroups and clonal complexes					
Year of isolation	Number	serogroup	ST	clonal complex	anatomical site
1960	70	A	4850	-	CSF
1960	84	A	1	ST-1 complex/subgroup I/II	CSF
1960	107	A	1	ST-1 complex/subgroup I/II	CSF
1970	1884	A	4942	-	CSF
1970	1940	A	1	ST-1 complex/subgroup I/II	CSF
1980	800048	A	1	ST-1 complex/subgroup I/II	CSF
1980	800289	A	1	ST-1 complex/subgroup I/II	blood
1980	800552	A	1	ST-1 complex/subgroup I/II	CSF
1980	801117	A	2205	ST-269 complex	CSF
1980	801170	A	1	ST-1 complex/subgroup I/II	CSF
1980	801303	A	1	ST-1 complex/subgroup I/II	CSF
1990	901399	A	5	ST-5 complex/subgroup III	CSF
1999	990615	W135	1286	ST-22 complex	CSF

2000	2000347		W135	22	ST-22 complex	blood
2000	2000472		B	4905	ST-162 complex	blood
2000	2000511		C	4255	ST-41/44 complex/Lineage 3	blood
2000	2000569		X	750	ST-750 complex	blood
2000	2000606		C	34	ST-32 complex/ET-5 complex	blood
2000	2001173		C	4428	ST-8 complex/Cluster A4	CSF
2000	2001359		B	4429	ST-8 complex/Cluster A4	CSF
2000	2001395		B	5261	ST-254 complex	CSF
2000	2001431		B	4738	ST-22 complex	blood
2000	2001509		C	2699	ST-8 complex/Cluster A4	CSF
2000	2001573		C	493	ST-18 complex	CSF
2000	2001668		B	2718	ST-18 complex	blood
2001	2010059		C	2698	ST-334 complex	CSF
2001	2010115		C	2699	ST-8 complex/Cluster A4	CSF
2001	2010118		C	60	ST-60 complex	CSF
2001	2010151		C	461	ST-461 complex	blood
2001	2010173		C	60	ST-60 complex	CSF
2001	2010237		B	2701	ST-254 complex	CSF
2001	2010351		W135	247	ST-11 complex/ET-37 complex	blood
2001	2010403		C	2699	ST-8 complex/Cluster A4	CSF
2001	2010511		C	2699	ST-8 complex/Cluster A4	CSF
2001	2010806		B	474	ST-376 complex	CSF
2001	2010903		B	3547	ST-60 complex	CSF
2001	2010904		B	3548	ST-18 complex	CSF
2001	2010958		B	35	ST-35 complex	blood
2001	2011060		C	2699	ST-8 complex/Cluster A4	CSF
2001	2011333		B	11	ST-11 complex/ET-37 complex	blood
2001	2011529		B	254	ST-254 complex	blood
2001	2011763		C	34	ST-32 complex/ET-5 complex	CSF
2001	2011833		C	3553	ST-269 complex	CSF
2001	2011940		W135	11	ST-11 complex/ET-37 complex	CSF
2001	2011991		W135	3422	ST-22 complex	CSF
2001	2012264		C	40	ST-41/44 complex/Lineage 3	blood
2001	2012303		C	269	ST-269 complex	CSF
2001	2012344		B	457	ST-35 complex	CSF
2001	2012653		C	2680	ST-8 complex/Cluster A4	CSF
2002	2020151		C	3486	ST-8 complex/Cluster A4	CSF
2002	2020745		C	2680	ST-8 complex/Cluster A4	CSF
2002	2020799		B	35	ST-35 complex	CSF
2002	2020894		W135	3334	ST-22 complex	blood
2002	2020907		C	1372	ST-8 complex/Cluster A4	blood
2002	2020994		C	2680	ST-8 complex/Cluster A4	CSF
2002	2021127		C	32	ST-32 complex/ET-5 complex	CSF
2002	2021237		C	213	ST-213 complex	CSF
2002	2021270		C	461	ST-461 complex	CSF
2002	2021338		B	289	ST-22 complex	CSF
2002	2021462		29E	60	ST-60 complex	skin biopsy
2002	2021497		C	2680	ST-8 complex/Cluster A4	CSF
2002	2021581		C	3522	ST-8 complex/Cluster A4	blood
2002	2021775		B	11	ST-11 complex/ET-37 complex	CSF
2002	2021786		B	5230	ST-18 complex	CSF
2003	2030162		C	337	ST-41/44 complex/Lineage 3	blood
2003	2030338		W135	22	ST-22 complex	CSF
2003	2030434		A	7	ST-5 complex/subgroup III	CSF
2003	2031235		B	60	ST-60 complex	blood
2003	2031855		B	4915	ST-162 complex	blood
2004	2040215		C	11	ST-11 complex/ET-37 complex	blood
2004	2040237		C	11	ST-11 complex/ET-37 complex	CSF
2004	2040355		B	153	ST-8 complex/Cluster A4	blood
2004	2040661		B	162	ST-162 complex	CSF
2004	2041268		B	4926	ST-35 complex	blood
2004	2041396		B	4930	ST-18 complex	CSF
2004	2041510		B	22	ST-22 complex	CSF
2005	2050046		B	32	ST-32 complex/ET-5 complex	blood
2005	2050093		B	461	ST-461 complex	blood
2005	2050115		B	4281	ST-269 complex	CSF
2005	2050123		C	11	ST-11 complex/ET-37 complex	blood
2005	2050145		B	34	ST-32 complex/ET-5 complex	blood
2005	2050157		W135	4932	ST-22 complex	blood
2005	2050273		B	32	ST-32 complex/ET-5 complex	CSF
2005	2050339		B	34	ST-32 complex/ET-5 complex	blood
2005	2050392		B	213	ST-213 complex	CSF
2005	2050457		B	4937	ST-269 complex	blood
2005	2050546		29E	198	ST-198 complex	blood
2005	2050635		B	269	ST-269 complex	CSF
2005	2050678		W135	3334	ST-22 complex	pericard fluid

2005	2050790		W135	1617	ST-22 complex	CSF
2005	2050793		B	3327	ST-865 complex	blood
2005	2050806		B	213	ST-213 complex	CSF
2005	2050894		B	213	ST-213 complex	oog
2005	2050912		B	5271	ST-60 complex	CSF
2005	2051026		C	60	ST-60 complex	CSF
2005	2051064		B	2660	ST-213 complex	blood
2005	2051166		W135	11	ST-11 complex/ET-37 complex	CSF
2005	2051356		C	283	ST-269 complex	blood
2005	2051372		B	461	ST-461 complex	blood
2005	2051511		B	754	ST-162 complex	blood
2005	2051589		B	5280	ST-60 complex	CSF
2005	2051624		B	11	ST-11 complex/ET-37 complex	CSF
2006	2060003		B	3050	ST-41/44 complex/Lineage 3	CSF
2006	2060004		B	571	ST-41/44 complex/Lineage 3	CSF
2006	2060021		B	40	ST-41/44 complex/Lineage 3	blood
2006	2060022		B	154	ST-41/44 complex/Lineage 3	blood
2006	2060062		B	461	ST-461 complex	CSF
2006	2060092		W135	22	ST-22 complex	CSF
2006	2060345		X	750	ST-750 complex	CSF
2006	2060871		B	1157	ST-1157 complex	CSF
2006	2060979		B	4484	ST-22 complex	CSF
2006	2061540		B	162	ST-162 complex	blood
2007	2071416		Y	23	ST23 complex	blood
2007	2071435		Y	784	ST92 complex	blood
Isolates form two anatomical sites per patient						
Year of isolation	**number**		**serogroup**		**clonal complex**	**anatomical site**
1992	920056	I	C	nd	nd	CSF
1992	920056	III	C	nd	nd	nasopharynx
1992	920154	II	B	nd	nd	blood
1992	920154	III	B	nd	nd	throat
1992	920293	I	B	nd	nd	CSF
1992	920293	II	B	nd	nd	blood
1992	920293	III	B	nd	nd	throat
1992	920525	II	B	nd	nd	blood
1992	920525	III	B	nd	nd	nasopharynx
1992	920550	I	B	nd	nd	CSF
1992	920550	II	B	nd	nd	blood
1992	920550	III	B	nd	nd	throat
1992	920618	I	C	nd	nd	CSF
1992	920618	III	C	nd	nd	nasopharynx
1992	920858	I	C	nd	nd	CSF
1992	920858	III	C	nd	nd	nasopharynx
1992	921206	I	B	nd	nd	CSF
1992	921206	III	B	nd	nd	throat
1992	921940	I	B	nd	nd	CSF
1992	921940	II	B	nd	nd	blood
1992	921940	III	B	nd	nd	throat
1992	922173	I	B	nd	nd	CSF
1992	922173	III	B	nd	nd	throat
1993	930164	I	B	nd	nd	CSF
1993	930164	IIIA	B	nd	nd	throat
1993	930164	IIIB	B	nd	nd	nose
1993	930524	I	B	nd	nd	CSF
1993	930524	III	B	nd	nd	throat
1993	930619	II	B	nd	nd	blood
1993	930619	III	B	nd	nd	nasopharynx
1993	931469	II	C	nd	nd	blood
1993	931469	III	C	nd	nd	throat
1994	940242	II	B	nd	nd	blood
1994	940242	III	B	nd	nd	nose
1994	940390	I	B	nd	nd	CSF
1994	940390	II	B	nd	nd	blood
1994	940401	II	B	nd	nd	blood
1994	940401	III	B	nd	nd	throat
1994	940605	IA	B	nd	nd	CSF
1994	940605	IIB	B	nd	nd	throat
1994	940605	III	B	nd	nd	throat
1994	940655	I	B	nd	nd	CSF
1994	940655	III	B	nd	nd	nasopharynx
1994	940895	I	B	nd	nd	CSF
1994	940895	III	B	nd	nd	sputa
1994	941612	II	B	nd	nd	blood
1994	941612	III	B	nd	nd	sputa
1994	941761	I	C	nd	nd	CSF

1994	941761	IIIA	C	nd	nd	throat
1994	941761	IIIB	C	nd	nd	nose
1995	951018	I	B	nd	nd	CSF
1995	951018	III	B	nd	nd	nose
1995	951432	II	C	nd	nd	blood
1995	951432	III	C	nd	nd	throat
1995	951596	II	B	nd	nd	blood
1995	951596	III	B	nd	nd	nasopharynx
1996	960441	IIA	C	nd	nd	blood
1996	960441	IIB	C	nd	nd	throat
1996	960441	III	C	nd	nd	throat
1996	961265	I	B	nd	nd	CSF
1996	961265	III	B	nd	nd	nose
1996	962030	I	B	nd	nd	CSF
1996	962030	III	B	nd	nd	throat
1997	970208	I	B	nd	nd	CSF
1997	970208	III	B	nd	nd	nasopharynx
1997	970710	I	B	nd	nd	CSF
1997	970710	III	B	nd	nd	nose
1997	971524	II	B	nd	nd	blood
1997	971524	III	B	nd	nd	eye
1997	971654	II	B	nd	nd	blood
1997	971654	III	B	nd	nd	throat
1997	971755	II	B	nd	nd	blood
1997	971755	III	B	nd	nd	throat
1997	971781	II	C	nd	nd	blood
1997	971781	III	C	nd	nd	throat
1997	971859	I	C	nd	nd	CSF
1997	971859	III	C	nd	nd	throat
1997	971982	I	B	nd	nd	CSF
1997	971982	III	B	nd	nd	nose
1998	981338	I	B	nd	nd	CSF
1998	981338	II	B	nd	nd	blood
1998	981338	III	B	nd	nd	throat
1998	981880	II	B	nd	nd	blood
1998	981880	III	B	nd	nd	nose
2000	2000202	I	B	nd	nd	CSF
2000	2000202	II	B	nd	nd	blood
2000	2000202	III	B	nd	nd	nose
2000	2001056	II	C	nd	nd	blood
2000	2001056	III	C	nd	nd	throat
Isolates from patients in the meningitis cohort						
Year of isolation	**Number**		**serogroup**	**ST**	**clonal complex**	**anatomical site**
1998	981781		B	41	ST-41/44 complex/Lineage 3	CSF
1998	981784		C	60	ST-60 complex	CSF
1998	981826		B	5444	ST-41/44 complex/Lineage 3	CSF
1998	981827		B	34	ST-32 complex/ET-5 complex	CSF
1998	981871		B	41	ST-41/44 complex/Lineage 3	CSF
1998	981915		B	2203	ST-41/44 complex/Lineage 3	CSF
1998	981936		B	41	ST-41/44 complex/Lineage 3	CSF
1998	981987		B	34	ST-32 complex/ET-5 complex	CSF
1998	982029		B	1374	ST-41/44 complex/Lineage 3	CSF
1998	982084		C	66	ST-8 complex/Cluster A4	CSF
1998	982102		B	41	ST-41/44 complex/Lineage 3	CSF
1998	982144		B	41	ST-41/44 complex/Lineage 3	CSF
1998	982153		B	42	ST-41/44 complex/Lineage 3	CSF
1998	982199		B	269	ST-269 complex	CSF
1998	982200		B	5445	ST-41/44 complex/Lineage 3	CSF
1998	982245		B	5098	ST-41/44 complex/Lineage 3	CSF
1998	982340		B	5446	ST-41/44 complex/Lineage 3	CSF
1998	982347		B	269	ST-269 complex	CSF
1999	990001		B	1163	ST-269 complex	CSF
1999	990005		B	32	ST-32 complex/ET-5 complex	CSF
1999	990007		B	2080	ST-41/44 complex/Lineage 3	CSF
1999	990030		B	2286	ST-41/44 complex/Lineage 3	CSF
1999	990056		C	51	ST-11 complex/ET-37 complex	CSF
1999	990062		B	41	ST-41/44 complex/Lineage 3	CSF
1999	990069		B	2016	ST-41/44 complex/Lineage 3	CSF
1999	990082		B	2016	ST-41/44 complex/Lineage 3	CSF
1999	990092		B	159	ST-41/44 complex/Lineage 3	CSF
1999	990104		B	5447	ST-41/44 complex/Lineage 3	CSF
1999	990121		B	34	ST-32 complex/ET-5 complex	CSF
1999	990134		B	5448	ST-41/44 complex/Lineage 3	CSF
1999	990135		B	41	ST-41/44 complex/Lineage 3	CSF
1999	990146		B	40	ST-41/44 complex/Lineage 3	CSF

1999	990149		B	41	ST-41/44 complex/Lineage 3	CSF
1999	990328		B	41	ST-41/44 complex/Lineage 3	CSF
1999	990334		B	5449	ST-41/44 complex/Lineage 3	CSF
1999	990492		B	32	ST-32 complex/ET-5 complex	CSF
1999	990502		B	4100	ST-41/44 complex/Lineage 3	CSF
1999	990576		B	571	ST-41/44 complex/Lineage 3	CSF
1999	990601		B	212	-	CSF
1999	990602		C	11	ST-11 complex/ET-37 complex	CSF
1999	990615		W135	1286	ST-22 complex	CSF
1999	990653		B	259	ST-32 complex/ET-5 complex	CSF
1999	990738		C	11	ST-11 complex/ET-37 complex	CSF
1999	990797		C	5450	ST-11 complex/ET-37 complex	CSF
1999	990808		B	259	ST-32 complex/ET-5 complex	CSF
1999	990815		B	41	ST-41/44 complex/Lineage 3	CSF
1999	990907		B	2707	ST-32 complex/ET-5 complex	CSF
1999	990947		C	11	ST-11 complex/ET-37 complex	CSF
1999	990975		B	269	ST-269 complex	CSF
1999	991027		B	4065	ST-32 complex/ET-5 complex	CSF
1999	991044		C	11	ST-11 complex/ET-37 complex	CSF
1999	991056		B	41	ST-41/44 complex/Lineage 3	CSF
1999	991093		B	5451	ST-32 complex/ET-5 complex	CSF
1999	991097		B	34	ST-32 complex/ET-5 complex	CSF
1999	991117		B	34	ST-32 complex/ET-5 complex	CSF
1999	991159		B	34	ST-32 complex/ET-5 complex	CSF
1999	991174		C	11	ST-11 complex/ET-37 complex	CSF
1999	991192		B	1403	ST-41/44 complex/Lineage 3	CSF
1999	991208		B	283	ST-269 complex	CSF
1999	991210		C	2680	ST-8 complex/Cluster A4	CSF
1999	991247		B	41	ST-41/44 complex/Lineage 3	CSF
1999	991275		B	32	ST-32 complex/ET-5 complex	CSF
1999	991344		B	42	ST-41/44 complex/Lineage 3	CSF
1999	991379		C	11	ST-11 complex/ET-37 complex	CSF
1999	991382		B	191	ST-41/44 complex/Lineage 3	CSF
1999	991397		B	5452	ST-41/44 complex/Lineage 3	CSF
1999	991511		B	3754	ST-41/44 complex/Lineage 3	CSF
1999	991598		Y	167	ST-167 complex	CSF
1999	991625		B	1374	ST-41/44 complex/Lineage 3	CSF
1999	991642		B	33	ST-32 complex/ET-5 complex	CSF
1999	991661		B	42	ST-41/44 complex/Lineage 3	CSF
1999	991705		B	1960	ST-41/44 complex/Lineage 3	CSF
1999	991712		B	42	ST-41/44 complex/Lineage 3	CSF
1999	991722		B	3621	-	CSF
1999	991765		B	32	ST-32 complex/ET-5 complex	CSF
1999	991774		B	1788	ST-41/44 complex/Lineage 3	CSF
1999	991853		B	40	ST-41/44 complex/Lineage 3	CSF
1999	991930		C	11	ST-11 complex/ET-37 complex	CSF
1999	991932		C	11	ST-11 complex/ET-37 complex	CSF
1999	992008		B	146	ST-41/44 complex/Lineage 3	CSF
1999	992062		C	11	ST-11 complex/ET-37 complex	CSF
1999	992073		Y	5453	ST-92 complex	CSF
1999	992076		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000020		B	4586	ST-41/44 complex/Lineage 3	CSF
2000	2000024		B	32	ST-32 complex/ET-5 complex	CSF
2000	2000041		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000070		B	42	ST-41/44 complex/Lineage 3	CSF
2000	2000100		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000101		B	42	ST-41/44 complex/Lineage 3	CSF
2000	2000131		B	269	ST-269 complex	CSF
2000	2000136		B	5454	ST-41/44 complex/Lineage 3	CSF
2000	2000149		B	269	ST-269 complex	CSF
2000	2000151		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000194		B	33	ST-32 complex/ET-5 complex	CSF
2000	2000201		B	2708	ST-41/44 complex/Lineage 3	CSF
2000	2000234		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000236		B	40	ST-41/44 complex/Lineage 3	CSF
2000	2000250		B	32	ST-32 complex/ET-5 complex	CSF
2000	2000297		B	34	ST-32 complex/ET-5 complex	CSF
2000	2000311		B	461	ST-461 complex	CSF
2000	2000345		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000373		C	461	ST-461 complex	CSF
2000	2000384		B	32	ST-32 complex/ET-5 complex	CSF
2000	2000488		B	2631	ST-41/44 complex/Lineage 3	CSF
2000	2000500		B	269	ST-269 complex	CSF
2000	2000589		B	4257	ST-41/44 complex/Lineage 3	CSF
2000	2000594		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000607		B	40	ST-41/44 complex/Lineage 3	CSF

2000	2000622		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000709		B	33	ST-32 complex/ET-5 complex	CSF
2000	2000726		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000732		B	40	ST-41/44 complex/Lineage 3	CSF
2000	2000739		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000749		B	32	ST-32 complex/ET-5 complex	CSF
2000	2000760		B	34	ST-32 complex/ET-5 complex	CSF
2000	2000779		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000803		B	3488	ST-41/44 complex/Lineage 3	CSF
2000	2000804		B	41	ST-41/44 complex/Lineage 3	CSF
2000	2000869		B	5455	ST-32 complex/ET-5 complex	CSF
2000	2000880		B	42	ST-41/44 complex/Lineage 3	CSF
2000	2000881		B	40	ST-41/44 complex/Lineage 3	CSF
2000	2000885		B	42	ST-41/44 complex/Lineage 3	CSF
2000	2000948		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000974		C	11	ST-11 complex/ET-37 complex	CSF
2000	2000987		B	40	ST-41/44 complex/Lineage 3	CSF
2001	2001043		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001212		C	11	ST-11 complex/ET-37 complex	CSF
2001	2001256		B	1374	ST-41/44 complex/Lineage 3	CSF
2001	2001297		B	34	ST-32 complex/ET-5 complex	CSF
2001	2001318		B	269	ST-269 complex	CSF
2001	2001329		C	2704	ST-11 complex/ET-37 complex	CSF
2001	2001346		C	2680	ST-8 complex/Cluster A4	CSF
2001	2001394		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001396		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001449		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001474		B	4018	ST-41/44 complex/Lineage 3	CSF
2001	2001477		C	11	ST-11 complex/ET-37 complex	CSF
2001	2001533		B	34	ST-32 complex/ET-5 complex	CSF
2001	2001553		B	2916	ST-41/44 complex/Lineage 3	CSF
2001	2001556		B	1163	ST-269 complex	CSF
2001	2001573		C	483	ST-18 complex	CSF
2001	2001615		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001632		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001633		C	11	ST-11 complex/ET-37 complex	CSF
2001	2001717		C	2704	ST-11 complex/ET-37 complex	CSF
2001	2001718		C	11	ST-11 complex/ET-37 complex	CSF
2001	2001782		B	32	ST-32 complex/ET-5 complex	CSF
2001	2001862		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001915		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001970		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2001976		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2002042		B	34	ST-32 complex/ET-5 complex	CSF
2001	2002060		B	32	ST-32 complex/ET-5 complex	CSF
2001	2002091		B	42	ST-41/44 complex/Lineage 3	CSF
2001	2002154		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2002173		C	3456	ST-11 complex/ET-37 complex	CSF
2001	2010002		B	2696	ST-32 complex/ET-5 complex	CSF
2001	2010024		B	40	ST-41/44 complex/Lineage 3	CSF
2001	2010080		B	42	ST-41/44 complex/Lineage 3	CSF
2001	2010082		B	42	ST-41/44 complex/Lineage 3	CSF
2001	2010115		C	2699	ST-8 complex/Cluster A4	CSF
2001	2010178		C	11	ST-11 complex/ET-37 complex	CSF
2001	2010216		B	3546	ST-41/44 complex/Lineage 3	CSF
2001	2010221		C	2680	ST-8 complex/Cluster A4	CSF
2001	2010259		B	269	ST-269 complex	CSF
2001	2010277		B	5406	ST-41/44 complex/Lineage 3	CSF
2001	2010306		B	34	ST-32 complex/ET-5 complex	CSF
2001	2010321		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2010353		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2010450		B	60	ST-60 complex	CSF
2001	2010513		C	11	ST-11 complex/ET-37 complex	CSF
2001	2010640		B	1475	ST-41/44 complex/Lineage 3	CSF
2001	2010688		B	34	ST-32 complex/ET-5 complex	CSF
2001	2010699		C	11	ST-11 complex/ET-37 complex	CSF
2001	2010749		B	42	ST-41/44 complex/Lineage 3	CSF
2001	2010760		C	11	ST-11 complex/ET-37 complex	CSF
2001	2010903		B	3547	ST-60 complex	CSF
2001	2010904		B	3548	ST-18 complex	CSF
2001	2010939		C	11	ST-11 complex/ET-37 complex	CSF
2001	2010999		B	3549	-	CSF
2001	2011023		B	3550	ST-41/44 complex/Lineage 3	CSF
2001	2011029		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2011043		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011060		C	2699	ST-8 complex/Cluster A4	CSF

2001	2011098		B	4258	-	CSF
2001	2011148		B	3551	ST-41/44 complex/Lineage 3	CSF
2001	2011150		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011212		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011215		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011233		B	3552	ST-32 complex/ET-5 complex	CSF
2001	2011246		B	3552	ST-32 complex/ET-5 complex	CSF
2001	2011332		B	274	ST-41/44 complex/Lineage 3	CSF
2001	2011334		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011337		B	259	ST-32 complex/ET-5 complex	CSF
2001	2011528		C	2704	ST-11 complex/ET-37 complex	CSF
2001	2011564		B	33	ST-32 complex/ET-5 complex	CSF
2001	2011595		B	5456	ST-269 complex	CSF
2001	2011728		C	4283	ST-11 complex/ET-37 complex	CSF
2001	2011745		B	33	ST-32 complex/ET-5 complex	CSF
2001	2011764		B	303	ST-41/44 complex/Lineage 3	CSF
2001	2011814		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011831		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011832		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011833		C	3553	ST-269 complex	CSF
2001	2011851		B	3554	ST-41/44 complex/Lineage 3	CSF
2001	2011929		C	11	ST-11 complex/ET-37 complex	CSF
2001	2011973		B	32	ST-32 complex/ET-5 complex	CSF
2001	2011979		B	33	ST-32 complex/ET-5 complex	CSF
2001	2012202		B	41	ST-41/44 complex/Lineage 3	CSF
2001	2012239		B	34	ST-32 complex/ET-5 complex	CSF
2001	2012278		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012280		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012303		C	269	ST-269 complex	CSF
2001	2012326		B	5408	-	CSF
2001	2012431		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012531		B	2700	-	CSF
2001	2012552		C	2680	ST-8 complex/Cluster A4	CSF
2001	2012598		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012602		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012620		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012640		B	2708	ST-41/44 complex/Lineage 3	CSF
2001	2012655		C	11	ST-11 complex/ET-37 complex	CSF
2001	2012673		C	2709	ST-11 complex/ET-37 complex	CSF
2002	2020047		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020094		B	269	ST-269 complex	CSF
2002	2020149		B	60	ST-60 complex	CSF
2002	2020150		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020151		C	3486	ST-8 complex/Cluster A4	CSF
2002	2020165		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020193		Y	3544	ST-174 complex	CSF
2002	2020207		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020226		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020276		B	3608	ST-41/44 complex/Lineage 3	CSF
2002	2020324		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020328		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020383		B	213	ST-213 complex	CSF
2002	2020416		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020417		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020434		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020435		C	3298	ST-11 complex/ET-37 complex	CSF
2002	2020449		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020473		B	41	ST-41/44 complex/Lineage 3	CSF
2002	2020503		C	11	ST-11 complex/ET-37 complex	CSF
2002	2020546		B	1374	ST-41/44 complex/Lineage 3	CSF
2002	2020547		B	5457	ST-364 complex	CSF
2002	2020561		C	1374	ST-41/44 complex/Lineage 3	CSF
2002	2020622		B	5458	ST-41/44 complex/Lineage 3	CSF
2002	2020707		C	8	ST-8 complex/Cluster A4	CSF
2002	2020745		C	2680	ST-8 complex/Cluster A4	CSF
2002	2020786		B	3515	ST-41/44 complex/Lineage 3	CSF
2002	2020798		B	275	ST-269 complex	CSF
2002	2020799		B	35	ST-35 complex	CSF
2002	2020811		B	1960	ST-41/44 complex/Lineage 3	CSF
2002	2020843		B	41	ST-41/44 complex/Lineage 3	CSF

Table S2. List of primers used for the sequencing of *lpxL1*.

Primer	Sequence coordinates (according to MC58 sequence)[a]
AvdE_02NMB1417F	1452953 - 1452971
344-2	1453690 - 1453709
AvdE_NMB1419R	1454685 - 1454669
670-1	1454693 - 1454674
AVDE-LPX1-101	1454977 - 1454960
AVDE_LPX1_100	1455059 - 1455039

[a]accession number AE002098.2.

Table S3.

Strain Number	Accession number
992073	FJ472279
9718866	FJ472280
9821956	FJ472281
2040760	FJ472282
2011169	FJ472283
2010151	FJ472284
2000569	FJ472285
2021270	FJ472286
2030162	FJ472287
2041268	FJ472288
2041396	FJ472289
2050093	FJ472290
2050806	FJ472291
2051372	FJ472292
2071416	FJ472293
2050392	FJ472294
970455	FJ472295
2050913	FJ472296
971523	FJ472297
2000311	FJ472298
2000607	FJ472299
2010640	FJ472300
2011764	FJ472301
2011833	FJ472302
2012202	FJ472303
2020434	FJ472304
2020622	FJ472305
2020799	FJ472306
990344	FJ472307
990576	FJ472308
991093	FJ472309
991174	FJ472310
991344	FJ472311
991382	FJ472312
992008	FJ472313
971859_I	FJ472314
971859_III	FJ472315
970710_I	FJ472316
970710_III	FJ472317
941761_I	FJ472318
941761_III	FJ472319
2040608_5#	FJ472320
2040608_3#	FJ472321
2011334_5#	FJ472322
2011334_3#	FJ472323

Chapter 5

Lipid A variants among meningococcal disease and carriage isolates; prevalence, age distribution and clinical impact

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Abstract

Background

Neisseria meningitidis is worldwide an important cause of invasive bacterial infections. The most important trigger of the pro-inflammatory cascade accompanying meningococcal disease is the lipid A component of lipopolysaccharide. Recently, naturally occurring meningococcal mutants with under-acylated lipid A due to an inactive *lpxL1* gene have been described in adult patients with meningococcal meningitis. Infection with these mutant lipid A strains caused less coagulopathy, presumably due to reduced activation of Toll-like receptor 4 (TLR4). The aim of this study was to explore the prevalence of lipid A variants among meningococcal disease and carriage isolates and to study the clinical impact of these mutants in all age-groups.

Methods and Findings

In a retrospective cohort study of patients with invasive meningococcal diseases in the Netherlands 448 isolates were screened for lipid A mutations. In addition 822 carrier isolates from a study conducted in Germany were included. In total 29 invasive lipid A variant strains were identified among disease isolates, in which prevalence was associated with age (4,4% in children under 5 years to 24.2% in adults 45-65 years of age). In patients over 5 years of age infections with lipid A variants were associated with increased underlying comorbidities (58% vs. 23%, respectively; $p<0.005$), less fever (39% vs. 72%, respectively; $p<0.01$), less septic shock (11% vs. 38%, respectively; $p=0.02$) and decreased IC admittance (20 vs. 45%, respectively; $p=0.04$) compared to wild-type strains. In children under 5 years of age no clinical differences were observed between mutant and wild-type isolates. Among carrier isolates 45 lipid A variants were found (5.5%). Forty-three were found among 71 isolates (61%) of clonal complex cc23. This clonal complex was not observed among disease strains.

Conclusions

Meningococci with lipid A mutations were more frequently found in children over 5 years and adults and associated in these age-groups with increased underlying comorbidities and less severe disease. By contrast, among carriage isolates lipid A mutations occur only very rarely, with the exception of one single clonal complex. These findings emphasize further study of lipid A variants in invasive disease and carriage, as such immune evasion may have implications for effectiveness of vaccination against meningococcal disease.

Introduction

N. meningitidis is an obligate commensal of the *human* nasopharyngeal mucosa and is found in the airways of 10-20% of the healthy population[1, 2]. Meningococcal colonization may result in an asymptomatic carrier state (which is most common), but can also cause invasive bacterial infections with disease syndromes varying from meningitis to fulminant septic shock with high mortality and morbidity rates[2, 3]. The incidence of meningococcal disease has its primary peak in children aged <5 years of age with up to 20 cases per 100,000 per year in infants under 1 year of age[4]. This increased susceptibility has been attributed to an immature adaptive immune system and lack of specific, functional protective antibodies[5, 6]. However, *N. meningitidis* remains a significant contributor to invasive bacterial disease in older children and adults with mortality rates varying from 3%-7% for meningitis to above 15% for meningococcal bacteremia[3, 7].

The major virulence factor in meningococcal disease is lipopolysaccharide (LPS) which is a gycolipid component of the outer membrane of Gram-negative bacteria[2, 8]. LPS is a potent activator of the innate immune system and is sensed through Toll-like receptor 4 (TLR4) in combination with co-receptors MD-2 and CD14[9]. TLR4 recognizes the lipid A moiety of the LPS molecule, and the composition of the lipid A determines how well it is recognized by TLR4. LPS also upregulates tissue factor activating the coagulation system which may lead to fulminant septic shock and disseminated intravascular coagulation[10, 11]. Recently, we reported that a surprisingly large fraction of meningococcal clinical isolates have LPS with underacylated lipid A due to mutations in the *lpxL1* gene[11], suggesting that under certain conditions lipid A variation is beneficial to the meningococcus. The resulting low-activity LPS may have an important role in virulence by aiding the bacteria to evade the innate immune system. We showed that in adults with meningococcal meningitis infection with lipid A variants results in a clinical phenotype with reduced coagulopathy and systemic inflammation[11].

Aim of this study was to explore the distribution and clinical impact of lipid A variants among a large consecutive cohort of patients with invasive meningococcal disease comprising all different disease syndromes and age groups. To seek evidence for the hypothesis that penta-acylated lipid A meningococci are selected during invasion, we explored the distribution and clinical impact of lipid A variants among a large cohort of patients with invasive meningococcal disease comprising different disease syndromes and age groups. We also explored the proportion of lipid A variants in carrier isolates and compared the distribution of lipid A variants among meningococcal genotypes (clonal complexes) of patient and carrier isolates.

Materials and Methods

Meningococcal isolates

The Netherlands Reference Laboratory for Bacterial Meningitis (NRLBM) is a laboratory-based nationwide surveillance system that collects bacterial isolates from blood, cerebrospinal fluid (CSF) and/or other normally sterile bodily fluids and receives >90% of the isolates from meningococcal invasive disease. All cases with invasive meningococcal disease reported by nine sentinel laboratories between June 1th 2001 and May 31th 2006 were included for analyses in this retrospective cohort study. These nine sentinel laboratories cover approximately 4.1 million inhabitants forming a representative proportion of circa 25% of the Dutch population. Meningococcal isolates from carriers were collected during a study on genotype, capsule expression and age/geographical distribution of meningococci conducted in Bavaria, Germany. Retropharyngeal swab sampling was conducted at schools and military camps. Serogrouping and MLST was performed as described elsewhere[12], clonal complexes were designated according to the online MLST-database[13].

Patient characteristics

Clinical information of invasive disease infections was retrospectively extracted from hospital records using an anonymous standard data collection form and was performed in accordance with the Dutch privacy legislation. Clinical syndromes were categorized in meningitis and non-meningitis. Meningitis was defined as a case with a CSF culture positive for *N. meningitidis* (or a positive CSF PCR) and/or with a clinical diagnosis of meningitis in combination with a blood culture positive for *N. meningitidis*. In case of bacteremia without focus no clinical focus was identified. Bacteremia with other focus was defined as a positive blood culture in combination with a clinical focus other than meningitis. Information about clinical presentation on admission was collected together with laboratory parameters from blood taken in the first 12 hours after hospital admission and data of the disease course during hospitalization. Information on the following parameters were collected: number of symptomatic days before admission, temperature, fever (temperature >38^{0}C) and presence of petechial rash at the time of admission, underlying comorbidities, development of septic shock (defined as clinical diagnosis or requiring IV-fluids), development of multiple organ failure (MOF) or disseminated intravascular coagulation (DIC) as reported by treating physician, need for active ventilation, length of hospital stay and rate of admission to the Intensive Care Unit (ICU). Case-fatality was defined as in-hospital death or death within 30 days after the first blood/CSF culture positive for *N. meningitidis*.

Lipid A mutation

To identify meningococcal isolates as lipid A variants, isolates were tested for their capacity to induce the pro-inflammatory cytokine IL-6, by using methods described previously[11]. In summary, serial dilutions of heat-inactivated bacterial suspensions were added to the human monocytic cell line Mono Mac 6 (MM6) and incubated 16 h at 37^0C in a humidified atmosphere containing 5% CO_2. IL-6 in supernatant was determined by ELISA. Of all strains causing a low induction of IL-6, the *lpxL1*-gene was sequenced to define the mutation.

Statistical analysis

Proportions were tested using Chi-square test or Fisher's exact tests as appropriate. Continuous outcomes were tested with nonparametric tests and presented in interquartile ranges (IQR). For each of the studied dichotomous outcomes a multivariable logistic regression model was fitted to estimate the association between determinants and the outcome. Those determinants that were univariately associated with the outcome were included in the multivariable logistic regression model, including potential confounders. Serogroup (B; C; others) and clonal complexes (cc11; cc32; cc41/44; others) were included as categorical determinants. Age was included as continuous determinant. For continuous outcome parameters linear regression models were used. Since age proved to be an important effect modifier, analyses were also performed in separate age-groups 0-4 years and ≥5 years. Clinical syndrome was included as an effect modifier to assess potential interaction. All statistical tests were two-tailed, p-values of less than 0.05 were considered significant. Statistical analyses were performed with SPSS 16.0 and Excel 2007.

Results

Study patients

In total 467 cases of meningococcal disease were reported by the nine sentinel laboratories to the NRLBM between June 2001 through May 2006. Hospital records could be traced for 458 (98%) patients (See Figure S1 for study flow chart). Meningitis was the most frequently diagnosed disease syndrome (314 cases; 67%), followed by 110 cases (24%) of bacteremia without focus and 15 cases (3%) of bacteremia with other focus.

Disease isolates

The isolates of 448 patients (96%) were available for further study on IL-6 induction. Of these, 349 (78%) were of serogroup B, 95 (21%) serogroup C and 4 (1%) belonged to other serogroups (29E and W135). Twenty-nine isolates (6.5%) had a decreased potential to induce IL-6. No Lipid A variants were found in the non-hospital record confirmed cases. Sequence analyses revealed *lpxL1* mutations in all of them (Figure S2). Nine different *lpxL1* mutations were found (Table 1). Of the 29 disease lipid A variant strains, 18 (62%) were of serogroup B, the remaining 11 strains (38%) were of serogroup C. The most prevalent clonal complexes among the lipid A variants were cc41/44 (11 isolates; 38%), cc11 (10 isolates, 34%) and cc461 (3 isolates, 10%) (Figure 2, Table S1).

Patient characteristics

The lipid A variants were distributed among all ages with a median age of 19.3 years (IQR 4.6-50.5) compared to 5.9 years (IQR 1.8-18.9) for wild type meningococci (p=0.007). The proportion of lipid A variants was lowest in children under 5 years of age (4.4%) and rose gradually to 24.2% among patients 45-64 years of age (Figure 1). Proportions of lipid A variants varied between 5.1% for meningitis to more than 10% for bacteremia without focus (p=0.04) (Table S2). All patients were hospitalized except for two patients infected with lipid A variant strains, who presented at the acute day ward but were sent home because of mild symptoms. In the medical record confirmed cases, blood was taken in the first 12 hours after admission in 96% (412/441) of the patients.

A milder clinical presentation on admission was seen after infection with lipid A variant strains, with less fever (56% vs. 77%; p=0.02) and less petechiae (50% vs. 68%; p=0.05) compared to infection with wild type strains. Also, several outcome parameters suggested a milder disease course in infections with a lipid A variant; fewer patients had active ventilation (4% versus 22% of wild type infected patients; p=0.02) and IC admittance was less frequent in patients with lipid A mutants (38% vs. 17%; p=0.02) (Table S2). Age proved to be an effect modifier; the association between lipid A variant

infection and fever was different for patients younger than 5 years of age and those older (p-value for interaction = 0.002). Also the data suggested a possible difference of the association between lipid A variant infection and sepsis for these subgroups (p-value for interaction = 0.082).

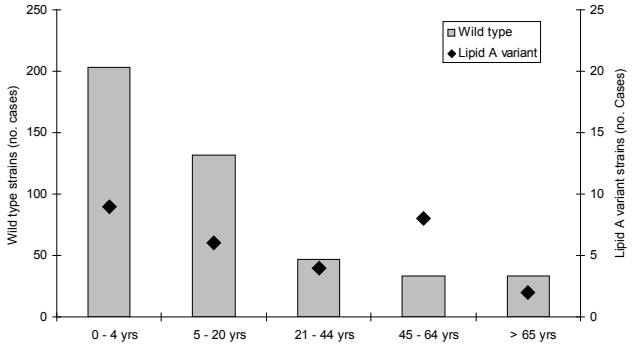

Figure 1. Distribution of lipid A variant and wild type strains among patients with meningococcal disease according to different age categories, N = 448.

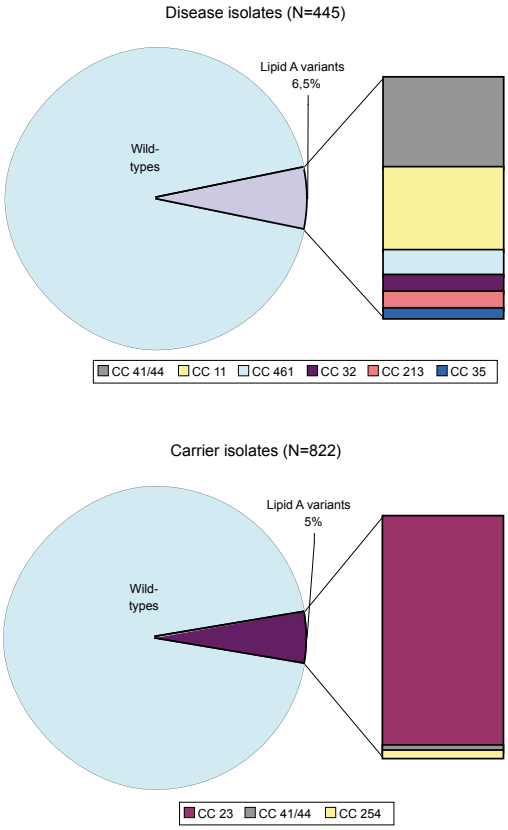

Figure 2. Clonal complex distribution of lipid A variants in disease (n=445) and carrier-isolates (n=822).

Table 1. Characteristics of lipid A variants in patients with meningococcal disease

Strain no.	Age (yrs)	Disease syndrome	Comorbidities	IC admittance	Mortality	Lipid A variant	ST	Clonal Complex
2022526	1 month	Meningitis				XI	461	461
2030161	7 months	Meningitis				III	571	41/44
2040084	1	Meningitis				V	5264	41/44
2050093	1	Bacteremia w/o focus				V	461	461
2041608	2	Meningitis				Deletion*	5413	41/44
2020348	3	Meningitis		X		IV	11	11
2012429	4	Meningitis				III	34	32
2020147	4	Meningitis				VIII	11	11
2021018	4	Bacteremia w/o focus				V	11	11
2020982	6	Meningitis	Fragile X syndrome, mental retardation			V	3732	11
2050247	9	Meningitis				V	41	41/44
2020382	14	Bacteremia w/o focus				V	41	41/44
2032251	14	Bacteremia w/o focus				III	7217	213
2031814	17	Bacteremia w/o focus				XII	7056	41/44
2021270	19	Meningitis				III	461	461
2011799	21	Meningitis	Osteogenesis Imperfecta	X		IV	11	11
2011852	30	Bacteremia w/o focus	Juvenile angiofibroma nasofarynx			V	11	11
2021197	39	Bacteriemia w/o focus	HIV positive			III	11	11
2030960	40	Meningitis				III	32	32
2040655	46	Bacteremia w/o focus				Conserved Amino Acid	571	41/44
2020799	49	Meningitis				Conserved Amino Acid	35	35
2040673	49	Meningitis	Multiple Sclerose and aphasia	X	X	III	41	41/44
2012536	51	Bacteremia w/o focus				III	11	11
2022289	53	Pneumonia	M. Crohn, Barret oesofagitis			X	11	11
2050499	58	Bacteremia w/o focus	Alcohol abuses, liver failure		X	Deletion*	5413	41/44
2050392	62	Meningitis	Intracerebral haematoma			VII	213	213
2050697	63	Meningitis	COPD, Epilepsy, Liver disease	X		V	41	41/44
2021152	76	Bacteremia w/o focus	COPD, AAA, Developed meningococcal disease after 77 days of hospitalization with complicated aorta-valve replacement and 30 days on ICU		X	V	11	11
2021179	80	Bacteremia w/o focus	Lungemboly and lunginfarction, COPD			IX	3755	41/44

* Deletion in *lpxL1*-gene to large to specify

Patients 0-4 years of age

Among infants younger than five years of age, only 9 of the 203 patients (4.4%) were infected by lipid A variant meningococci. No differences were seen in clinical manifestation at admission between the group of cases infected with lipid A variants and those infected with wild type strains (Table 2). Total white blood cell counts at presentation were higher in infants infected with lipid A variants compared to wild-type infected infants (medians, 22.3 vs. 13.0 x 10^9/L; p=0.006) as well as total and mature neutrophil counts and hemoglobine concentration (Table 3). No differences were observed for CRP and thrombocyte counts. During admission no differences were seen in development of septic shock, IC admission, or in mortality. Multivariable analyses showed infection with lipid A variants to be associated with higher white blood cell counts (p=0.03).

Patients 5 years of age and older

Among patients aged 5 years and older, 20 (8.2%) of 245 cases were infected by lipid A variant meningococci. Underlying comorbidities were present in 58% (11/19) of the patients with lipid A variants versus 23% (50/214) in patients infected with wild type meningococci (p<0.005). Patients infected with lipid A variants were more often diagnosed with bacteremia without focus and less with meningitis. A milder clinical presentation on admission was seen after infection with a lipid A variant, with less fever (7/18 [39%] vs. 131/182 [72%]; p=0.006) and less petechiae (6/19 [32%] vs. 139/212 [66%]; p=0.005) compared to infection with wild type strains (Table 2). White blood cell counts and thrombocyte counts were not different between patients infected with lipid A variants and wild type meningococci. Immature neutrophil counts (medians, 0.9 vs. 2.3 x 10^9/L; p=0.05) and levels of CRP (medians, 163 vs. 112 mg/dl; p = 0.05) were lower after lipid A variant infections (Table 3). The same was observed for serum lactate (medians, 1.9 vs. 4.1 μmol/l; p=0.03), but this was tested only in a small proportion of patients. During the course of infection, fewer patients with infection due to lipid A variants developed septic shock (2/19 [11%] vs. 81/214 [38%]; p=0.02). Patients were also less likely to have mechanical ventilation (0/19 [0%] vs. 43/203 [21%]; p=0.03) and IC admittance was less frequent in patients with lipid A variants (4/20 [20%] vs. 92/205 [45%]; p=0.04). No difference was seen in mortality between both groups. The association between lipid A variant infection and fever was similar for patients with and patients without clinical meningitis (p-value for interaction 0.918). Also the association between lipid A variant infections and petechiae was similar between these subgroups (p-value for interaction 0.335). Multivariable analyses showed that infection with lipid A

Table 2. Clinical manifestation on admittance and disease course in patients with meningococcal disease due to wild type or lipid A variant strains; Sorted by age-groups 0-4 years and ≥5 years of age.

	0-4 years			≥5 years		
	Wild type	**Lipid A variant**	**p-value[a]**	**Wild type**	**Lipid A variant**	**p-value[a]**
Patient characteristics	**N=194**	**N=9**		**N=225**	**N=20**	
Male, *n (%)*	109/194 (56%)	4/9 (44%)	0.51	109/225 (48%)	8/20 (40%)	0.49
Underlying comorbidity, *n (%)*	21/192 (11%)	0/9 (0%)	0.60	50/214 (23%)	11/19 (58%)	**0.002**
Clinical manifestation at admission						
Disease syndrome: Meningitis, *n (%)*	149/192 (78%)	7/9 (78%)	1.00	149/218 (68%)	9/20 (45%)	**0.05**
Disease syndrome: Bacteremia w/o focus, *n (%)*	40/192 (21%)	2/9 (22%)	1.00	58/218 (27%)	10/20 (50%)	0.037
Symptoms before admittance < 1 day, *n (%)*	48/180 (27%)	1/8 (13%)	0.68	40/198 (20%)	0/15 (0%)	**0.08**
Symptoms before admittance ≥ 1 week, *n (%)*	9/171 (5%)	0/7 (0%)	1.00	15/183 (8%)	3/14 (21%)	0.12
Fever; temperature >38 °C, *n (%)*	122/148 (82%)	7/7 (100%)	0.60	131/182 (72%)	7/18 (39%)	**0.006**
Petechiae, *n (%)*	132/185 (71%)	8/9 (89%)	0.45	139/212 (66%)	6/19 (32%)	**0.005**
Disease Course						
Septic Shock, *n (%)*	89/192 (46%)	5/9 (56%)	0.74	81/214 (38%)	2/19 (11%)	**0.02**
DIS / MOF, *n (%)*	19/183 (10%)	0/9 (0%)	0.60	24/205 (12%)	1/19 (5%)	0.70
Active ventilation, *n (%)*	43/183 (24%)	1/9 (11%)	0.69	43/203 (21%)	0/19 (0%)	**0.03**
Days in hospital, median (IQR)	9 (8-11)	8 (8-11)	0.48	10 (8-14)	13 (7-18)	0.56
IC admittance, *n (%)*	58/188 (31%)	1/9 (11%)	0.28	92/205 (45%)	4/20 (20%)	**0.04**
Mortality, *n (%)*	10/185 (5%)	0/9 (0%)	1.00	23/205 (11%)	3/20 (15%)	0.71

[a] Proportions were tested with Chi-square test or Fisher's exact tests, as appropriate, all p values are 2 sided. Continuous outcomes were tested with nonparametric tests; IQR, Interquartile range. Clinical manifestations that show (trend towards) a significant correlation with lipid A variant meningococci are depicted in bold.

Table 3. Blood investigations within 12 hours after admittance in patients with meningococcal disease due to wild type of lipid A variant strains.

	0-4 years			≥5 years		
	Wild type	**Lipid A variant**	**p-value**	**Wild type**	**Lipid A variant**	**p-value**
Parameters	N=194	N=9		N=225	N=20	
Infectious						
White cell count, 10^9/L (IQR)	13.0 (6.5-20.4)	22.3 (17.0-29.0	**0.006**	17.8 (11.8-25.1)	15.8 (12.1-17.3)	0.18
Neutrophil count, 10^9/L (IQR)	8.7 (4.2-16.1)	22.0 (10.6-23.9)	**0.02**	15.4 (9.6-22.5)	11.1 (7.0-15.9)	**0.10**
-Immature	1.3 (0.5-3.6)	6.4 (2.7-14.1)	0.26	2.3 (0.8-4.1)	0.9 (0.1-2.0)	**0.05**
-Mature	6.5 (2.7-14.1)	13.9 (11.2-22.4)	**0.03**	14.3 (8.7-19.8)	9.9 (6.3-13.9)	**0.08**
Lymfocyte count, 10^9/L (IQR)	1.9 (1.3-3.2)	2.0 (1.0-4.4)	0.92	1.1 (0.7-1.6)	0.9 (0.6-1.5)	0.65
CRP, mg/dl (IQR)	140 (66-211)	198 (120-247)	0.19	163 (101-249)	112 (45-212)	**0.05**
Coagulation						
Thrombocyte count, 10^9/L (IQR)	246 (190-308)	320 (237-374)	**0.08**	197 (148-247)	208 (144-252)	0.91
PT, sec (IQR)	-	-	-	18 (14-21)	3 (2-14)	**0.02**
aPTT, sec (IQR)	-	-	-	39 (32-49)	43 (34-60)	0.58
Other parameters						
Hemoglobine, mmol/l (IQR)	7.0 (6.6-7.5)	7.9 (7.0-8.2)	**0.04**	8.1 (7.5-8.6)	8.2 (7.6-9.0)	0.92
Sodium, mmol/l (IQR)	135 (133-137)	134 (130-137)	0.24	136 (134-139)	136 (134-138)	0.58
Potassium, mmol/l (IQR)	3.9 (3.5-4.2)	3.7 (3.2-4.1)	0.50	3.6 (3.2-3.9)	4.0 (3.6-4.5)	**0.003**
Glucose, mmol/l (IQR)	6.5 (5.0-7.7)	6.9 (5.6-8.4)	0.39	7.3 (6.2-8.7)	7.5 (6.5-10.6)	0.38
Lactate, μmol/l (IQR)	-	-	-	4.1 (2.3-6.0)	1.9 (0.8-1.9)	**0.03**
Creatinine, μmol/l (IQR)	41 (30-59)	48 (30-58)	0.82	89 (67-121)	76 (65-108)	0.33
Urea, mmol/l (IQR)	4.8 (3.6-6.2)	3.9 (3.7-5.0)	0.38	5.9 (4.6-8.3)	6.0 (4.5-9.5)	0.88

[a] P values were calculated by nonparametric tests; all p values are 2 sided. IQR, Interquartile range. Clinical manifestations that show (trend towards) a significant correlation with lipid A variant meningococci are depicted in bold.

variants was independently associated with different clinical outcomes (Table 4). Lipid A variant infected patients were more likely to have underlying comorbidities (OR 4.1; 95% CI 1.3-12.6), and developed less fever (OR 0.3; 95% CI 0.1-0.8), less petechiae (OR 0.3; 95% CI 0.1-0.8) and less septic shock (OR 0.2; 95% CI 0.04-0.9). Linear multivariable analyses confirmed that in patients after infection with lipid A variants levels of CRP were on average 94 mg/dl lower (95% CI -152 to -34; p=0.002); temperature at admission was on average 0.7 ^{0}C lower (95% CI -1.3 to -0.03; p=0.04).

Table 4. Multivariable analyses for infection with lipid A variants on different outcome variables in patients ≥5 years of age.

Outcome variable	Adjusted OR (95% CI)	p-value
Clinical characteristics [a]		
Disease syndrome: Meningitis	0.37 (0.13-1.04)	0.06
Fever; temperature >38 ^{o}C	0.27 (0.09-0.77)	0.02
Petechiae	0.28 (0.09-0.82)	0.02
Septic Shock	0.19 (0.04-0.87)	0.03
IC admittance	0.20 (0.06-0.68)	0.01
Patient characteristics [b]		
Underlying comorbidities	4.10 (1.34-12.60)	0.01

[a] Odd ratio adjusted for age, gender, underlying comorbidities, serogroup and clonal complex.
[b] Odds ratio adjusted for age, gender, serogroup and clonal complex

Carrier isolates

Of 822 meningococcal carrier isolates, 45 strains (5.5%) had a lower potential to induce IL-6. All of these had a mutation in *lpxL1*; 3 types of mutations (III, V and VI) were observed. The 45 lipid A variants among carrier isolates were almost exclusively of serogroup Y (38, 84%), 6 (13%) were of unknown serogroup and one (2%) lipid A variant strain had serogroup B. We found no lipid A variant carrier strains of serogroup C. Of the 45 lipid A variants among carrier isolates, 43 (96%) belonged to cc23, a clonal complex not found among our invasive isolates (Figure 2).

Discussion

In this report we show that the prevalence of lipid A variants in a large retrospective cohort of patients with invasive meningococcal disease in the Netherlands is age-dependent and highest in the age category 45-64 year (24%). In addition, older children and adults infected with lipid A variants endure milder symptoms with less systemic inflammation which occurs independent of disease syndrome. In carrier isolates a different distribution of lipid A variants was found, mainly belonging to one single clonal complex, namely clonal complex cc23.

Variation in LPS-structure and lipid A variance has been described for several bacteria allowing pathogens to evade host immunity[14]. *Yersinia* and *Pseudomonas* species modulate their LPS structures which results in diminished TLR4 responses[14, 15]. For *N. meningitidis* the importance of LPS detection is illustrated by the finding that individuals with rare mutations in TLR4 are more prone to meningococcal disease[16]. We previously suggested that *N. meningitidis* is also a pathogen modulating its lipid A structure under selective pressure with *lpxL1* mutations resulting in penta- instead of hexa-acylated lipid A[11]. In this report we show that infections by these lipid A variant strains occur frequently and do not show the age-dependent decrease in incidence as infections by wild type strains. This resulted in highest proportions of lipid A variant strains among patients 45-64 year of age. The incidence of meningococcal disease caused by wild type strains among persons >5 years of age decreases, presumably due to the development of specific, functional antibodies[5, 6]. Influence of LPS variation on the adaptive immune system through its effect on TLR4 signaling has been described for *B. parapertussis*. Recently, Wolfe et al described that in animals passively immunized with antibodies, this bacterium can survive and grow efficiently because its LPS is less stimulatory for TLR4[17]. When TLR4 agonist was added, *B. parapertussis* was cleared. Secondly, a connection at receptor level between TLR4 and FcRIII pathways has been described, where TLR4 signalling proved to be essential for FcRIII signalling[18]. These findings can form an explanation for the absence of an age-dependent decline in disease due to meningococcal lipid A variant strains, since pre-existing specific immune responses, especially bactericidal antibodies are hampered by TLR4 evasion. The potential association between the development of specific immunity and variant lipid A frequency however should be further investigated; as such immune evasion may have implications for effectiveness of vaccination against meningococcal disease.

We found differences in disease course for lipid A variant infections when all ages were taken together in a single analysis. However, subgroup analyses showed these differences were only present in the older age groups, as age was an important

effect modifier. In children under five year of age, the numbers of lipid A variants in our study are too small to draw any firm conclusions about clinical impact. No differences or trends towards differences in clinical picture were seen after infection with lipid A variant strains in comparison to wild-type strains. In young children, both the adaptive and innate immune components are not yet fully matured and innate responses to LPS in neonates have been described to be impaired as well[19, 20]. Probably, due to this overall poor maturation of the immune system, in young children the lipid A variants show fewer differences in clinical picture and do not have a selective advantage in comparison to wild type strains[17]. However, also host mechanisms like complement and leukocyte activation can be of influence[21]. It has been advocated that less activation of TLR4 opens possibilities for treatment of fulminant septic shock[22]. However, the probable difference in TLR4-activation by naturally occurring lipid A variants and its clinical impact in the different age categories questions the impact of these new treatment strategies in the youngest patients and supports further research.

In this study we further substantiate the clinical significance of lipid A variants for meningococcal infections in children over 5 years and adults. We show that infection with lipid A variant strains is an independent determinant of a mild disease course in non-meningitis meningococcal infections. Systemic infection seems to be less severe because of less fever, lower serum CRP and less development of septic shock and IC admittance. Also patients with lipid A variant infections tended to have longer symptoms before hospital admission, possibly due to a delayed host response as described for *B. parapertussis* in a animal infection model[17]. The lower amount of petechiae and the reduced protrombin time in patients infected with lipid A variants supports the notion that the lipid A variants have reduced potential for cytokine induction and activation of tissue-factor mediated coagulopathy[8]. In contrast to our earlier findings, thrombocyte counts did not differ, possibly due to differences in age-distribution and disease syndrome. However, subgroup analyses did not show different impact of lipid A variants on clinical characteristics in patients with meningitis or bacteremia without focus. The percentage of underlying comorbidities in patients with lipid A variant infection was higher than in wild type meningococcal infection. A potential correlation with impaired immune responses could not be fully determined because we had no data on medication use, for example corticosteroids in COPD. Possibly a reduced immune status or higher frequency of respiratory infections is of influence for lipid A variants to successfully overcome mucosal barriers[23, 24]. Underlying comorbidities can explain the non-difference in mortality between lipid A variants and wild type strains, because all three deceased patients with lipid A variants had underlying comorbidities.

Except for isolates of cc23, a low proportion (0.3%) of lipid A variants was found among meningococcal carrier isolates. In carriage, the innate immune system plays a crucial role in immediate protection against invasion by a strong first line defence through epithelial and phagocytic cells, along with complement factors and antimicrobial peptides. Our results suggest that penta-acylated lipid A is not advantageous to meningococci when they reside on the mucosa of the nasopharynx. This might be due to low TLR4 expression on mucosal surface; i.e. epithelial cells compared to for example white blood cells. Moreover, mucosa produce a significant amount of cationic antimicrobial peptides (CAMPs)[25]. Meningococci expressing under-acylated lipid A on their surface have an increased susceptibility to CAMPs[26], providing an explanation for the decreased frequencies of lipid A variants among isolates from carriers observed in this study. A high frequency of lipid A variants was observed among serogroup Y isolates from carriers, in particular among cc23, suggesting that the disadvantage of expressing penta-acylated lipid A is not present or compensated in these meningococci. Underlying mechanisms for this high prevalence are currently unknown and should be further investigated. This may be relevant as a recent study in the USA and Israel described high disease rates of cc23[27]. In our study no invasive cc23 cases were found, in line with a study of disease-associated and carriage meningococcal isolates in three European countries[28].

Some limitations of our study should be acknowledged. First, the numbers of lipid A variants are still relatively small, leaving some uncertainties for comparative analyses. Especially for the youngest age-category small numbers result in limited precision and preclude firm conclusions. However, for the older age-categories differences after infection with lipid A variant strains or wild-type strains seem consistent for most parameters tested, also after correcting for other potential predictors. Second, clinical data were collected retrospectively. In only 2% of the cases no clinical information was available and most clinical parameters and essential laboratory values were available in more than 80% of the cases. However, some laboratory parameters like lactate and protrombin time should be interpreted with caution since only for a limited number of cases data were available. Third, serogroup distribution is highly regional[2, 4]. Also cases in clusters or localized outbreaks with different serogroups or clonal complexes have been described. It is possible that the clonal complex distribution of the carrier isolates and disease isolates can be affected by time and place of collection.

In conclusion, we show that a significant proportion of meningococcal disease is caused by meningococci with penta-acylated lipid A, while frequencies of lipid A variants in carriage isolates were very low with the exception of cc23. These findings shed new light on the adaptation of meningococci when interacting with its host, and emphasize

further study of this naturally occurring lipid A variant in invasive disease and carriage, as such immune evasion may have implications for effectiveness of vaccination against meningococcal disease. We show that meningococcal disease due to lipid A variant meningococci is very common among adults and vulnerable patients with underlying comorbidities. Conceivably, due to inefficient stimulation of TLR4, lipid A variant meningococci poorly stimulate the inflammatory response and are better able to avoid host immunity. Hence, older children and adults infected with lipid A variants endure milder symptoms with less systemic inflammation. In young children these clinical differences were less apparent. These findings underline the importance of research for new treatment strategies for meningococcal disease.

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Supporting information

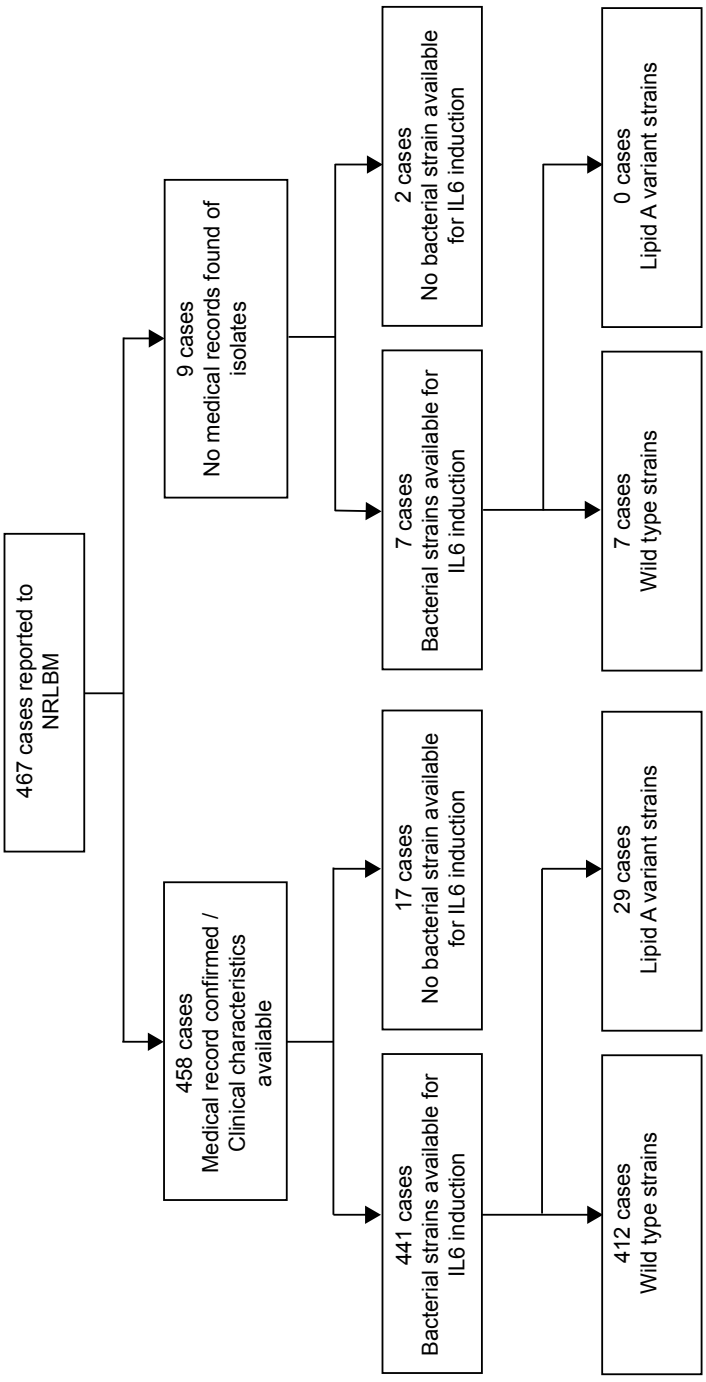

Figure S1. Flow diagram of meningococcal disease isolates submitted to thavailability of medical records and IL6 induction.

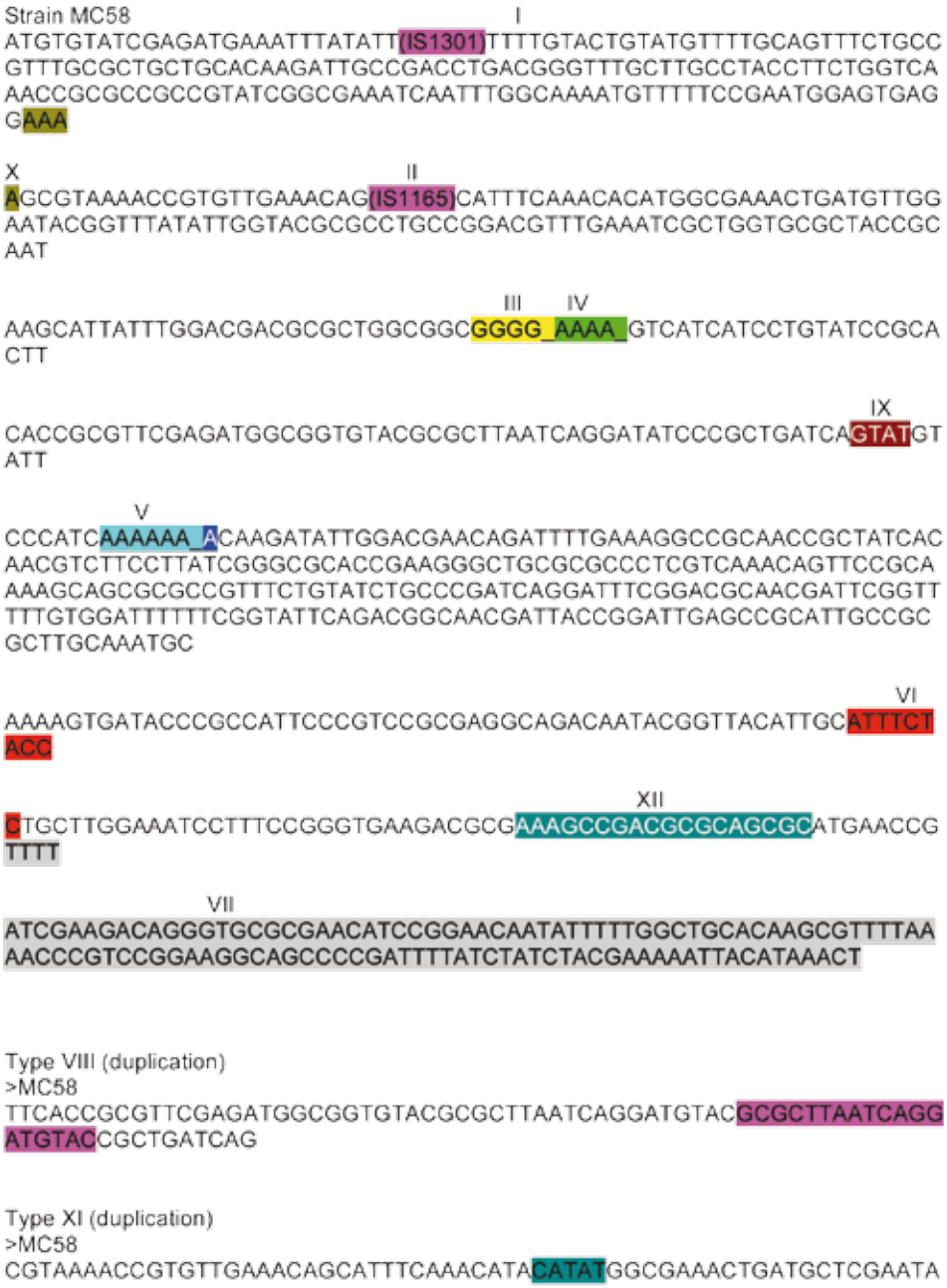

Figure S2. Type and position of mutations in the *lpxL1*-gene of strain MC58
Strain MC58.

Table S1. Frequencies of lipid A variants in clonal complexes of disease and carrier meningococcal strains.

Clonal Complex	Proportion of lipid A variants Disease isolates N=448 (%)	Carrier isolates N=822 (%)
cc41/44	11/217 (5%)	1/138 (1%)
cc11	10/76 (13%)	0/8 (0%)
cc461	3/3 (100%)	0/6 (0%)
cc32	2/71 (2,8%)	0 (-)
cc213	2/15 (13%)	0/3 (0%)
cc35	1/3 (33%)	0/45 (0%)
cc23	0 (-)	43/71 (61%)
cc254	0/1 (0%)	1/16 (6%)
Others	0/62 (0%)	0/535 (0%)

Table S2. Clinical manifestation on admittance and disease course in patients with meningococcal disease due to wild type or lipid A variant strains.

Patient characteristics	All ages Wild type N=419	Lipid A variant N=29	p-value
Age at presentation, years (IQR)	5.9 (1.8-18.9)	19.3 (4.6-50.5)	**0.007**
Male, *n (%)*	218/419 (52%)	12/29 (41%)	0.27
Underlying comorbidity, *n (%)*	71/406 (17%)	11/28 (39%)	**0.004**
Clinical manifestation at admission			
Clinical syndrome: Meningitis, *n (%)*	298/410 (73%)	16/29 (55%)	**0.04**
Clinical syndrome: Bacteremia w/o focus, *n (%)*	98/410 (24%)	12/29 (41%)	0.04
Symptoms before admittance < 1 day, *n (%)*	88/378 (23%)	1/23 (4%)	**0.03**
Symptoms before admittance ≥ 1 week, *n (%)*	24/354 (7%)	3/21 (14%)	0.19
Fever, *n (%)*	253/330 (77%)	14/25 (56%)	**0.02**
Petechiae, *n (%)*	271/397 (68%)	14/28 (50%)	**0.05**
Clinical Course during admission			
Septic Shock, *n (%)*	170/406 (42%)	7/28 (25%)	**0.08**
DIS / MOF, *n (%)*	43/345 (11%)	1/28 (4%)	0.34
Active ventilation, *n (%)*	86/386 (22%)	1/28 (4%)	**0.02**
Days in hospital, median (IQR)	9 (8-12)	9 (7-16)	0.80
IC admittance, *n (%)*	150/393 (38%)	5/29 (17%)	**0.02**
Mortality, *n (%)*	33/390 (8%)	3/29 (10%)	0.73

[a] Proportions were tested with Chi-square test or Fisher's exact tests, as appropriate, all p values are 2 sided. Continuous outcomes were tested with nonparametric tests; IQR, Interquartile Range. Clinical manifestations that show (trend towards) a significant correlation with lipid A variant meningococci are depicted in bold.

Chapter 6

The structure of *Neisseria meningitidis* lipid A determines outcome in experimental meningococcal disease

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Abstract

Lipopolysaccharide (LPS), a major component of the meningococcal outer membrane, is sensed by the host through activation of Toll-like receptor 4 (TLR4). Recently, we demonstrated that a surprisingly large fraction of *Neisseria meningitidis* disease isolates are lipid A mutants, due to inactivating mutations in the *lpxL1* gene. The *lpxL1* mutants activate human TLR4 much less efficiently than wild type bacteria, which may be advantageous by allowing them to escape from the innate immune system. Here we investigated the influence of lipid A structure on virulence in a mouse model of meningococcal sepsis. One limitation however is that murine TLR4 recognizes *lpxL1* mutant bacteria much better than human TLR4. We show that an *lpxL2* mutant, another lipid A mutant lacking an acyl chain at a different position, activates murine TLR4 less efficiently than the *lpxL1* mutant. Therefore, the *lpxL2* mutant in mice might be a better model for infections with *lpxL1* mutants in humans. Interestingly, we found that the *lpxL2* mutant is more virulent in mice than the wild type strain, whereas the *lpxL1* mutant is actually much less virulent than the wild type strain. These results demonstrate the crucial role of *N. meningitidis* lipid A structure in virulence.

Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of virtually all Gram-negative bacteria[1]. Many species contain a common form that consists of a highly variable surface exposed polysaccharide and a more conserved lipid A portion that anchors the molecule to the bacterial outer membrane. It has been known for a long time that LPS has a wide variety of biological activities even at very low concentrations. These properties gave LPS its alternative name "endotoxin". The anticipated receptor for mediating the biological effects of LPS remained elusive for many years[2]. Now we know that lipid A, the bioactive component of the LPS, is recognized by the innate immune system through Toll-like receptor 4 (TLR4). Lipopolysaccharide binding protein (LBP) and CD14 facilitate the transfer of LPS to to the co-receptor MD-2[3]. Binding of LPS to the TLR4/MD-2 receptor complex leads to dimerization of its extracellular domains and recruitment of adaptor proteins to the intracellular domains. This triggers a signaling cascade and subsequent activation of the innate immune system[4]. Among the Toll-like receptors, TLR4 is unique in that it utilizes both the adaptor proteins MyD88 and TRIF. Activation of these proteins eventually leads to induction of pro-inflammatory cytokines and type I interferon, respectively. TLR4 is important for the protection against many Gram-negative bacterial pathogens[5-9].

Several studies have shown that the prototypical *E. coli* lipid A with 6 acyl chains of 12 to 14 carbons in length gives optimal activation of human TLR4, whereas changing the number or the length of the acyl chains, or altering the charge of the lipid A reduce the activity of LPS[10-12]. In addition, there are species-specific differences in recognition of lipid A by TLR4/MD-2[13-16]. The murine TLR4/MD-2 complex seems to be more promiscuous than human TLR4/MD-2 in recognizing various lipid A molecules, but also for murine TLR4/MD-2 recognition, lipid A with 6 acyl chains is optimal.

Lipid A was once thought to be an invariant component, but recent studies have shown that the variety in lipid A structures is actually quite large. These structural alterations have an impact on how well LPS is sensed by the host. Therefore, it seems that bacteria have evolved ways to manipulate the pathogen associated molecular pattern recognized by the host innate immune system to modulate host interactions to their benefit. For example, *Salmonella* and *Pseudomonas* species can modulate their lipid A structure in response to the host environment[14, 17-21]. Other human pathogens such as *H. pylori*, *L. pneumophila*, *Y. pestis* and *Francisella spp.* also have lipid A moieties that are poorly recognized by human TLR4, which likely contributes to their ability to cause disease in humans[22].

The Gram-negative bacterium *Neisseria meningitidis* is a frequent commensal of the human upper respiratory tract[23]. Occasionally, the bacterium becomes invasive causing the serious conditions meningitis and/or sepsis in otherwise healthy individuals. Meningococcal lipid A consists of 6 acyl chains and is highly biologically active. It has a major contribution to the induction of the excessive pro-inflammatory responses seen in meningococcal disease[24, 25]. Moreover, levels of circulating *N. meningitidis* LPS are directly correlated to the morbidity and mortality of meningococcal sepsis[26, 27].

Recently, we demonstrated that a surprisingly large fraction (about 9%) of meningococcal disease isolates have lipid A with only 5 acyl chains, due to inactivating mutations in the acyl-transferase gene *lpxL1*[28]. These LPS mutants induced much less pro-inflammatory cytokines in different cell types and this effect is TLR4-dependent. The high frequency of the *lpxL1* mutants suggests there must be a benefit for the bacterium to have underacylated lipid A under certain conditions. A reasonable hypothesis would be that the *lpxL1* mutants are better capable of evading the innate immune defenses of the host, because of the reduced capability of their lipid A to activate TLR4.

In this study we wanted to compare the virulence of the *N. meningitidis lpxL1* mutant with the wild type strain in a mouse model of meningococcal sepsis. However, while *lpxL1* LPS is a poor activator of human TLR4/MD-2, it is still a significant activator of murine TLR4/MD-2[29]. Therefore, we also included an *lpxL2* mutant in our study, a mutant defective in the other gene required for the addition of a secondary acyl chain to the lipid A moiety[30]. Here we show that the *lpxL2* mutant is a weaker activator of murine TLR4/MD-2 than the *lpxL1* mutant. Interestingly, the *lpxL2* mutant was much more virulent in the mouse model of meningococcal sepsis than the wild type strain. On the other hand, the *lpxL1* mutant was completely avirulent. These results demonstrate the importance of *N. meningitidis* lipid A structure for virulence.

Materials and Methods

Animals

Female specific-pathogen-free C57BL/6JIco mice were purchased from Charles River Laboratories and were housed under specific-pathogen-free conditions. Mice were acclimatized for approximately 1 week and were 6 to 8 weeks old at the start of the experiment. Animal experiments were approved by the Institute's Animal Ethics Committee.

Bacterial strains and growth conditions

The *lpxL1* and *lpxL2* mutants were generated from the parent strain H44/76, immunotype L8 as described previously[30]. The construction of the *lpxA* mutant is also described elsewhere[31]. Strains were grown on GC medium base (Difco laboratories) supplemented with IsoVitaleX (Becton Dickinson) overnight at 37°C in 5% CO_2 in a humid atmosphere. For stimulation of cell lines, bacteria were suspended from plate in PBS and the A_{620} was determined. Next, the bacteria were heat inactivated at 56ºC for 30 min. For infection experiments, bacteria were suspended from plate in TSB medium (tryptic soy broth, Becton Dickinson) at an A_{620} of approximately 0.075 and grown at 37°C to log phase (A_{620} of 0.2-0.3). To determine CFUs, serial dilutions of bacterial suspensions in PBS were plated on GC agar and grown overnight at 37°C in 5% CO_2. The next day the number of colonies was counted. LPS from these strains was isolated by the hot-phenol extraction method as described previously[32].

Outer membrane profile analysis

N. meningitidis strains L8 H44/76, L8 *lpxL1*, and L8 *lpxL2* were grown in TSB liquid medium at 37°C to late log phase. Then the bacteria were heat inactivated at 56°C for 1 h. These were used for isolation of outer membrane complexes by sarcosyl extraction as described previously[33]. The quantity of protein was determined with the bicinchoninic acid protein assay reagent (Pierce), with bovine serum albumin as a standard. Protein profiles were analyzed by SDS-PAGE. Gels were stained with Coomassie.

In vitro growth of bacteria in whole mouse blood

Whole blood from C57BL/6JIco mice was collected in 1 ml Lithium Heparin tubes (Greiner Bio-One) and pooled. Strains H44/76, *lpxL1*, and *lpxL2* were grown until log phase as described above and diluted to $1x10^8$ CFU/ml in RPMI 1640 medium (Gibco BRL). Next, bacteria where suspended in 1 ml whole mouse blood at a final concentration of $1x10^3$ CFU/ml or $1x10^5$ CFU/ml and incubated for 4 hours at 37°C, 80 rpm in a humid

atmosphere. Each hour a sample of 10 μl was taken and serial dilutions in RPMI were made, which were plated on GC agar and grown overnight at 37°C in 5% CO_2. The next day the number of colonies on the plates were determined to calculate the CFUs in the samples. This was also done for the original stock of $1x10^8$ CFU/ml bacteria in RPMI to determine the actual number of bacteria that was used.

Cell lines

For experiments and/or maintenance, the mouse macrophage cell line J774A.1 was suspended in IMDM (Gibco BRL) supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin, 300 µg/ml l-glutamine (Gibco BRL), and 10% heat-inactivated fetal calf serum (FCS) (Gibco BRL). For experiments and maintenance of HEK-293 cells stably transfected with mouse TLR4A, MD-2, and CD14 (Invivogen), DMEM (Gibco BRL) was used, supplemented with 10% FCS, 10 μg/ml blasticidin (Invivogen), and 50 μg/ml Hygromycin B (Invivogen).

ELISA

J774A.1or HEK-293 mTLR4/MD-2/CD14 cells were seeded in 96-well flat-bottom plates at 2-3 x 10^5 cells in 200-300 μl per well. Cells were stimulated with heat-inactivated bacteria or purified LPS and incubated o/n at 37 °C in a humidified atmosphere containing 5% CO_2. Cytokine concentrations in the culture supernatants were quantified with ELISA. Mouse IL-6 was determined with the mouse IL-6 ELISA set (BD Biosciences), mouse IP-10 with mouse IP-10 ELISA kit (R&D systems), and human IL-8 with PeliPairTM reagent sets (Sanquin).

Animal model

Female C57BL/6JIco mice 6 to 8 weeks old were randomly distributed in groups of 3 mice per group. Animals were injected intraperitoneally with 100 μl of 20 mg/ml Iron-Dextran (Sigma) in PBS a few hours before infection and again 24 hours later. Strains H44/76, *lpxL1*, and *lpxL2* were grown to log phase as described above and diluted to the desired dose in PBS. Mice were injected i.p. with 100 μl of bacterial suspension. Four different doses of each strain were used: $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$ CFU per mouse. Animal health was monitored twice a day over a period of 48 hours and a health score was assigned to each mouse on each time point. Score was as follows: healthy = 0, slightly ruffled fur = 1, ruffled fur, active = 2, ruffled fur, inactive = 3, ruffled fur, inactive, crouched = 4, very sick, not eating or drinking, no movement after stimulation = 5, death = 6. Mice with a score of 5 were killed to limit suffering. Two hours and 19

hours after infection, blood samples (50 μl) were taken from the tail vein and collected in 1 ml Lithium Heparin tubes (Greiner Bio-One). Serial dilutions in PBS were plated to determine CFUs. The remaining blood was separated by centrifugation to obtain the plasma, which was centrifuged again with 0.22 μm filter Ultrafree-MC (Millipore) to eliminate possible remaining bacteria. Plasma was stored at -20ºC for later use.

Determination of cytokine levels in plasma

Plasma samples of mice 2 hours and 19 hours after infection were analyzed using a Bio-Plex system (Bio-Rad) for determination of cytokines. A 6-plex Bio-Plex assay (Bio-rad) containing beads for mouse IL-1β, IL-6, IL-10, IL-12p70, RANTES, and TNF-α was used.

Statistical analysis

Before statistical analysis, data from CFU values were $\log_{10}$ converted, which normalized their distribution. One-way analysis of variance (ANOVA) was performed followed by the post-hoc Bonferroni's multiple comparison test to analyze differences in means (GraphPad Prism 4). For each dose, H44/76 was compared with *lpxL1* and *lpxL2*. Differences were considered significant at p-values of <0.05.

Results

Characterization of the lpxL mutants

The *lpxL1* and *lpxL2* mutants were generated in strain H44/76, immunotype L8. Their LPS structures are shown in Fig. 1A. The *lpxL1* mutant has penta-acylated lipid A missing the secondary acyl chain at the 2'-position. The *lpxL2* mutant also has penta-acylated lipid A, but lacks the secondary acyl chain at the 2-position. Lipid A was directly extracted from whole bacteria by the ammonium-isobutyrate method and analyzed by nanoelectrospray tandem mass spectrometry to verify the mutant structures[28]. Both the L8 *lpxL1* and L8 *lpxL2* LPS were found to have >95% penta-acyl lipid A (results not shown). The growth of the *lpxL1* and *lpxL2* mutants was compared to that of the parental strain in whole blood of C57BL/6 mice. The blood was incubated with two doses of each strain, $1x10^3$ CFU/ml and $1x10^5$ CFU/ml, and the number of bacteria in the blood after 1, 2, 3, and 4 hours was determined (Fig 1B-C). For both doses, growth was very similar for all strains in whole blood. Finally, the outer membrane protein profiles were compared between the different strains (Fig. 1D). Minor differences in the expression of Opa proteins were visible; however, in our experience the presence or absence of particular Opa proteins does not affect virulence in the i.p. mouse model (van den Dobbelsteen, unpublished results). Overall the outer membrane profiles did not show major differences, suggesting that the *lpxL* mutations did not have unanticipated effects on the expression of proteins possibly involved in the virulence of *N. meningitidis*.

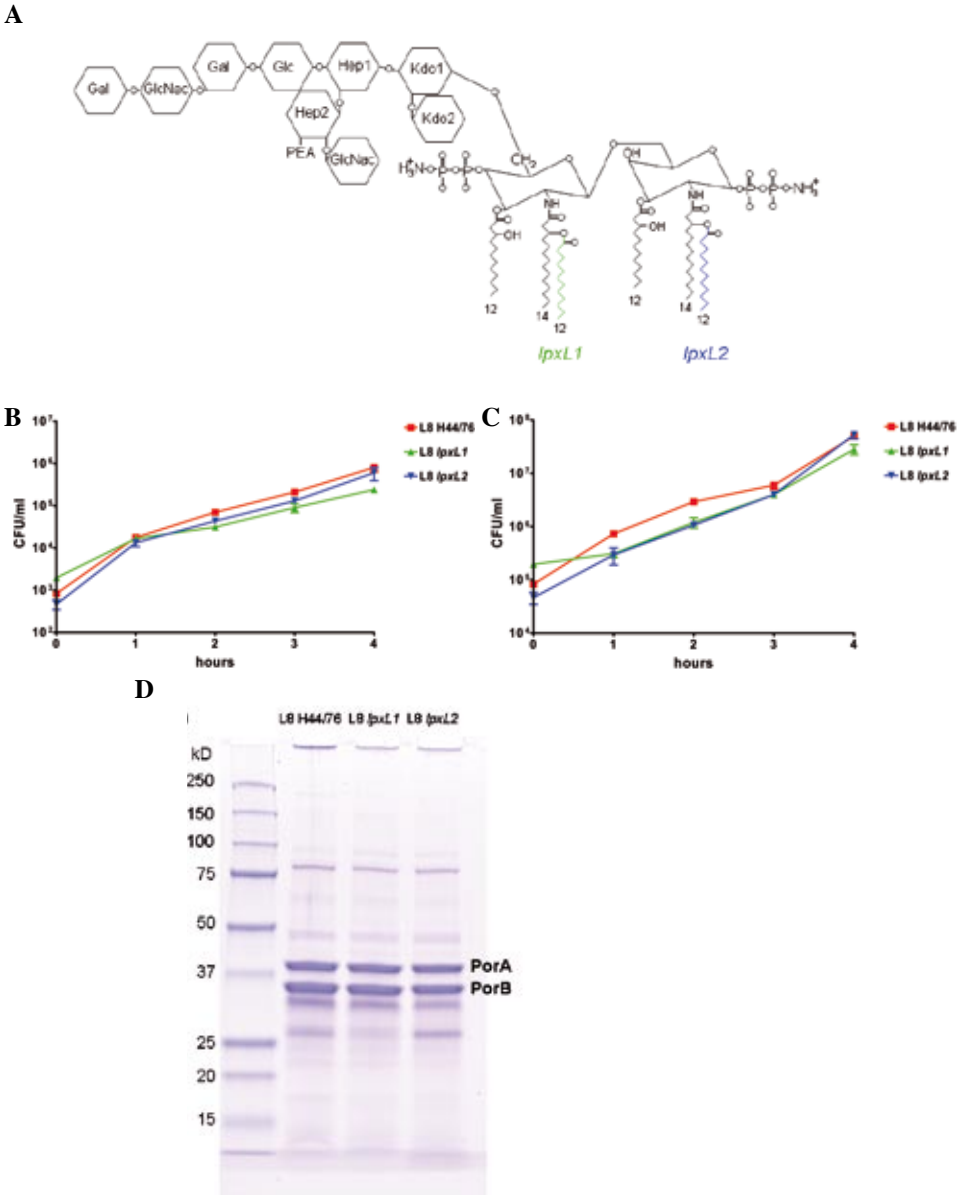


Figure 1. Characterization of *N. meningitidis* strains L8 H44/76, L8 *lpxL1*, and L8 *lpxL2*. (A) Schematic structure of LPS from the wild type strain H44/76, immunotype L8. The acyl chains missing in the *lpxL1* and *lpxL2* mutants are indicated with green and blue, respectively. (B,C) Growth of strains H44/76, *lpxL1*, and *lpxL2* in whole blood from C57BL/6JIco mice. Results of one out of two independent experiments are shown. Data are expressed as means of triplicates, error bars indicate S.E.M. The outcome for an initial dose of approximately $1x10^3$ CFU/ml is shown in panel B, and for an initial dose of approximately $1x10^5$ CFU/ml in panel C. (D) Outer membrane complex proteins of strains H44/76, *lpxL1*, and *lpxL2* separated by SDS-PAGE and stained with Coomassie.

In vitro biological activity of lpxL mutants

HEK293 cells stably transfected with murine TLR4, MD-2, and CD14 were stimulated with different doses of heat inactivated whole bacterial strains: H44/76, the *lpxL1* mutant, the *lpxL2* mutant, and the *lpxA* mutant, which is completely LPS-deficient[31]. In addition, purified wild type LPS, LpxL1 LPS, and LpxL2 LPS was tested. Activation of the LPS receptor complex was assessed by measuring IL-8 production of the HEK293 cells with ELISA (Fig. 2). As expected, wild type bacteria strongly activated the murine LPS receptor complex, while *lpxA* mutant bacteria did not activate the receptor complex at all. Compared to the wild type strain, *lpxL1* mutant bacteria were about 10-fold less efficient in activating murine TLR4/MD-2 and *lpxL2* mutant bacteria about 100-fold. Similar results were obtained with purified LPS from these strains (Fig.2).

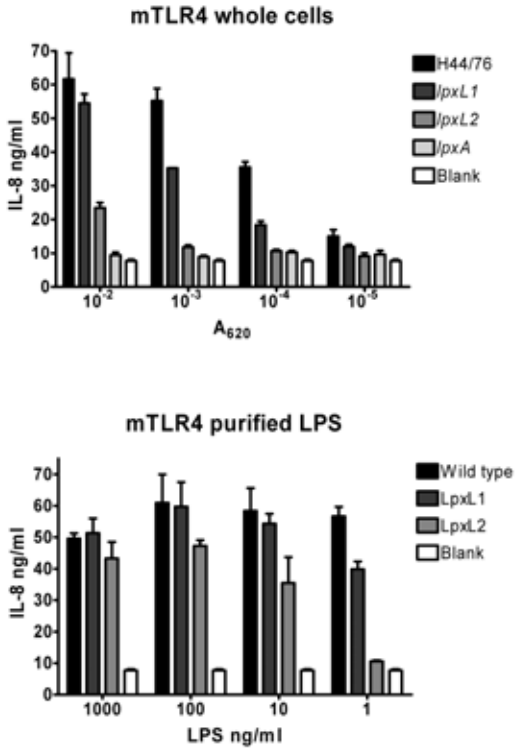


Figure 2. Comparison of murine TLR4 activation by the *lpxL* mutants and wildtype strain. HEK293 cells transfected with mouse TLR4, MD-2, and CD14 were stimulated with different concentrations of heat inactivated whole H44/76 bacteria, *lpxL1* bacteria, *lpxL2* bacteria, *lpxA* bacteria, or purified LPS from these strains. After overnight incubation the IL-8 concentration in the supernatant was measured by ELISA. Results of one representative experiment of three independent experiments are shown. Data are expressed as means of triplicates, error bars indicate S.E.M.

The bacteria also contain other components that can be recognized by pattern recognition receptors that contribute to the innate immune response[34]. Therefore, we also investigated the influence of recognition of *lpxL* lipid A with immune cells that also express other pattern recognition receptors. The murine macrophage cell line J774A.1 was stimulated with titrations of strains *H44/76*, *lpxL1*, *lpxL2*, or *lpxA*. We measured IL-6 and IP-10 induction (Fig. 3).

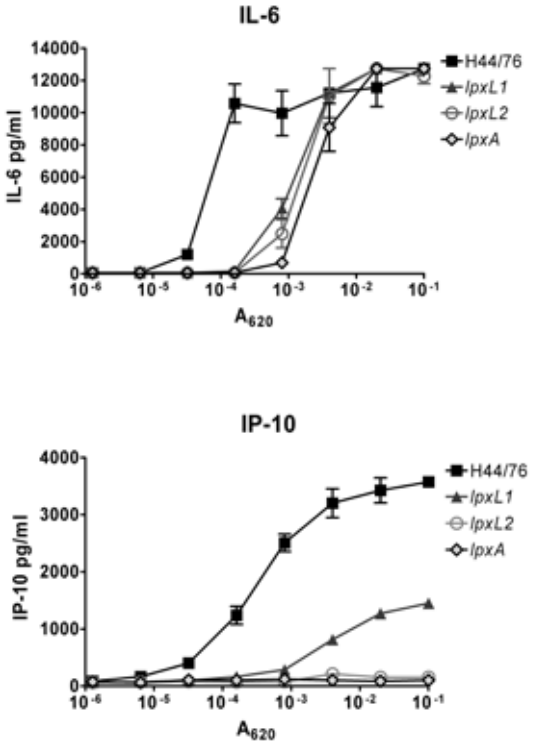


Figure 3. Activation of J774A.1 cells with H44/76, *lpxL1*, *lpxL2*, and *lpxA*. J774A.1 cells were stimulated overnight with titrations of heat inactivated strains H44/76, *lpxL1*, *lpxL2*, and *lpxA*. Concentrations of IL-6 and IP-10 in the supernatant were determined with ELISA. Results of one representative experiment of three independent experiments are shown. Data are expressed as means of triplicates, error bars indicate S.E.M.

IL-6 is induced by the MyD88-dependent pathway and can therefore also be induced by other TLRs, such as TLR2. In contrast, IP-10 is induced by the TRIF-dependent pathway. Therefore, LPS should be the only component of the bacteria capable of inducing IP-10. In agreement with this, differences between the lipid A mutants were most pronounced for IP-10, which was not induced at all by the *lpxL2* and *lpxA* mutants, and about 25-fold less by the *lpxL1* mutant compared to the wild type. For IL-6 all the lipid A mutants were

less active than the wildtype, but differences among them were small (Fig.3). Taken together, these results demonstrate that the wildtype strain is the most potent activator of mouse immune cells and that the *lpxL2* mutant is the weakest activator. As expected, these effects were TLR4-dependent.

Mouse model of meningococcal sepsis

Next, we compared the virulence of the wild type strain, the *lpxL1* mutant, and the *lpxL2* mutant in a mouse model of meningococcal sepsis. We anticipated that the results might be different between different dosages. Therefore, the mice were challenged i.p. with 4 different doses of each strain: $1x10^4$, $1x10^5$, $1x10^6$, and $1x10^7$ CFU/mouse. There were 3 female C57BL/6 mice per group. Since *N. meningitidis* needs iron for growth, but can not sequester iron from murine proteins[35], mice were injected i.p. with iron-dextran a few hours before injection with bacteria and again 24 hours later. Animal health was monitored twice a day over a period of 48 hours and a health score was assigned to each mouse on each time point. A score of 0 means no visible symptoms, a score of 6 means death. To limit suffering, mice that were severely ill (a score of 5) were euthanized. In our experience animals that reach that stage will not recover, but die later on. Animals that received the two lowest doses of the wild type strain did not show any signs of illness, animals that received $1x10^6$ CFU of H44/76 had moderate symptoms, and animals that received the highest dose of H44/76 were severely ill after 19 and 25 hours (Fig. 4A, B). Interestingly, even the lowest dose of the *lpxL2* mutant gave the mice moderate symptoms and mice that received higher doses of the *lpxL2* mutant had more severe symptoms. On the other hand, none of the mice that received the *lpxL1* mutant showed any signs of illness, not even with the highest dose. After 19 hours all animals were still alive and none had a score higher than 4. However, after 25 hours two mice were dead (one in the group that received $1x10^6$ CFU *lpxL2* mutant and one in the group that received $1x10^7$ CFU *lpxL2* mutant) and others had a score of 5, so they were euthanized (Fig. 4C). Only the highest dose of H44/76 was lethal, whereas lower doses of the *lpxL2* mutant were lethal to some but not all mice in the group. In contrast, the highest dose of the *lpxL2* mutant was less lethal than the highest dose of H44/76 or lower doses of the *lpxL2* mutant. Obviously, all mice that received the *lpxL1* mutant survived. Moreover, all mice that survived the first 25 hours were practically symptom free after 43 hours (data not shown).

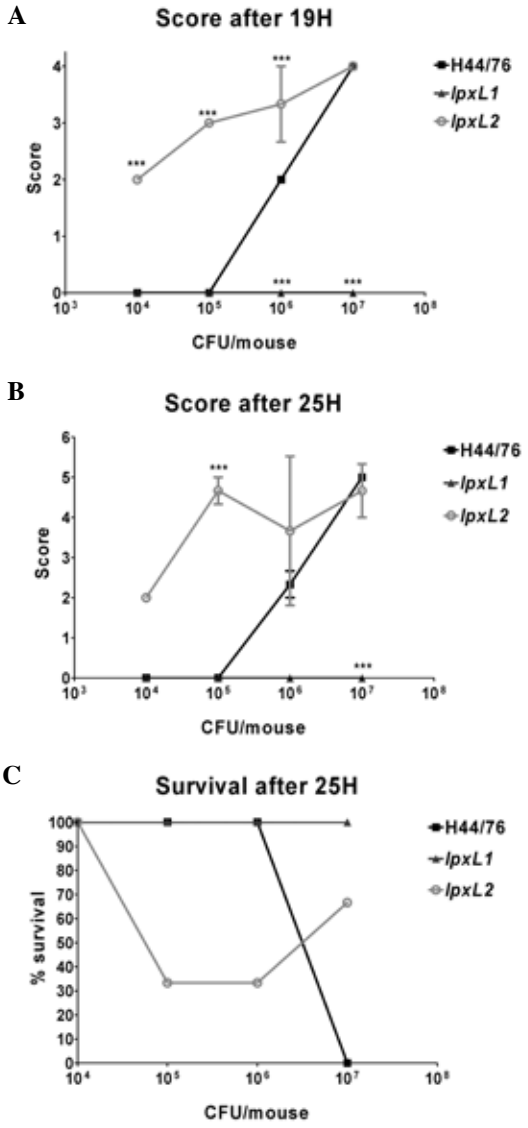


Figure 4. Disease severity of mice infected with strain H44/76, *lpxL1*, or *lpxL2*. After infection the health of the animals was monitored regularly and a heath score was given on each time point. The score ranged from healthy (0) to death (6). There were three mice per group. Health scores 19 hours after infection (A) and 25 hours after infection (B) are shown. Percentage of survivors for each group after 25 hours (C). Animals with a score of 5 that were killed at this time point were counted as non-survivors. Results of one representative experiment of two independent experiments are shown. Data are expressed as means of three mice, error bars indicate S.E.M. An asterisk indicates that the group receiving the *lpxL* mutant was significantly different from the group receiving the same dose of wild type strain H44/76. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Meningococcal growth in vivo

A sample of blood was taken from the animals after 2 and 19 hours to determine the bacterial load in the circulation (Fig. 5). Already after 2 hours the difference in fitness between the strains was visible. At the lower doses, more *lpxL2* mutant bacteria were found in the blood than wild type bacteria. However, there were less *lpxL1* mutant bacteria in the blood compared to the wild type strain. After 19 hours, none of the mice that received the *lpxL1* mutant had any bacteria in their blood, consistent with no signs of sickness. Only

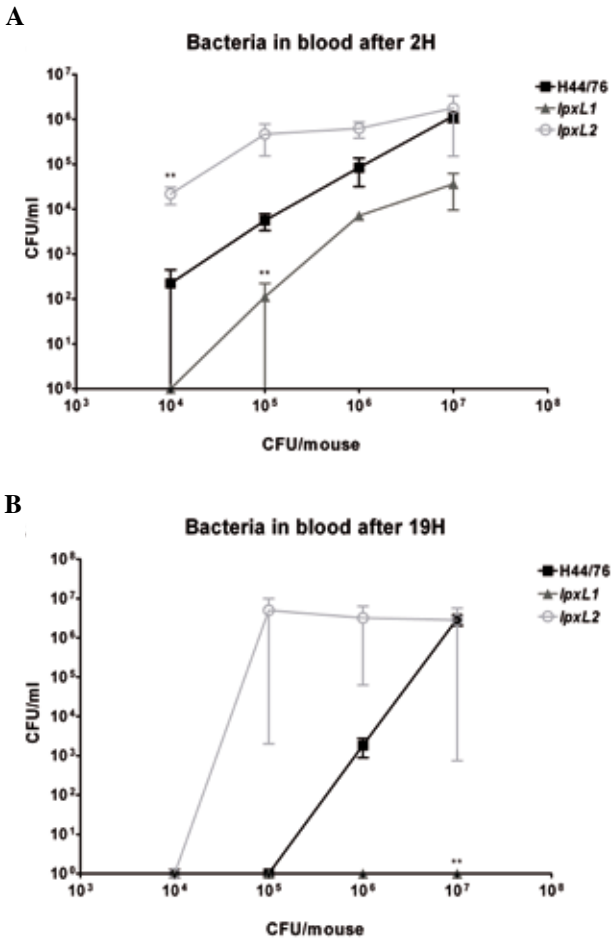


Figure 5. Number of bacteria in blood after infection. A sample of blood was taken from mice infected with strains H44/76, *lpxL1*, or *lpxL2*, 2 hours (A) and 19 hours (B) after administration. Serial dilutions of blood in PBS were plated to determine CFUs. Results of one representative experiment of two independent experiments are shown. Data are expressed as means of three mice, error bars indicate S.E.M. An asterisk indicates that the group that received an *lpxL* mutant was significantly different from the group that received the same dose of wild type strain H44/76. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

the mice that received the two highest doses of H44/76 had bacteria in their blood after 19 hours. However, mice that received an initial dose of $1x10^5$ CFU of the *lpxL2* mutant or higher had a high bacterial load at that time point. Overall, the number of bacteria in the blood correlated strongly with illness severity. These results demonstrate that at lower doses the *lpxL2* mutant survived better *in vivo* than the wild type strain. In contrast, the *lpxL1* mutant was more easily cleared by the mice than the wild type strain.

Murine cytokine response

Also the concentration of a number of cytokines important in the innate immune response was measured in the serum after 2 and 19 hours. The levels of the following cytokines were determined: IL-1β, IL-6, TNF-α, IL-12p70, IL-10, and RANTES. The levels of all cytokines 2 hours after challenge correlated very well with the number of bacteria in the blood (Fig. 6A). A high number of bacteria gave high levels of cytokines and vice versa. At lower doses the *lpxL2* mutant induced more cytokine production than the wild strain. The *lpxL1* mutant induced less cytokine production than the wild type strain. Surprisingly, there was not much difference in the cytokine inducing capabilities of the different strains when the bacterial load in the blood was approximately equal, although the wild type strain was clearly more biologically active than the *lpxL* mutants *in vitro* (Fig. 3). For example, the initial dose of $1x10^7$ CFU gave approximately the same number of wild type and *lpxL2* bacteria in the blood after 2 hours (Fig. 5A). Yet, wild type bacteria did not induce higher levels of cytokines after 2 hours than *lpxL2* mutant bacteria. One important exception however was TNF-α, which was much higher in mice that received wild type bacteria (Fig. 6A). The levels of cytokines in the blood 19 hours after challenge also correlated strongly with bacterial load (Fig. 6B). Again *lpxL2* bacteria induced higher levels of cytokines than wild type bacteria at the lower doses, whereas cytokines were practically undetectable in blood of animals infected with *lpxL1* bacteria.

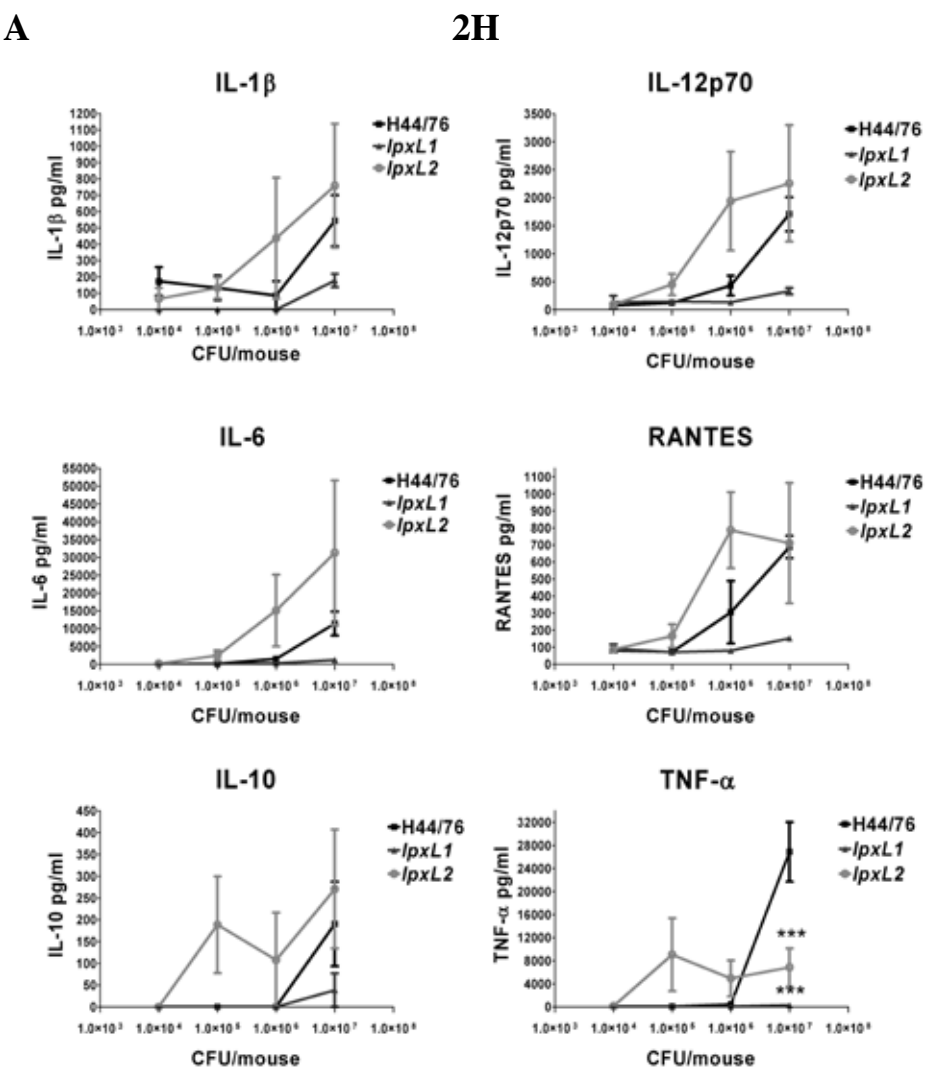
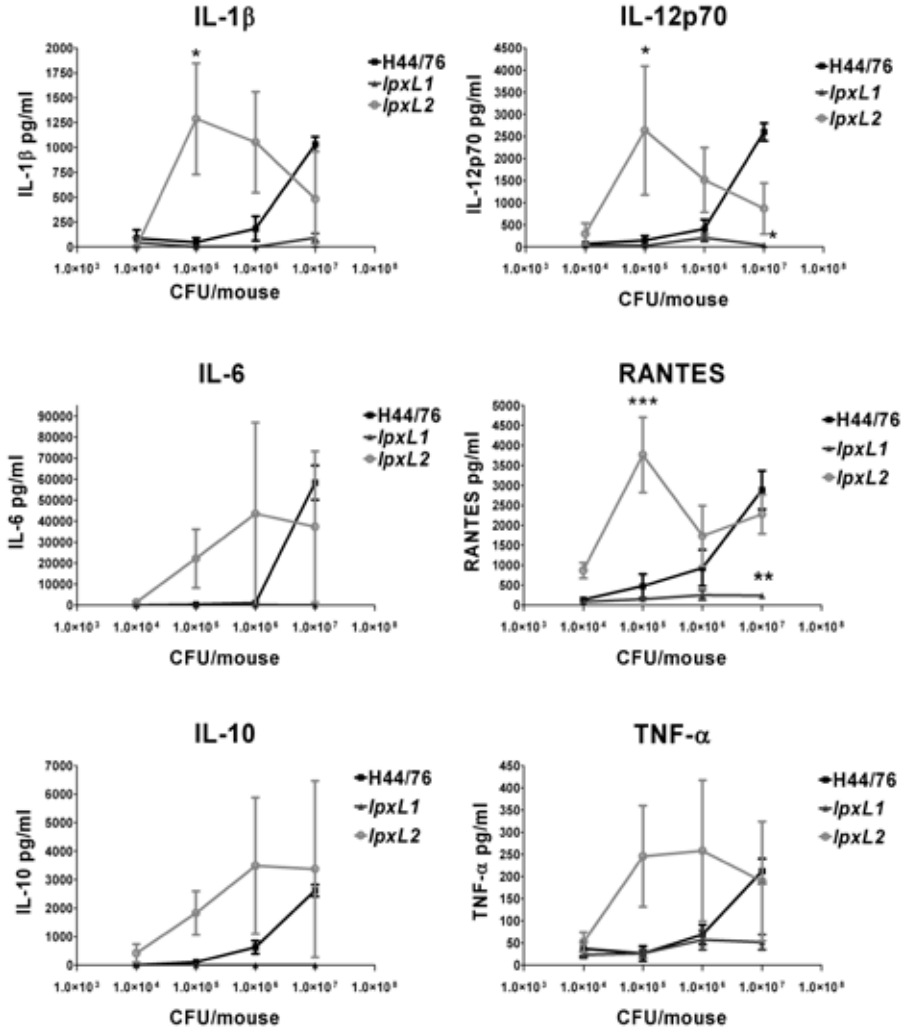


Figure 6. Cytokine levels in plasma of mice after infection with H44/76, *lpxL1*, or *lpxL2*. Two hours (A) or 19 hours (B) after infection with H44/76, *lpxL1*, or *lpxL2*, a blood sample was taken from the mice. Cytokines levels in the plasma were determined with an 6-plex Bio-Plex assay (Bio-rad) containing beads for mouse IL-1β, IL-6, IL-10, IL-12p70, RANTES, and TNF-α. Data are expressed as means of three mice, error bars indicate S.E.M. An asterisk indicates that the group that received an *lpxL* mutant was significantly different from the group that received the same dose of wild type strain H44/76. * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

B **19H**


IL-1β
IL-1β pg/ml
CFU/mouse
H44/76
lpxL1
lpxL2
IL-12p70
IL-12p70 pg/ml
IL-6
IL-6 pg/ml
RANTES
RANTES pg/ml
IL-10
IL-10 pg/ml
TNF-α
TNF-α pg/ml

Discussion

Our study demonstrates that the *lpxL2* mutant was much more virulent in mice than the wild type strain, in contrast to the *lpxL1* mutant, which was completely avirulent. The higher virulence was reflected by a higher bacterial load, higher cytokine levels, and more severe symptoms. Overall, there was a very strong correlation between these three parameters. Also in humans it was found that meningococcal disease patients who died had higher bacterial loads than patients who survived[36]. Other parameters indicative for disease severity were also associated with bacterial load in the quoted study. Presumably, a higher bacterial load leads to higher cytokine levels. In humans, disease severity and mortality are also associated with higher cytokine levels[37].

Why is the *lpxL2* mutant more virulent than the wild type strain in our mouse model? We demonstrate that *lpxL2* mutant bacteria activate murine TLR4/MD-2 much less efficiently than wild type bacteria. Moreover, *lpxL2* mutant bacteria were less active on murine macrophages compared to wild type bacteria. The importance of TLR4 in protection against Gram-negative bacterial pathogens has been widely demonstrated in mice[5-9]. Similarly, others have demonstrated that virulence in mice of certain Gram-negative bacteria can be related to the low activity of their LPS[38-40]. For example, the plague bacillus *Yersinia pestis* normally produces tetra-acyl LPS at mammalian body temperature, which is poorly recognized by TLR4. The wild type strain was virulent in mice in contrast to a modified strain that produced hexa-acyl LPS at 37ºC. However, the modified strain was fully virulent in TLR4-deficient mice, demonstrating the importance of evasion of TLR4 activation for this bacterium[38]. Possibly, also *lpxL2* mutant bacteria evade recognition by the innate immune system, which promotes their survival in mice. TLR4-deficient mice have indeed been demonstrated to be more susceptible to experimental meningococcal infection[41]. Also in humans the importance of LPS detection by the host is illustrated by the finding that individuals with rare mutations in TLR4 are more prone to meningococcal disease[42-44].

Then why is the *lpxL1* mutant less virulent than the wild type strain in mice, although it is less well recognized by TLR4? Other host defense mechanisms are also important in the protection against *N. meningitidis*. The complement system plays a major role, demonstrated by the fact that individuals with complement deficiencies are very susceptible to infection with *Neisseria* species[45]. Another important defense mechanism includes antimicrobial peptides[46]. Having lipid A with six acyl chains or more can protect bacteria from antimicrobial peptides[22, 47]. Indeed it has been demonstrated that *N. meningitidis* lipid A mutants are less resistant to such peptides[48]. LPS structure is also known to influence the complement sensitivity of *N. meningitidis*[49]. However, whether the

acylation pattern of lipid A has any effect on complement sensitivity has not been studied to our knowledge. Thus, the *lpxL1* mutant may be less virulent than the wildtype strain because of increased sensitivity to other host defense mechanisms than TLR4. Likely, the *lpxL2* mutant is also more sensitive to these other defense mechanisms, but that is widely compensated by the evasion of TLR4 recognition. On other hand, the *lpxL1* mutant might still be recognized by TLR4 well enough to clear the bacteria.

We show that *in vitro* the induction of IL-6 is not much different between the *lpxL1* and *lpxL2* whole bacteria, but that IP-10 induction is much lower after *lpxL2* mutant stimulation compared to the *lpxL1* mutant. Presumably, activation of other pattern recognition receptors such as TLR2 contributes to IL-6 production via the MyD88 pathway, which compensates for the difference in biological activity of *lpxL1* and *lpxL2* LPS. However, the *lpxL1* and *lpxL2* mutant clearly differ in their ability to induce IP-10, likely because IP-10 is induced via the TRIF pathway, which is only activated by LPS. Activation of the TRIF pathway leads to the production of type I IFN[4]. Interestingly, it has been demonstrated that type I IFN treatment of mice infected with *Salmonella typhimurium* leads to reduced lethality[50].

Somewhat in contrast to our findings Plant et al. reported that a serogroup C wild type strain and its isogenic *lpxA* mutant induced similar amounts of cytokines and caused equivalent disease severity in mice[51]. Moreover, TLR4-/- mice were protected from disease, rather than more susceptible. Differences in experimental design might be an explanation. They used $1x10^8$ and $5x10^8$ CFU per mouse and no exogenous iron source. Thus higher doses were used than our highest dose. We also found little differences between the wild type strain and the *lpxL2* mutant at the highest dose. Possibly, the *lpxL2* mutation has an advantage at lower bacterial numbers, because TLR4 recognition can be evaded. However, at high bacterial numbers other pattern recognition receptors get sufficiently activated. At high bacterial loads having TLR4 is probably only a disadvantage for the host, because it contributes significantly to the excessive production of pro-inflammatory cytokines, which can be lethal.

Two hours after challenge with $1x10^7$ wild type bacteria the mice did not have higher levels of cytokines in their blood than mice challenged with $1x10^7$ *lpxL2* mutant bacteria, even though the measured bacterial load was comparable and the wild type strain was shown to be more potent *in vitro*. Possibly, with such high numbers of bacteria in the blood, cytokine production has reached a plateau and the other pattern recognition receptor ligands compensate for the difference in LPS activity. One exception was TNF-α, which was much higher in the blood of mice challenged with the wild type strain, suggesting that specifically TNF-α production is more dependent on LPS. Interestingly,

it has been demonstrated previously that the levels of LPS in the cerebrospinal fluid of patients with meningococcal disease correlated with levels of TNF-α, but not with levels of IL-6 or IL-1[52].

We recently reported that a surprisingly large fraction of *N. meningitidis* disease isolates have mutations in *lpxL1* [28]. The *lpxL1* mutants activate human TLR4 much less efficiently than wild type bacteria. Therefore, an explanation for the high frequency of *lpxL1* mutations could be that it creates an advantage for the bacteria, because they can evade TLR4 recognition and subsequent clearing by the innate immune system. In the present study we wanted to test the influence of lipid A structure on virulence in a mouse model of meningococcal sepsis. However, a complicating factor is that *lpxL1* mutant bacteria activate murine TLR4 much better than human TLR4. Here we show that an *lpxL2* mutant activates murine TLR4 less efficiently than the *lpxL1* mutant. Moreover, we demonstrate that the *lpxL2* mutant is more virulent in mice than the wild type strain, in contrast to the *lpxL1* mutant, which is much less virulent. How the *lpxL2* mutant behaves in mice might be a good model of how the *lpxL1* mutant behaves in humans. There are also differences however. We previously showed that meningitis patients infected with an *lpxL1* mutant were less severely ill than patients infected with a wild type strain[28]. But in the present study, mice infected with *lpxL2* mutant bacteria had higher morbidity and mortality than mice infected with equivalent amounts of wild type bacteria, at least at the lower bacterial doses used. Of course, the mouse model differs in several crucial respects from a human infection, including (i) the different route of infection, (ii) the absence of any pre-existing specific immunity, and (iii) the differential activity of many host-specific factors such as Opa adhesins, iron-sequestering proteins and complement regulators[35]. However, in spite of these differences our results clearly demonstrate the crucial role of *N. meningitidis* lipid A structure in virulence, possibly through its effect on the degree of TLR4 activation. Moreover, our results suggest that the lipid A structure is important in the bacteria's defense against other host immune mechanisms as well.

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Chapter 7

Summarizing discussion

The ideal adjuvant

Can adjuvant activity and toxicity be dissected?

During evolution multiple mechanisms have evolved to protect organisms from parasitic infections[1]. Even unicellular organisms such as bacteria contain enzymes to counteract viral infections. Thus one could argue that the immune system is almost as ancient as life itself, which suggests that it is of vital importance. Indeed, individuals with severe immune deficiencies like AIDS are extremely susceptible to opportunistic infections. However, the immune system also has a dark side. Several diseases such as autoimmune disorders and allergies are caused by an overactive immune system. In many infections, including meningococcal disease, much of the resulting pathology is caused by excessive activation of the immune system. In fact, injection of molecules derived from microbes such as LPS can be lethal due to overstimulation of the immune system[2]. These toxic effects are mediated by pro-inflammatory cytokines such as TNF-α.

Clearly extreme caution is needed before application of immune-stimulating adjuvants to improve immunity after vaccination. This particularly holds for immunization of healthy subjects against infectious diseases that only affect a minority of the human population. The ideal adjuvant would activate the immune system without provoking any pathology. However, the clear link between artificial immune activation and toxicity makes the development of such an adjuvant a daunting task. Indeed, many compounds have been shown to have adjuvant activity in animal models, but their toxicity renders them unsuitable for human use. In fact, aluminium salts (alum) are still the only adjuvants approved for human vaccination in the USA[3]. Alum enhances the humoral immune response, which generally provides protection against extracellular pathogens. But alum is not very useful for improving cellular immunity required to combat intracellular pathogens. Thus new adjuvants are needed to steer the immune response in other directions than alum.

We have demonstrated previously that LPS isolated from the *N. meningitidis lpxL1* mutant induces much less pro-inflammatory cytokines *in vitro* compared to wildtype LPS. On the other hand, *lpxL1* LPS was shown to have comparable adjuvant activity to wildtype LPS in mice[4-6]. Thus it seems possible to reduce adjuvant toxicity without compromising adjuvant activity, which indicates that the pathways upstream of these two properties can at least in part be separated. The two major pathways activated after LPS recognition are the MyD88 pathway and the TRIF pathway[7]. Activation of the MyD88 pathway eventually leads to induction of pro-inflammatory cytokines, whereas activation of the TRIF pathway leads to production of type I IFN. Since pro-inflammatory cytokines

mediate the toxic effects of LPS, an LPS variant with reduced MyD88 activating properties could have the best adjuvant characteristics. It has been reported that different LPS variants can differ in their ability to activate the MyD88 pathway versus the TRIF pathway[8]. The LPS derivative monophosphoryl lipid A (MPL), which has low toxicity but good adjuvant activity, preferentially activates the TRIF pathway[9]. Unfortunately, we could not find any differences in induction of the MyD88 pathway versus the TRIF pathway between different LPS variants including *lpxL1* LPS and MPL (unpublished results). An alternative explanation may be that LPS with low biological activity induces adjuvant activity rather than toxicity. In this scenario there is no differential activation of MyD88 and TRIF, but equal moderate induction of both pathways not sufficient to induce toxicity

Role of type I IFN

In Chapter 2 we compared a panel of TLR agonists (TLR1-9) and a NOD2 ligand for their ability to enhance immunogenicity of outer membrane complexes (OMCs) derived from the LPS-deficient *N. meningitidis lpxA* mutant in mice. We found that only agonists of TLR3, 4, 7 and 9 improved immune responses against LPS-deficient meningococcal OMCs, in contrast to TLR2, 5, and NOD2 agonists. The adjuvant effect was characterized by higher levels of antigen-specific IgG2a and IgG2b, while amounts of antigen-specific IgG1 were barely influenced by the adjuvants. The immunoglobulin classes IgG2a and IgG2b in contrast to IgG1 can activate complement. In agreement with this, we found that agonists of TLR3, 4, 7, and 9 enhanced levels of antibodies that activate complement-mediated killing of *N. meningitidis* in the mice sera. Bactericidal antibodies are currently considered the most important correlate of protection in meningococcal disease[10]. Therefore, the current consensus is that a vaccine against *N. meningitidis* should elicit these antibodies. Class switching to IgG2a and IgG2b requires help from Th1 cells[11-13]. Thus to generate bactericidal antibodies a meningococcal vaccine should induce a Th1 response. Indeed, ligands of TLR3, 4, 7, and 9 have been shown to skew the T cell response towards Th1[14-17]. A critical role in this process might be played by type I IFN. Activation of TLR3, 4, 7, and 9 leads to induction of type I IFN, in contrast to activation of TLR2, 5, or NOD2[18].

N. meningitidis OMV vaccines and *B. pertussis* whole-cell vaccines contain LPS. Therefore, these vaccines can induce type I IFN via the TRIF pathway downstream of TLR4. In Chapter 3 we investigated the contribution of the TRIF pathway to the immunogenicity of these vaccines by comparing adaptive immune responses after immunization of TRIF-deficient mice and wildtype C57BL/6 mice. For both vaccines we

found that in particular antigen-specific levels of the Th1-dependent IgG2b and IgG2c isotypes were much lower in TRIF-deficient mice after immunization. Moreover, T cells derived from immunized TRIF-deficient mice produced less Th1 cytokines, but more Th2 cytokines after restimulation with antigen. Finally, TRIF-deficient mice had significant lower levels of bactericidal antibodies after *N. meningitidis* OMV vaccination. Thus activation of the TRIF pathway seems to be required to generate the desired Th1 response after vaccination. Since type I IFN is induced by the TRIF pathway, these data again are consistent with an important role of these cytokines in Th1 polarization.

It has been demonstrated previously that induction of type I IFN is essential for the adjuvant activity of poly I:C and complete Freund's adjuvant, as their enhancement of the antibody response was diminished in mice deficient for the type I IFN receptor[19]. In addition, administration of purified recombinant type I IFN alone also enhanced antibody responses. Recently, others compared several TLR ligands in their ability to enhance Th1 $CD4^+$ T cell responses to a HIV vaccine in mice[20]. They found that poly I:C was the most effective adjuvant. Moreover, it was shown that poly I:C was the most potent inducer of type I IFN, which was essential for DC maturation and development of $CD4^+$ Th1 immunity. Type I IFN can stimulate Th1 immunity in several ways. It may act indirectly by promoting the secretion of the Th1-enhancing cytokines IL-12 and IFN-γ. IL-12 plays a crucial role in Th1 priming[21], while IFN- γ increases responsiveness to IL-12 and activates the Th1 transcription factor T-bet[22, 23]. Production of IL-12p70, the bioactive form of IL-12, by DCs indeed depends on type I IFN[24]. In addition, Longhi et al. showed that type I IFN activates NK cells to produce IFN-γ *in vivo*[20]. Moreover, IFN-γ producing NK cells promote Th1 polarization[25]. Despite these effects, Longhi et al. reported that mice deficient in IL-12p40 or the IFN- γ receptor have normal T cell responses after immunization with a vaccine plus poly I:C adjuvant[20]. Possibly, there is redundancy in these pathways and type I IFN can induce Th1 responses via either pathway. Alternatively, another mechanism may be responsible for type I IFN-mediated Th1 immunity. Finally, it has been found that activation of TLR3, 7, and 9 on DCs enables them to cross-present exogenously acquired antigens and prime antigen-specific $CD8^+$ T cells to differentiate into cytotoxic T cells[26]. Interestingly, as described above TLR3, 7 and 9 induce type I IFN, which has been shown to enhance cross-presentation by DCs[27].

In conclusion, it seems that to direct the immune response towards a Th1 response and/or $CD8^+$ T cell response the use of an adjuvant that induces type I IFN should be considered. Additionally, since type I IFN is induced by another pathway (TRIF) than pro-inflammatory cytokines (MyD88) after TLR4 activation, it might be possible to select LPS variants with retained adjuvant activity and reduced toxicity. Indeed, as described

above there are data that support the claim that the low-toxicity vaccine adjuvant MPL preferentially activates the TRIF pathway[9].

Enhancing the specificity of the adjuvant

In Chapter 2 we also compared the immunogenicity of OMCs isolated from a wildtype *N. meningitidis* strain with a mixture of OMCs derived from the LPS-deficient mutant and wildtype meningococcal LPS. These preparations are practically the same except for the presentation form of the LPS. Nevertheless, wildtype OMCs induced much higher antibody responses. It seems that the effectiveness of an adjuvant can be greatly improved if it is physically linked to the antigen. Others have observed this phenomenon as well. For example, the adjuvant effect of CpG is much stronger when mixed with antigen in the emulsion incomplete Freund's adjuvant, cross-linked with antigen via biotin-avidin bridges, or by coencapsulating adjuvant and antigen in liposomes[28]. Moreover, it has been demonstrated that the TLR11 ligand profilin, present in the parasite *Toxoplasma gondii*, is an immunodominant antigen in the $CD4^+$ T cell response against the parasite in mice. Interestingly, the immunogenicity of profilin depends on TLR11 activation, which suggests that antigens in association with TLR ligands are preferentially presented to T cells[29]. Similarly, antigens phagocytosed by DCs depend on TLR ligands within the phagocytosed cargo for optimal antigen presentation[30]. Finally, it has been found that DCs that are indirectly activated by inflammatory cytokines produced by bystander cells, but not directly with TLR ligands, cannot promote effective T cell responses[31]. Together these data suggest that the adjuvant should directly activate the cell that is also presenting the antigen.

A reduction of adjuvant toxicity and improved adjuvants effects may be further achieved by specific targeting of the adjuvant-antigen complex to the cell type involved in antigen presentation. For example, the oligosaccharide of LPS from a *N. meningitidis lgtB* mutant binds to the C-type lectin DC-SIGN on human dendritic cells[32]. The effectiveness of an adjuvant could be enhanced by physically linking the adjuvant to the antigen and/or targeting the adjuvant to the desired cell type. By increasing the specificity of the adjuvant less activation of bystander cells will occur, which will likely decrease the toxicity of the adjuvant.

Differential role of TLR2 and TLR4

N. meningitidis OMV vaccines and *B. pertussis* whole-cell vaccines contain several PRR ligands including the TLR4 agonist LPS and TLR2 ligands such as lipoproteins[33-37]. The vaccines have been successfully employed to prevent infection with these pathogens in

the human population[38-40]. Moreover, agonists of TLR2 and TLR4 have been shown to have adjuvant activity in numerous studies in mice[5, 41-44]. In Chapter 3 we investigated the contribution of TLR2 activation and LPS signalling to the immunogenicity of *N. meningitidis* OMVs and a whole-cell pertussis vaccine in mice. To this end, immune responses were compared after immunization of TLR2-/-, TRIF-deficient, and wildtype C57BL/6 mice. In addition, to study the role of TLR4, immune responses were compared between TLR4-deficient C3H/HeJ mice and wildtype C3H/HeOuJ mice. We found that the mice that were impaired in LPS signalling (C3H/HeJ and TRIF-deficient mice) showed impaired immune responses after vaccination with both vaccines. Surprisingly, both vaccines induced normal or even higher responses in TLR2-/- mice.

The finding that mice deficient in LPS signalling showed impaired immune responses after immunization with *N. meningitidis* OMVs corresponds with the results shown in Chapter 2 where we show that LPS-deficient OMCs induced much lower antibody responses than wildtype OMCs or LPS-deficient OMCs mixed with LPS. Together these data suggest that LPS is essential for optimal immunogenicity of an *N. meningitidis* outer membrane vaccine. However, although LPS-deficient OMCs hardly induced antigen-specific antibodies apart from IgG1, antibody responses and bactericidal antibody levels were only slightly lower in TLR4 deficient C3H/HeJ mice compared to wildtype C3H/HeOuJ mice, and this difference did not reach statistical significance. This discrepancy might relate to differences in mouse strains, as BALB/c mice were immunized with LPS-deficient OMCs and C3H mice with *N. meningitidis* OMVs. Also differences between OMCs and OMVs might contribute to these observations. Yet, these differences are unlikely to fully explain the large dissimilarity between the response to LPS-deficient OMCs in wildtype mice and wildtype OMVs in TLR4-deficient mice. Therefore, it seems that LPS also contributes to the induction of antibody responses via a TLR4-independent mechanism. Possibly, antibodies directed against LPS play a role, or LPS may target the vaccine to DCs by binding for example to CD14 or a C-type lectin. In addition, we show in Chapter 3 that T cells from TLR4-deficient mice immunized with *N. meningitidis* OMVs do not produce detectable levels of cytokines after antigen restimulation. These results suggest that TLR4 activation is particularly important for the generation of T cell responses and plays a smaller role in the antibody response.

TLR2 ligands have been shown to have adjuvant activity in numerous studies[43-46]. Moreover, LPS-deficient *N. meningitidis* activates TLR2[35-37]. We also observed that LPS-deficient OMCs induce pro-inflammatory cytokines in wildtype murine macrophages, but not in macrophages derived from TLR2-/- mice (unpublished results). However, our results indicate that LPS-deficient OMCs barely induced antibody responses with the

exception of IgG1 (Chapter 2). Moreover, we found that adaptive immune responses are not impaired in TLR2-/- mice after immunization with *N. meningitidis* OMVs or a whole-cell pertussis vaccine (Chapter 3). In contrast, some responses were even higher in the TLR2-/- mice, especially the production of T cell cytokines. These results indicate that TLR2 activation suppresses adaptive immune responses instead of activating them. In agreement with this, others found that the TLR2 ligand zymosan induced DCs and macrophages to produce high levels of the anti-inflammatory cytokines IL-10 and TGF-β and low levels of IL-6 and IL-12p70. As a consequence these antigen-presenting cells induced a T-regulatory response. These effects were TLR2-dependent[47, 48]. In addition, Tregs have been shown to express TLR2. Therefore, TLR2 ligands can activate Tregs directly, which leads to their expansion[49]. Thus TLR2 ligands within the *N. meningitidis* OMVs and whole-cell pertussis vaccine may activate Tregs. This would explain why TLR2-/- mice show normal or even elevated responses after immunization compared to wildtype mice. Some Treg subsets exert their regulatory function through the production of IL-10[50]. However, we show that spleen cells of immunized TLR2-/- mice produce normal amounts of IL-10 after antigen restimulation. It is also possible that in our study TLR2 activation induced another subset of Tregs that did not secrete IL-10. Whether vaccination with *N. meningitidis* OMVs or whole-cell pertussis vaccine induces T-regulatory responses and whether TLR2 plays a role should be further investigated in the future. Confusingly, TLR2 activation has also been reported to induce a Th1 response[26, 51], or Th2 response[52-54]. How TLR2 activation can lead to all these different responses is currently not clear, but it might depend for example on the experimental setup, the immunization route, the nature of the ligand, whether the ligand is recognized by TLR2/TLR1 or TLR2/TLR6, or which cell type is targeted.

Evidence for influence of TLRs on immunogenicity of vaccines in humans

It has been widely demonstrated that activation of TLRs in mice leads to the induction of adaptive immune responses[26]. Of course these results can not be automatically extrapolated to humans. The possibility that TLRs play a less important role in immunity in humans compared to mice is illustrated by the finding that individuals deficient in the adaptor protein MyD88 are more susceptible to only a small number of pyogenic bacteria[55]. In contrast, MyD88-/- mice are vulnerable to infection with a broad range of pathogens. Surprisingly little is known about the role of TLRs in the immunogenicity of vaccines in humans, despite the fact that many licensed vaccines contain TLR ligands[56]. The best evidence for the importance of TLR activation in vaccination of humans comes from a study on the *Borrelia burgdorferi* OspA vaccine[45]. OspA is a lipoprotein that activates

TLR2/TLR1. Immunogenicity of the vaccine in mice depends on these TLRs, as mice deficient in TLR2 or TLR1 showed impaired immune responses. Interestingly, individuals with very low antibody titers after vaccination had lower cell-surface expression of TLR1, and OspA stimulation induced less pro-inflammatory cytokines in macrophages of these low responders. More recently others investigated the association between human polymorphisms in genes involved in LPS signalling and antibody titers after vaccination with a whole cell pertussis vaccine. A polymorphism in TLR4 was associated with lower antibody titers[57]. In addition, polymorphisms in other genes in the TLR4 signalling pathway were associated with antibody titers in children after vaccination with this vaccine[58]. These data support a role for TLR4 in the immunogenicity of the whole cell pertussis vaccine also in humans.

It is important to establish how licensed vaccines confer immunity after vaccination, because most vaccines do not induce sufficient immunity in all vaccinated subjects, which is presumably related to differences in their immune system. Individuals at the extremes of life are not only more susceptible to infections, they also often respond poorly to vaccination. Moreover, TLR function has been reported to be suboptimal in these age groups[59, 60]. Also environmental factors can influence TLR function. For example, TLR2 and CD14 expression was found to be elevated in farmers' children compared to other children[61]. Once it is known which pathways that contribute to the immunogenicity are activated by a certain vaccine, we can think of strategies to enhance immunity in non-responders. One possibility is to give this group a different adjuvant that improves immunity via alternative pathways.

What is the desired immune response after vaccination?

In most immunization studies, including ours, immune responses are measured shortly after vaccination. It is often concluded that higher responses are beneficial, but this might be too simple. More important questions to address are whether the vaccine protects against disease, how long it will protect, and whether it is safe. However, it is not easy to find answers to these questions. In the first place because we are interested in the immune response in humans after vaccination, but the large majority of studies use animal models. For numerous pathogens a reliable animal model does not exist and even a good model cannot substitute for the complexity of the real-life situation. Studies on immunological memory in animals and humans are time-consuming and, as a consequence, expensive. Furthermore, memory studies in humans might be complicated by the fact that individuals can receive natural boosters by encountering the pathogen. Another limitation of human studies is that only blood cells can be tested, while some memory cells probably reside in

other locations such as the bone marrow. Finally, even if a vaccine is shown to be safe in a large clinical trial with thousands of people, there is still the possibility that it can induce rare adverse effects in a small subset of the population. In conclusion, whether a vaccine is successful can only be fully determined after it is approved and millions of individuals have been immunized.

Regarding safety, an interesting issue is whether we should suppress Tregs in vaccination or not. In Chapter 3 we show that some immune responses were higher in TLR2-/- mice after immunization with *N. meningitidis* OMVs and a whole-cell pertussis vaccine. In addition, it has been demonstrated that TLR2 activation can induce Tregs[47, 48]. Suppose that *N. meningitidis* OMVs and whole-cell pertussis vaccine indeed induce Tregs via TLR2, would it be better to exclude TLR2 ligands from these vaccines to generate higher adaptive immune responses after vaccination? Overstimulation of the immune system can result in severe inflammation and collateral tissue damage. To minimize damage during infection, the immune response to pathogens is regulated by a variety of host suppressor mechanisms, including the production of anti-inflammatory cytokines by innate immune cells in response to PRR ligands[62]. However, it is now becoming clear that the adaptive immune system may also help to control infection-induced immunopathology through the generation of antigen-specific Tregs. Under these circumstances induction of Tregs is beneficial. On the other hand, there is accumulating evidence that pathogens that cause chronic infections exploit Tregs to subvert the protective immune responses of the host. For example, in hepatitis C virus (HCV) infection IL-10 secreting $CD4^+$ and $CD8^+$ Treg cells have been demonstrated, which may inhibit HCV-specific T cells in chronically infected individuals[62]. On the other hand, it has been suggested that HCV-specific Tregs that produce IL-10 and home to the liver help to reduce liver inflammation. Furthermore, HCV infected patients with reduced numbers of Tregs often develop an autoimmune syndrome, called mixed cryoglobulinemia. Thus, while Tregs may prevent pathogen clearance they could also prevent immunopathology and development of autoimmunity.

Naturally occurring meningococcal lipid A variants

Role of meningococcal lipid A structure in virulence

The *N. meningitidis lpxL1* mutant was initially studied after artificially constructing a strain in which this gene was inactivated. To our surprise, we later found that *lpxL1* inactivation has also occurred naturally, as described in Chapters 4-5. This provided us with the unique opportunity to study its activity in a natural setting i.e. human patients. As described in Chapter 4 we tested over 400 meningococcal disease isolates for their ability to induce cytokines *in vitro*. Interestingly, we found that around 9% of these patient isolates induced much lower levels of cytokines than the other strains. All the low cytokine inducing strains, with one exception, had underacylated lipid A due to inactivating mutations in the *lpxL1* gene. Remarkably, among adults with meningitis, patients that were infected with *lpxL1* mutants had milder symptoms than patients infected with wildtype strains. Several different types of mutations in *lpxL1* were found and the majority of mutations were reversible, making phase variation likely. The high frequency and reversibility of *lpxL1* mutations suggests that switching *lpxL1* on-and-off is a mechanism often used by *N. meningitidis* to adapt to changing conditions within the human host. Moreover, the fact that only a proportion of strains have inactivating mutations in *lpxL1* suggests that there are also disadvantages for having underacylated lipid A.

Since the innate immune system senses Gram-negative bacteria through TLR4, one can imagine that bacteria that can evade TLR4 recognition have better changes to survive in the host. The most straightforward method to avoid TLR4 recognition is to modulate the LPS molecule, which is sensed by this receptor. In Chapter 4 we show that naturally occurring *lpxL1* mutants activate human TLR4 much less efficient than wildtype strains. Furthermore, the observation that individuals with rare TLR4 mutations are more susceptible to meningococcal disease suggests that TLR4 plays an important role in the host defence against *N. meningitidis*[63]. These results indicate that meningococci may inactivate *lpxL1* to avoid detection by the innate immune system through TLR4.

To further investigate the influence of lipid A structure on meningococcal virulence, we compared the wildtype strain, *lpxL1* mutant, and *lpxL2* mutant in a mouse model of meningococcal disease as described in Chapter 6. One limitation of the mouse model is the species-specific difference between humans and mice in recognition of lipid A by TLR4-MD-2[64-67]. In general, lipid A with 4 or 5 acyl chains is poorly recognized by human TLR4-MD-2, but it still activates murine TLR4-MD-2 relatively well. Indeed it has been demonstrated that *lpxL1* LPS is a good activator of murine TLR4-MD-2, but a weak activator of human TLR4-MD-2[68]. In Chapter 6 we report that the *lpxL2* mutant activates murine TLR4-MD-2 less well than the *lpxL1* mutant. Thus, in terms of TLR4 activation,

the *lpxL2* mutant in mice more closely resembles the *lpxL1* mutant in humans than the *lpxL1* mutant itself. Interestingly, the *lpxL2* mutant was more virulent in the mouse model of meningococcal disease than the wildtype strain. Surprisingly, the *lpxL1* mutant was completely avirulent. These results demonstrate that alteration in lipid A structure can have both negative and positive effects on virulence, which suggests that multiple host defence mechanisms interact with LPS. We propose that the *lpxL2* mutant is more virulent than the wildtype strain, because it can evade TLR4 recognition. TLR4-deficient mice have indeed been shown to be more susceptible to experimental meningococcal infection[69]. The recognition of *lpxL1* mutant LPS by TLR4, while reduced, may still be sufficient to trigger innate immunity-mediated protection. The reduced virulence of the *lpxL1* mutant compared to the wildtype strain may than result from the fact that lipid A makes meningococci more susceptible to other host defence mechanisms. For example, it has been demonstrated that *lpxL1* mutants are less resistant to antimicrobial peptides[70]. LPS structure is also known to influence the complement sensitivity of *N. meningitidis*[71], but the impact of the acylation pattern of meningococcal lipid A has not been studied to our knowledge. However, *Bordetella bronchiseptica* with lipid A that lacks a palmitoyl group is less virulent in mice due to increased susceptibility to antibody-mediated complement lysis[72].

Although our results with the mouse model provide interesting insights in the relation between lipid A structure and meningococcal virulence, it does not give us a definitive answer on the question whether *lpxL1* mutants are selected in the human host because they can escape TLR4 recognition. Possibly, this issue could be addressed with humanized mice. An example of such a humanized rodent model is the bone marrow/liver/thymus (BLT) mouse[73]. To obtain these mice, nonobese diabetic (NOD)/SCID mice, which lack T and B cells, are surgically implanted with fetal thymic and liver organoids. Next, the mice undergo a bone marrow transplant, receiving human stem cells that are autologous to their human liver/thymus implants. As a result, these mice show an impressive range of human cells in their blood, organs, and tissues. Apart from human TLR4 recognition, another advantage of this model would be of course that many more aspects of the human host that are relevant for meningococcal disease are present.

lpxL1 mutants in carriage

TLRs target conserved components of microbes that have important functions. Therefore, there are limitations to structural alterations of these components that can be tolerated by the microbes. For example, LPS has an important function in outer membrane stability. It has been observed that most species of Gram-negative bacteria that inhabit mucosal

surfaces produce hexa-acyl LPS, which suggests that such a structure is beneficial at the mucosa. Indeed it has been demonstrated that having lipid A with six acyl chains or more can protect bacteria from antimicrobial peptides[74, 75]. Also for *N. meningitidis* is has been demonstrated that lipid A mutants are more susceptible to such peptides[70]. Therefore, we hypothesized that the frequency of *lpxL1* mutations would be lower among strains isolated from the respiratory tract of carriers compared to invasive isolates. In Chapter 5 we screened 822 carrier strains for their ability to induce IL-6 *in vitro*. We found that 45 strains (5.3%) were impaired in cytokine induction. As expected, all these strains had inactivating mutations in the *lpxL1* gene. Thus, overall the frequency of *lpxL1* mutations was not much lower among carrier isolates. However, whereas *lpxL1* mutants isolated from patients were not associated with a particular clonal complex or serogoup, almost all *lpxL1* mutant carrier isolates (43 of 45) were of clonal complex cc23. Most *lpxL1* mutants (38) among clonal complex cc23 belonged to serogroup Y, the other five were of unknown serogroup. In conclusion, with the exception of clonal complex cc23, *lpxL1* mutants were extremely rare (0.3%) among meningococcal carrier isolates. Therefore, it seems that for the large majority of meningococcal clonal complexes having hexa-acyl lipid A in the human respiratory tract is beneficial. Possibly, lipid A with 6 acyl chains protects the bacteria from the antimicrobial peptides within the mucosa. In contrast, *lpxL1* mutants were exceptionally common (61%) among clonal complex cc23 carrier isolates. The reason for this extremely high frequency is not clear at present. It would be interesting to investigate susceptibility of these mutants to antimicrobial peptides. Possibly, strains of clonal complex cc23 manage to somehow compensate for underacylated lipid A. Another interesting aspect of these mutants is that only two different types of mutations were found in the *lpxL1* gene, whereas in total over 10 different types of *lpxL1* mutations have been identified. Moreover, one of these two mutations was a deletion of 10 nucleotides in *lpxL1*. It is very unlikely that this same mutation occurred independently more than once. Therefore, these mutants presumably all originated from a single clone. Together, these results suggest that there is less dynamic on-and-off switching of *lpxL1* in the carrier isolates of clonal complex cc23 compared to the patient isolates.

Influence of age on host response to lpxL1 mutants

In Chapter 4 we examined the impact of infection with *lpxL1* mutants on the clinical course of adults with meningitis. However, young children and adolescents have the highest risk for contracting meningococcal disease. Moreover, other clinical manifestations than meningitis can occur after *N. meningitidis* infection. Therefore, we investigated the prevalence of *lpxL1* mutants in all age-groups and all forms of meningococcal disease in

Chapter 5. We found that *lpxL1* mutant infections are associated with age. The frequency of *lpxL1* mutants among strains isolated from individuals above the age of 5 was 8.2%, which is quite comparable with the frequency that we found in our first study described in Chapter 4. However, the prevalence of *lpxL1* mutants among isolates from patients younger than 5 was just 4.4%. Moreover, the patients above the age of 5 infected with *lpxL1* mutants clearly had milder symptoms than patients infected with a wildtype strain, whereas this difference between *lpxL1* mutants and wildtype strains was not seen in patients younger than 5 years.

Children below the age of 5 are most susceptible to meningococcal disease[76]. We indeed observed that most cases were in the age group of 0 to 4 years. An immature adaptive immune system and lack of specific, functional protective antibodies are believed to contribute to this increased susceptibility[77]. With increasing age the incidence of meningococcal disease decreases, probably due to improved specific immunity. Infections with *lpxL1* mutants do not show this age dependent decrease. The frequency of *lpxL1* mutations was highest among strains isolated from patients in the age group 45-64 years (24.2%). Therefore, it seems that *lpxL1* mutations are particularly advantageous for the meningococcus in the presence of pre-existing immunity. Interestingly, a relation between TLR4 activation and the humoral immune response has been shown for the very closely related species *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*. *B. pertussis* and *B. bronchiseptica* have LPS that is well recognized by TLR4. Moreover, mice require TLR4 for the clearance of these species. In contrast, *B. parapertussis* contains LPS that poorly activates TLR4 and TLR4-deficient mice are not more susceptible to *B. parapertussis* infection[33]. Furthermore, *B. bronchiseptica* is rapidly cleared from the lungs by adoptive transfer of antibodies. This rapid antibody-mediated clearance was shown to be controlled by TLR4-dependent recruitment of leukocytes[78]. Remarkably, *B. pertussis* is able to avoid rapid antibody-mediated clearance by inhibiting TLR4-dependent leukocyte recruitment to the lungs via pertussis toxin[79]. Finally, *B. parapertussis* was shown to avoid clearance by inefficient TLR4 stimulation, as addition of a potent TLR4 agonist led to rapid antibody-mediated clearance of the bacterium in wildtype mice but not in TLR4-deficient mice[80]. It would be interesting to determine whether *lpxL1* mutant *N. meningitidis* is able to avoid immunity via similar mechanisms. If the *lpxL1* mutation indeed enables meningococci to escape antibody-mediated immunity, it may have important implications for vaccine development, as current strategies are focused on the generation of bactericidal antibodies. In this light it should be noted that the ability of *B. pertussis* and *B. parapertussis* to avoid antibody-mediated clearance probably contributes to their capability to re-infect the same human host[81]. It can be speculated that the re-emergence of infections with these bacteria

in vaccinated populations is related to this phenomenon.

Finally, we found in Chapter 5 that *lpxL1* mutant infections were not prevalent among children younger than 5 years. Secondly, the few young patients that were infected with *lpxL1* mutants did not have milder symptoms than patients infected with wildtype strains. Inability of the innate immune system of young children to distinguish *lpxL1* LPS from wildtype LPS could explain these results. It would explain the relatively low incidence of *lpxL1* mutant infections in this group, because *lpxL1* mutants do not have the advantage of evasion of TLR4 recognition. In addition, since *lpxL1* and wildtype LPS would induce inflammation and coagulation to the same degree, no differences in clinical symptoms would be observed. Interestingly, immune cells from human newborns show impaired pro-inflammatory Th1-polarizing responses after LPS stimulation[82]. This likely contributes to the increased risk of newborns to contract infectious diseases. Importantly, these results also suggest that an LPS adjuvant may be less suitable for vaccination of very young children.

Conclusions and perspectives

The results presented in this thesis indicate that induction of type I IFN after vaccination with a meningococcal outer membrane vaccine or a whole-cell pertussis vaccine is required for optimal immunogenicity. Induction of pro-inflammatory cytokines via the MyD88-pathway seems less important for adjuvant activity, as we observed that LPS-deficient meningococcal OMCs that activate MyD88 via TLR2 are weakly immunogenic. As pro-inflammatory cytokines are thought to contribute to toxicity and type I IFNs mediate adjuvant activity, the design of the ideal adjuvant seems within reach. LPS variants that preferentially activate the TRIF pathway may be good adjuvant candidates. TLR3 ligands may also hold promise as adjuvant as TLR3 solely activates the TRIF-dependent pathway. We have indeed noticed that the TLR3 ligand poly I:C induces high levels of type I IFN and only very low levels of pro-inflammatory cytokines *in vitro* (unpublished results). The effectiveness and safety of an adjuvant can be further improved by targeting the adjuvant to the desired cell type such as DCs and by physically linking the adjuvant and antigen. Whether *lpxL1* mutant LPS is an adjuvant suitable for human use is an important issue that should be addressed. It is clearly a good adjuvant in mice, but *lpxL1* mutant LPS is less well recognized by human TLR4-MD-2. In any case, it is very unlikely to be toxic in humans as patients infected with *lpxL1* mutants had milder symptoms than patients infected with a wildtype strain.

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Appendices

Nederlandse Samenvatting

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Nederlandse Samenvatting

De meningokok (*Neisseria meningitidis*) is een bacterie die voorkomt in de keel/ neusholte van ruwweg anderhalf miljoen Nederlanders zonder dat deze mensen daar last van hebben. Af en toe verspreidt de bacterie zich echter naar de bloedbaan en/of hersenvliezen, waardoor bloedvergiftiging en hersenvliesontsteking kunnen ontstaan. Jaarlijks worden in Nederland rond de 150 mensen met zo'n ziektebeeld opgenomen. Het is dus vrij zeldzaam, maar van deze patiënten overlijdt ongeveer 10% en nog eens 20% houdt er blijvende schade aan over. De heftige ontstekingsreactie in deze ziektes wordt voornamelijk veroorzaakt door een belangrijke bouwsteen van de meningokok, lipopolysaccharide (LPS). Immuuncellen bezitten een eiwit op hun celmembraan dat specifiek aan LPS kan binden als een sleutel op een slot. Dit eiwit luistert naar de naam Toll-like receptor 4 (TLR4). Als LPS aan TLR4 bindt, krijgt de immuuncel het signaal om allerlei stoffen uit te scheiden die een ontsteking veroorzaken. De bedoeling van die ontsteking is om de bacteriën op te ruimen, maar doordat het immuunsysteem grof geschut inzet, wordt ook het eigen lichaam beschadigd. Het is zelfs zo dat het merendeel van de gebruikelijke symptomen in deze ziektes niet direct door de bacterie zelf worden veroorzaakt, maar door een overactief immuunsysteem.

Behalve TLR4 hebben onze cellen nog veel meer receptoren die structuren afkomstig van microben kunnen herkennen. Daarvan zijn receptoren van de Toll-like receptorfamilie het bekendst. Tot deze familie behoren naast TLR4 ook andere receptoren. TLR3 kan bijvoorbeeld dubbelstrengs RNA van bepaalde virussen herkennen, TLR5 bindt aan een eiwit dat sommige bacteriën als een soort propeller gebruiken om voort te bewegen, en TLR9 'ziet' DNA van sommige bacteriën en virussen. Net als bij TLR4 geeft de ontmoeting tussen deze Toll-like receptors en hun structuur, die zij specifiek kunnen herkennen, een signaal af aan de immuuncellen dat iets niet in de haak is. Vervolgens gaan de immuuncellen stoffen maken die het immuunsysteem activeren om de indringer uit te schakelen. Samen kunnen Toll-like receptors zo ongeveer alle pathogenen herkennen. De maatregelen die het immuunsysteem neemt om de indringer op te ruimen hangt af van welke Toll-like receptor wordt geactiveerd, want de strategie om de ongewenste klant uit te schakelen verschilt bijvoorbeeld tussen een virus of een parasiet.

Zoals bij de meeste pathogenen, zit er nogal wat variatie tussen verschillende meningokokkenstammen. De bacterie wil natuurlijk niet opgeruimd worden door ons immuunsysteem en door wat te variëren in zijn uiterlijk kan de bacterie het immuunsysteem op het verkeerde been zetten. Je zou het kunnen vergelijken met een gezochte crimineel die zijn snor laat staan in een poging om uit handen te blijven van de politie. Bijna alle

meningokokkenstammen die geïsoleerd worden uit patiënten hebben een kapsel. Dat is een soort jasje gemaakt van bepaalde suikers dat de bacterie omringt en beschermt tegen bepaalde afweermechanismen van ons immuunsysteem. Meningokokken kunnen verdeeld worden in een paar hoofdtypes (serogroepen) gebaseerd op verschillen in de structuur van dit kapsel. Zes verschillende serogroepen (A, B, C, W-135, X, en Y) zijn verantwoordelijk voor bijna alle gevallen van meningokokkenziekte. Van al deze kapsels, behalve type B, zijn vaccins gemaakt die bescherming geven tegen die serogroepen na vaccinatie. Het type B- kapsel wekt helaas nauwelijks een immuunreactie op en is dus niet bruikbaar als vaccin. Jammer genoeg komt type B nou juist het meeste voor in Nederland. Daarom is het Nederlands Vaccin Instituut bezig met de ontwikkeling van een alternatief vaccin tegen deze serogoep. Dit vaccin bestaat uit de buitenmembraan van de bacterie. Aangezien LPS een belangrijk bestanddeel van de buitenmembraan is, bevat het vaccin dus ook LPS. Maar omdat LPS een heftige ontstekingsreactie kan veroorzaken, is een dergelijk vaccin wellicht niet ideaal om mensen mee in te enten. Al dient wel vermeld te worden dat tijdens de productie van het vaccin de LPS- concentratie door bepaalde methodes aanzienlijk wordt verlaagd. Bovendien zijn al miljoenen mensen gevaccineerd met dit vaccin zonder veel vervelende bijwerkingen. Toch zou het met het oog op mogelijke bijwerkingen beter zijn als er helemaal geen LPS in het vaccin zit. Daarom zijn onderzoekers bij het Nederlands Vaccin Instituut op zoek gegaan naar manieren om de LPS structuur van de meningokok te veranderen. Waarschijnlijk zou een veranderde LPS structuur minder goed worden herkend door immuuncellen en daardoor minder ontstekingsreacties veroorzaken.

Tot ieders verbazing bleek het mogelijk om in het laboratorium een meningokok te creëren met helemaal geen LPS door een gen uit schakelen dat essentieel is voor de LPS synthese. Dit was verrassend omdat altijd was aangenomen dat alle bacteriesoorten deze belangrijke bouwsteen nodig hebben om te kunnen overleven. De buitenmembraan van deze stam zonder LPS zou geschikt kunnen zijn als vaccin, omdat nu de veroorzaker van ontstekingen niet meer aanwezig is. Helaas bleek dat als muizen ingeënt werden met een vaccin gemaakt van deze stam, de resulterende immuunreactie niet voldoende was om bescherming tegen meningokokkenziekte te geven. Kennelijk moet een vaccin dus een stof zoals LPS bevatten die het immuunsysteem wakker schudt om adequaat te reageren na vaccinatie. Zulke hulpstoffen worden adjuvantia genoemd. Dat zijn stoffen die de werkzaamheid van het vaccin verbeteren. Omdat stoffen die het immuunsysteem activeren de werkzaamheid van het vaccin verbeteren, maar ook meer ontsteking veroorzaken, is het lastig om bij het ontwikkelen van een nieuw adjuvant hierin de juiste balans te vinden.

Het doel van het onderzoek, beschreven in dit proefschrift, was om uit te zoeken of structuren die herkend worden door andere Toll-like receptoren dan TLR4 ook bruikbaar zijn als adjuvant voor een vaccin tegen meningokokken. Verder wilden we het mechanisme begrijpen waarom sommige structuren die herkend worden door deze receptoren als adjuvant kunnen dienen, terwijl andere structuren niet geschikt zijn. Daarnaast hebben we de invloed onderzocht van verandering van de LPS structuur op de immuunreactie na infectie van mensen en muizen met meningokokken.

In hoofdstuk 2 hebben we verscheidene structuren die herkend worden door Toll-like receptors (TLR1-9) vergeleken in hun capaciteit om de immuunreactie te verbeteren tegen een buitenmembraanvaccin van de LPS-loze meningokok in muizen. We hebben gevonden dat structuren die herkend worden door TLR3, TLR4, TLR7, en TLR9 hiertoe in staat waren. Dus naast LPS zouden ook stoffen die TLR3, TLR7, en TLR9 activeren gebruikt kunnen worden als adjuvant om de werkzaamheid van een vaccin tegen meningokokken te verbeteren. Sommige van deze stoffen worden al gebruikt voor bijvoorbeeld therapieën bij mensen en hiervan is bekend dat er geen nare bijwerkingen zijn. Daarnaast laten we zien dat bepaalde LPS varianten waarvan de structuur is aangepast ten opzichte van normaal LPS ook goed werken als adjuvant, terwijl deze LPS varianten minder ontsteking veroorzaken, vermoedelijk door slechtere binding aan TLR4.

Het buitenmembraanvaccin tegen meningokokken type B met in het vaccin normaal LPS is al toegediend aan miljoenen mensen en is in staat gebleken om te beschermen tegen meningokokkenziekte. De buitenmembraan van deze bacterie bevat onder andere LPS en lipoproteïnen, die herkend worden door respectievelijk TLR4 en TLR2. De bijdrage van deze stoffen aan de werkzaamheid van het vaccin zijn nooit goed uitgezocht. In hoofdstuk 3 onderzoeken we muizen met inactivaties in genen verantwoordelijk voor de herkenning van LPS (bijvoorbeeld TLR4) en lipoproteïnen (TLR2). Verschillende soorten muizen (normale en mutanten) werden ingeënt met het buitenmembraanvaccin tegen meningokokken. Daarna werd de immuunreactie vergeleken tussen de verschillende muistypes. Hieruit bleek dat herkenning van LPS door de muis belangrijk is voor een goede immuunreactie na vaccinatie. Tot onze verrassing lieten muizen met een inactivatie van TLR2 een normale immuunreactie zien. Blijkbaar draagt LPS bij aan de werkzaamheid van het vaccin in tegenstelling tot lipoproteïnen.

In hoofdstuk 4 tonen we aan dat rond 9% van patiënten uit Nederland met meningokokkenziekte geïnfecteerd is met een stam met een gemuteerde vorm van LPS. Bijna al deze isolaten, op één uitzondering na, hadden een mutatie in hetzelfde gen. Van dit gen wisten we al dat het een bepaald onderdeel toevoegt aan het LPS molecuul. In de mutanten ontbreekt dit onderdeel. Deze aangepaste LPS structuur kan veel minder goed

binden aan TLR4. Het gevolg is dat de LPS mutanten immuuncellen minder goed kunnen activeren, waardoor er een geringere ontstekingsreactie ontstaat. Vermoedelijk ontstaan deze mutanten omdat ze detectie door het immuunsysteem beter kunnen vermijden dan normale meningokokken. Verder laten we zien dat volwassenen met hersenvliesontsteking die geïnfecteerd zijn met zo'n LPS mutant mildere symptomen hebben dan normaal. Dit komt goed overeen met onze observatie dat deze LPS mutanten minder ontsteking veroorzaken.

De overgrote meerderheid van mensen die geïnfecteerd worden met meningokokken dragen de bacterie alleen een tijdje in de keel/neusholte zonder daar ooit ziek van te worden. Naast hersenvliesontsteking zijn er nog enkele andere aandoeningen mogelijke na infectie met meningokokken. Bovendien, hebben kinderen de grootste kans om ziek te worden. Daarom hebben we in hoofdstuk 5 onderzocht hoe het zit met LPS mutant infecties in alle groepen waar we in hoofdstuk 4 nog niet naar hadden gekeken. Hieruit bleek dat naarmate mensen ouder worden ze relatief vaker met een LPS mutant zijn geïnfecteerd. Oudere mensen hebben vaak meer immuniteit opgebouwd tegen een ziekteverwekker zoals de meningokok dan jongere mensen. Mogelijk is de LPS mutatie extra voordelig voor de bacteriën in individuen die al immuniteit hebben tegen meningokokken. Verder vonden we net als in hoofdstuk 4 dat oudere mensen mildere symptomen vertonen dan normaal als ze geïnfecteerd zijn met een LPS mutant. Opmerkelijk genoeg was dit niet het geval bij patiënten jonger dan 5 jaar. Wat we ook totaal niet hadden verwacht was de verdeling van LPS mutanten in meningokokken geïsoleerd uit de keel/neusholte van dragers. Bij de isolaten uit patiënten was de verdeling van LPS mutanten gelijk verdeeld over de verschillende genetische varianten van de meningokok. Bij dragerstammen behoorden juist bijna alle LPS mutanten tot een groep van meningokokken die genetisch zeer sterk verwant zijn.

Het feit dat LPS mutanten vaak voorkomen bij patiënten suggereert dat de meningokok er baat bij heeft om de structuur van LPS aan te passen. In hoofdstuk 6 hebben we onderzocht wat de invloed van de LPS structuur is op de mogelijkheid van de meningokok om muizen ziek te maken. Hiertoe hebben we muizen geïnfecteerd met drie verschillende stammen: de normale stam, de LPS mutant die regelmatig mensen infecteert, en een LPS mutant die we zelf gemaakt hebben, die eenzelfde onderdeel mist aan het LPS molecuul, maar op een andere positie. We laten zien dat die laatste LPS mutant muizen veel zieker maakt dan normaal, terwijl de LPS mutant die regelmatig mensen infecteert de muizen juist minder ziek maakt. Dit toont aan dat de LPS structuur een grote invloed heeft op de capaciteit van de bacterie om een infectie te veroorzaken.

Samenvattend, onze resultaten laten zien dat LPS een dominante rol speelt in

meningokokkenziekte en immunitieit tegen de bacterie. Het goede nieuws is dat de structuur van LPS kan worden aangepast of worden vervangen door andere stoffen die een minder ernstige ontsteking veroorzaken, maar de immuuncellen nog wel genoeg activeren voor een goede reactie na vaccinatie.

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Curriculum vitae

Floris Fransen was born on the 10th of June 1979 in Deventer. In 1998, he graduated at the Staring College (VWO) in Lochem. In the same year he started to study Biology at the Utrecht University. In the final phase of his studies he did two internships and wrote one literature study. The first internship was performed at the Molecular Microbiology group, Utrecht University under supervision of prof. dr. Jan Tommassen and dr. Peter van Ulsen. During this time he characterized the autotransporter protein NalP of the Gram-negative bacterium *Neisseria meningitidis*. After that he wrote a literature study under supervision of dr. Adri Thomas from the Department of Developmental Biology at Utrecht University. The title of this literature study was: "Important molecular biological factors of the next influenza pandemic". For his second internship he went to Hamilton, Montana, USA. There he did research at the Laboratory of Human Bacterial Pathogenesis, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health under supervision of dr. Philip Stewart and dr. Patricia Rosa. Here he investigated the role of the *Borrelia burgdorferi* BB0323 gene in outer membrane integrity. After receiving his Master of Science degree in 2005, he started his PhD project at The Netherlands Vaccine Institute under supervision of dr. Peter van der Ley and prof. dr. Claire Boog. This project was performed in collaboration with prof. dr. Jos van Putten from the Department of Immunology and Infectious Diseases, Utrecht University. During his PhD he received an EMBO short-term fellowship to study the interactions between dendritic cells and meningococcal LPS variants under supervision of prof. dr. Colin Watts at the Cell Biology and Immunology department, University of Dundee, UK. As of the 1st of April 2010, he will start working as a post-doc at the European Institute of Oncology in Milan in the group of dr. Maria Rescigno, studying host-pathogen interactions during *Salmonella* infection of the gut with two-photon microscopy.

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