Agonists of Toll-Like Receptors 3, 4, 7, and 9 Are Candidates for Use as Adjuvants in an Outer Membrane Vaccine against *Neisseria meningitidis* Serogroup B[∇]

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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 lipopolysaccharide (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 Toll-like receptor (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 immunoglobulin G (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.

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 (44, 56). These vaccines contain lipopolysaccharide (LPS), which is a powerful adjuvant but is also 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 (50). However, when outer membrane complexes (OMCs) of this mutant were used to immunize mice, the resulting antibody response was too low to be protective (51), demonstrating that an adjuvant such as 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 wild-type *N. meningitidis* LPS in immunization experiments (52, 55).

After the finding of TLR4 as the LPS receptor many more pattern recognition receptors and their corresponding pathogen-associated molecular patterns have been discovered in recent years (53). The TLR family is the best-studied family of pattern recognition receptors and recognize a broad spectrum of pathogen-associated molecular patterns 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- κ B and consequently the induction of many genes involved in immunity (26). Besides the MyD88-dependent pathway, TLR3 and TLR4 signaling 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 (IFN). TLR7/8 and TLR9 are also capable of activating type I IFN, 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 (22, 57). This process involves phagocytosis, increased surface expression of costimulatory molecules, redistribution of major histocompatibility complex (MHC) molecules from intracellular compartments to the cell surface, a switch in chemokine receptor ex-

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pression, and secretion of cytokines and chemokines (2). After antigen processing and expressing epitopes in an MHC-restricted manner, mature DCs can stimulate naive T cells to differentiate into effector cells. Depending on the TLR ligand, the immune response may be skewed toward cytotoxic T-cell responses or particular classes of (Th) T helper responses (40). 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 the present 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 immunoglobulin G (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 toward 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 specific-pathogen-free BALB/c mice were purchased from Harlan Europe (Horst, The Netherlands) and were housed under specific-pathogen-free conditions. Mice were acclimatized for approximately 1 week and were 8 to 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 IsoVitalX (Becton Dickinson) overnight at 37°C in 5% CO₂ in a humid atmosphere. Next, the bacteria were transferred from plate to Trypticase soy broth liquid medium and grown at 37°C, 5% CO₂ to late log phase. Then the bacteria were heat inactivated at 56°C for 1 h. These were used for isolation of OMCs by sarcosyl extraction as described previously (54). The quantity of protein was determined with the bicinchoninic acid protein assay reagent (Pierce), with bovine serum albumin as a standard.

Adjuvants. *N. meningitidis* L3 LPS, *lpxL1* mutant LPS, and *B. pertussis* strain Tohama LPS were isolated by the hot-phenol extraction method as described previously (59). FSL-1, Pam₃CSK4, poly(I-C), monophosphoryl lipid A (MPL), *B. subtilis* flagellin, Imiquimod, Loxoribine, and muramyl dipeptide (MDP) were purchased from Invivogen. CpG DNA ODN1826 (5'-TCCATGACGTTCTCTG ACGTT-3') with phosphorothioate bases was synthesized by Genosys.

Immunizations. Mice were immunized subcutaneously on days 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 of phosphate-buffered saline (PBS). The adjuvants used and their amounts in weight and molar units are listed in Table 1. The mice that received H44/76 OMCs, pLAK33 OMCs alone, or pLAK33 OMCs in combination with FSL-1, Pam₃CSK4, poly(I-C), MPL, L3, or LpxL1 were bled on day 42. Mice that 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 -20°C.

Antigen-specific antibodies. Antigen-specific IgG, IgG1, IgG2a, and IgG2b antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well microtiter plates (Immulon 2; Nunc) were coated overnight at room temperature with 100 µl/well H44/76 P1.7,16 OMCs (4 µg/ml PorA) in PBS. Sera were diluted 1:50, and from this threefold 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 37°C for 1 h. Again, plates were washed, and either anti-mouse IgG-horseradish peroxidase (HRP), anti-mouse IgG1-HRP, anti-mouse IgG2a-HRP, or anti-mouse IgG2b-HRP (Southern Biotechnology Associates, Inc.) diluted 1:5,000 in PBS-0.1% Tween 80-1% skimmed milk (Protifar; Nutricia, The Netherlands) was added at 100

TABLE 1. Weight and molar units per mouse

Adjuvant	Per mouse	
	Wt (µg)	Molar units (nmol)
FSL-1	10	6
	1	0.6
Pam ₃ CSK4	50	33.12
	5	3.31
Poly(I-C)	100	
	10	
MPL	20	13.11
	2	1.31
L3	1	0.26
	0.1	0.026
LpxL1	10	2.76
	1	0.28
Flagellin	10	0.31
	1	0.031
<i>B. pertussis</i> LPS	10	2.47
	1	0.25
Imiquimod	100	417
	10	41.7
Loxoribine	85	250
	8.5	25
CpG DNA	127	20
	12.7	2
MDP	100	203
	10	20.3

µl/well. Plates were incubated at 37°C for 1 h. After a washing step, 100 µl of peroxidase substrate (3,3',5,5'-tetramethylbenzidine with 0.01% H₂O₂ in 0.11 M sodium acetate buffer [pH 5.5]) was added per well. After 20 min, the reaction was stopped by the addition of 100 µl of 2 M H₂SO₄/well. 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 an optical density of 0.6. The results are expressed as log₁₀ titers.

Total IgE. Total IgE levels in the sera were measured by ELISA. Flat-bottom 96-well microtiter plates were coated with 100 µl per well of 2 µg of rat anti-mouse IgE monoclonal antibody (BD Biosciences)/ml in PBS, followed by incubation overnight at 4°C. Plates were blocked with 200 µl per well of PBS and 1% skimmed milk at room temperature for 1 h. Next, the plates were incubated for 1 h at room temperature with the sera of the immunized mice, the serum of a naive mouse, and purified mouse IgE clone C48-2 (BD Biosciences) as a standard, all diluted in PBS-1% skimmed milk-0.05% Tween 20. The plates were then incubated with 100 µl per well of 2 µg of biotinylated anti-mouse IgE (BD Biosciences)/ml in PBS for 1 h at room temperature. Next plates were incubated at room temperature for 30 min with 100 µl per well streptavidin-HRP (Sanquin) diluted 1:5,000 in PBS-1% skimmed milk-0.05% Tween 20. Peroxidase substrate was used as a substrate, and the reaction was stopped with 2 M H₂SO₄. 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* H46/76 P1.7,16 (10⁴ 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 Iso-VitalX (Becton Dickinson) and grown overnight at 37°C in 5% CO₂ 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 log₁₀ titers.

Statistics. Before statistical analysis, data from each experiment were log₁₀ converted, which normalized their distribution. In every graph the mean of log₁₀ titers of eight mice per group, including the standard errors of the mean (SEM) are presented, except for IgE for which unconverted data are shown. One-way analysis of variance 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).

FIG. 1. Antigen-specific total IgG levels. Mice were immunized with the following adjuvants: FSL-1 (TLR2/TLR6); Pam₃CSK4 (TLR2/TLR1); poly(I-C) (TLR3); *N. meningitidis* wild-type L3, LpxL1 LPS, *B. pertussis* LPS, and MPL (TLR4); *B. subtilis* flagellin (TLR5); Imiquimod and Loxoribine (TLR7); CpG DNA (TLR9); and MDP (NOD2). The sera of immunized mice (eight per group) were analyzed by ELISA for antigen-specific IgG. The data are expressed as means $\pm$ the SEM of log₁₀ 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. LPS Bp, *B. pertussis* LPS.

RESULTS

Antigen-specific total IgG levels. The ligands used in the present study were as follows: FSL-1 (recognized by TLR2/TLR6 heterodimer); Pam₃CSK4 (recognized by TLR2/TLR1 heterodimer); poly(I-C) (TLR3); different forms of LPS (TLR4), i.e., *N. meningitidis* wild-type L3, mutant penta-acylated *lpxL1* LPS, *Bordetella pertussis* LPS, and MPL; *B. subtilis* flagellin (TLR5); Imiquimod and Loxoribine (TLR7); CpG DNA (TLR9); and 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 examine dose dependency and mixed with 5 μ g of LPS-deficient OMCs in PBS. The BALB/c mice were then immunized subcutaneously with these formulations, LPS-deficient OMCs alone (pLAK33), or wild-type *N. meningitidis* OMCs (H44/76) as a positive control. Mice were immunized on days 0 and 28. At 42 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 significantly ($P < 0.05$) higher IgG levels than did pLAK33 alone: MPL (2 μ g), L3 (1 μ g), LpxL1 (10 and 1 μ g), LPS *B. pertussis* (10 μ g), and Imiquimod (100 μ g). Poly(I-C) (100 μ g) and CpG DNA (2 nmol) also seemed to enhance IgG levels, but not significantly. There was little difference in response to 10 or 1 μ g 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-depen-

FIG. 2. Antigen-specific antibody subclasses. The sera of immunized mice (eight per group) were analyzed by ELISA for the presence of antigen-specific IgG1 (A), IgG2a (B), and IgG2b (C). The data are expressed as means $\pm$ the SEM of log₁₀ titers. An asterisk indicates that the immunoglobulin levels of a group were significantly different ($P < 0.05$) from pLAK33-immunized mice. The dashed line indicates the level of immunoglobulin from pLAK33-immunized mice. LPS Bp, *B. pertussis* LPS.

dent effect on IgG levels. As demonstrated in previous studies (52, 55), LpxL1 had an effect on the IgG levels comparable to that of 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.

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 interleukin-4 (IL-4) are involved in isotype switching from IgM and IgD to IgG1 and IgE, while the Th1 cytokines IL-2 and gamma IFN (IFN- γ) induce class switching to IgG2a and IgG2b (37, 38). To assess whether the TLR ligands influence the balance between Th1- and Th2-dependent immunoglobulin production, mouse sera were analyzed on the presence of IgG1, IgG2a, and IgG2b antibody subclasses specific for *N. meningitidis* outer

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

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 μ g) induced significantly 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 that for total IgG (Fig. 2B and 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, whereas the other ligands showed a dose-dependent effect. Since these agonists improved IgG2a and IgG2b levels but not so much the IgG1 levels, they demonstrated a skew toward Th1-dependent immunoglobulin 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 (5, 21, 24, 25).

Total IgE levels. To further establish Th2-dependent immunoglobulin 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 naive 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 naive mouse. This suggests that in the absence of LPS the *N. meningitidis* vaccine skews the balance more toward 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 that other studies have also found that TLR2 ligands can induce Th2 responses (1, 11, 42). 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

FIG. 4. Total IgE levels. Amounts of IgE antibodies were determined in sera of immunized mice (eight per group, except for CpG DNA 2 nmol [seven mice]) by ELISA. The data are expressed as the means $\pm$ the SEM of nanograms of IgE/milliliter. 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. LPS Bp, *B. pertussis* LPS.

FIG. 5. Serum bactericidal antibodies. Serial dilutions of sera of immunized mice (eight mice per group) were incubated with *N. meningitidis* and rabbit complement. The highest dilution that killed $\geq 90\%$ of bacteria was determined. The number of responders is indicated in the graph. A responder was defined as a mouse whose serum killed $\geq 90\%$ at at least a 1:2 dilution. The data are expressed as means $\pm$ the SEM of log₁₀ titers.

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 immunoglobulin 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. An exception to 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.

Serum bactericidal antibodies. We have shown that TLR ligands elevated the production of certain antibody subclasses. However, this 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 (17). Therefore, sera of the immunized mice were analyzed in 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.

We also assessed the correlation coefficient between the 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 did the IgG1 level with bactericidal activity. Indeed, IgG2a and IgG2b, but not IgG1, isotypes are known for their ability to activate complement (27).

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 can one assume that the adjuvant (LPS) is coinorporated into the antigen complex. The LPS content in OMCs is between 5 and 10%, which is similar to the LPS content in OMVs (49). 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 to the combination of LPS-deficient OMCs and *N. meningitidis* wild-type LPS, because these two formulations are practically the same except for 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 ovalbumin were emulsified in

TABLE 2. Correlation between serum bactericidal activity and antibody isotypes

Antibody isotype (amt or concn)	Correlation coefficient ^a				
	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 ₃ CSK4 (50 µg)	-	-	-	-	-
Pam ₃ CSK4 (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)	-	-	-	-	-
<i>B. pertussis</i> LPS (10 µg)	0.92	0.76	0.96	0.91	-0.58
<i>B. pertussis</i> LPS (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)	-	-	-	-	-

^a The 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:

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

incomplete Freund's adjuvant, cross-linked by biotin-avidin bridges, or coencapsulated 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 and Medzhitov have demonstrated that antigens phagocytosed by DCs depend on TLR ligands within the phagocytosed cargo for efficient presentation (4). Similarly, Sporri and Reis e Sousa 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 (48). 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 (14). 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 significantly 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 immunoglobulin production (43, 47). Others have demonstrated that TLR ligands, even the ones that induce a Th1 response, can augment antigen-specific IgG1 levels (12, 24). 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 (19, 20, 39) and other reports that showed that TLR2 ligands induce a Th2 response (1, 11, 42, 42). It seems reasonable that the level of Th2-dependent IgG1 induced by LPS-deficient OMCs cannot be augmented by Th1 polarizing adjuvants. Similar results were found by Vasilakos et al. (58). These researchers examined the effects of Th1 adjuvants R-848 and CpG DNA on antibody production induced in the presence of the Th2 adjuvant alum. It was found that IgG2a but not IgG1 levels induced by alum-ovalbumin 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, since it has been demonstrated that CpG directly inhibits IgG1 and IgE switching in purified B cells, while promoting the production of IgG2a (32, 33). Previously, it was assumed that CpG influenced antibody class switching only by its ability to induce Th1-skewing cytokines, such as IL-12, IL-18, IFN-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 in 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. Since bactericidal activity depends on class switching to Th1-dependent antibodies, these results were not unexpected, because these agonists are known to induce Th1 polarization (5, 21, 24, 25). 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 ability to induce type I IFN. Activation of TLR3, TLR4, TLR7, or TLR9, in contrast to the activation of TLR2, TLR5, or NOD2, is reported to induce production of type I IFN (46). 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 (31). 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 (18). Finally, production of IL-12p70 by DCs depends on type I IFN (15). DC-derived IL-12p70, the bioactive form of IL-12, stimulates IFN- γ production in naive Th cells and, therefore, strongly promotes Th1 responses (9).

There are several possible explanations for the apparent lack of adjuvant activity of the TLR2, TLR5, and NOD2 agonists in our model. Therefore, we cannot 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 (8, 10, 13, 23, 29, 30, 35, 36). 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 (13). In that formulation, however, MDP is dissolved in a water-in-oil emulsion, and others have also found that MDP alone barely improves responses (29, 36). 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 (19, 20, 39). 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 (34, 45). 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 (30). 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, since flagellin is reported to promote a Th2-type response (8, 10), 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, Freund's complete adjuvant, Freund's incomplete adjuvant, and Ribi adjuvant mount robust antibody responses in mice completely deficient in TLR signaling (16). 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, since 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 cannot 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 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 (41). Moreover, our data show that immunization with bacterium-derived OMCs enhance antibody responses mainly by the 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* serogroup B vaccine. Wild-type LPS from *N. meningitidis* is very toxic (6). In fact, LPS levels in the blood of patients, who suffer from septicemia 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, such as Imiquimod, CpG DNA, and in particular MPL, have already been tested extensively in humans and were found to be safe (7, 14, 28).

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