Chapter 6

Supplemention of whole-cell pertussis vaccines with lipopolysaccharide analogs: a novel strategy for modulating vaccine efficacy and reactogenicity

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Abstract

Lipopolysaccharide (LPS) is one of the main constituents of the Gram-negative bacterial outer membrane. Besides being an endotoxin, LPS also possesses a powerful adjuvant activity. Previously, it has been shown that changes in the chemical composition of the lipid A domain of LPS modulate its biological activity. An example of this is monophosphoryl lipid A (MPL), an LPS analog that has been shown to be a non-toxic immunostimulatory compound. Moreover, several LPS analogs have been shown to function as LPS antagonists. In the present study, we show that supplementation of whole-cell pertussis (wP) vaccines with LPS analogs modulates their efficacy and reactogenicity. We show that addition of MPL to a wP vaccine increases vaccine efficacy without changing vaccine reactogenicity. Furthermore, we show that *Neisseria meningitidis* LpxL2 LPS, an LPS species derived from *N. meningitidis* by insertionally inactivating the *lpxL2* gene, functions as an LPS antagonist and that addition of this LPS not only decreases the *in vivo* reactogenicity of the wP vaccine, but also increases vaccine efficacy.

Introduction

Whooping cough, or pertussis, is a severe, highly-contagious respiratory tract disease that is caused by the Gram-negative bacterium *Bordetella pertussis*. Introduction of whole-cell pertussis (wP) vaccines in the second half of the 20th century led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after its introduction, it became clear that wP vaccines displayed a considerable reactogenicity, which was caused by the presence of potent immunomodulatory agents, such as lipopolysaccharide (LPS) and pertussis toxin. Hence, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the late 1980s and early 1990s. Nowadays, aP vaccines are broadly used in the industrialised countries and have been shown to be highly effective. Nevertheless, vaccination with aP vaccines has, compared to vaccination with wP vaccines, some important drawbacks, including high production costs, which prohibits their use in developing countries, the potential to select for escape mutants more rapidly, and the fact that aP vaccination evokes a Th2 immune response, whereas wP vaccination and natural infection induce a Th1 immune response (Ausiello *et al.*, 1997; Ryan *et al.*, 1997; Ryan *et al.*, 1998; van den Berg *et al.*, 2001). Therefore, the development of safer wP vaccines remains an issue of importance.

An important factor contributing to the reactogenicity of wP vaccines is the presence of LPS or endotoxin. LPS is a large glycolipid that forms one of the major components of the Gram-negative bacterial outer membrane. It is usually composed of a highly variable O-antigen, a less variable core oligosaccharide, and a highly conserved lipid moiety, designated lipid A. The structure of lipid A consists of a phosphorylated glucosamine disaccharide substituted with a variable number of acyl chains (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also possesses a powerful adjuvant activity. Both these properties are based upon the recognition of the LPS by the host TLR4/MD-2 receptor complex and the subsequent activation of NF-κB (reviewed in Pålsson-McDermott and O'Neill, 2004; O'Neill, 2006). Due to its adjuvant activity LPS potentially forms a valuable vaccine component. However, the pyrogenicity and toxicity of LPS have precluded its development as a therapeutic compound in humans.

The endotoxic activity of LPS is dependent on the composition of the lipid A moiety (Homma *et al.*, 1985). Previous studies have indicated that the phosphate groups, as well as the number and length of the acyl chains are critical determinants of the endotoxic activity (Loppnow *et al.*, 1989; Raetz and Whitfield, 2002; Steeghs *et al.*, 2002; Geurtsen *et al.*, 2006). These studies have stimulated the development

of LPS derivatives with potentially useful properties. Some of these derivatives have been shown to exert a strongly reduced endotoxic activity, whereas their adjuvant and immunostimulatory properties were retained. One of the best known examples is monophosphoryl lipid A (MPL). MPL has been developed both as an adjuvant for application in human vaccines and as a prophylactic drug against septic shock (Rudbach *et al.*, 1994; Baldridge and Crane, 1999). The reduced endotoxic activity of MPL has been attributed to a reduced capacity to induce the secretion of pro-inflammatory cytokines, such as IL-6, IL-1β, and TNFα (Gustafson and Rhodes, 1994; Henricson *et al.*, 1993; Okemoto et al, 2006). In addition, MPL has been shown to increase the secretion of the anti-inflammatory cytokine IL-10 by murine macrophages (Salkowski *et al.*, 1997). Another example is the LPS from *Porphyromonas gingivalis*. This LPS has been shown to be non-toxic and to block *in vitro* secretion of TNFα by both human and murine macrophage cell lines stimulated with *Escherichia coli* LPS (Henricson et al., 1992; Golenbock *et al.*, 1991; Takayama *et al.*, 1989). Furthermore, it has been shown to act *in vivo* as an LPS antagonist (Qureshi *et al.*, 1991).

To create novel LPS species with potentially useful properties the possibilities of manipulating the LPS biosynthesis route have been explored. Recently, it has been shown that *Neisseria meningitidis* strains deficient in the late acyltransferases LpxL1 or LpxL2 display a dramatically decreased endotoxic activity when tested for their capability to stimulate human macrophages (van der Ley *et al.*, 2001). However, when the LPSs from these strains were tested for their adjuvant activity in a mouse model, the mainly tetra-acylated *lpxL2* mutant LPS proved to be only moderately immunogenic, whereas the penta-acylated *lpxL1* mutant LPS was still capable of boosting the immune response up to wild-type levels (van der Ley *et al.*, 2001).

In the present study, we investigated whether supplementation with either a non-toxic, non-antagonistic LPS-analog or a non-toxic, antagonistic LPS-analog beneficially influences the efficacy and/or reactogenicity of wP vaccines and thus facilitates the development of safer wP vaccines.

Materials and Methods

Bacterial strains and growth conditions

Unless otherwise notified, *B. pertussis* strain B213, a streptomycin resistant derivative of *B. pertussis* strain Tohama (Kasuga *et al.*, 1953) was grown at 35°C on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood (Tritium, Veldhoven, the Netherlands).

In vitro endotoxic activity assay

Stimulation of IL-6 production was tested with the human macrophage cell line MM6 (Ziegler-Heitbrock *et al.*, 1998). The macrophages were seeded in 24-wells plates ($1x10^6$ cells/well) in 200 µl of IMDM medium (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 (Gibco BRL). The cells were stimulated with 100 µl of serial dilutions of a whole-cell suspension of wild-type *B. pertussis* strain B213 or purified *B. pertussis* LPS in the absence or presence of 1 µg/ml *N. meningitidis* LpxL2 LPS (van der Ley *et al.*, 2001). The whole-cell suspension and *B. pertussis* LPS was prepared as described (Geurtsen *et al.*, 2006) After stimulation for 16-18 h at 37°C in a humidified atmosphere containing 5% CO_2, IL-6 concentrations in the culture supernatants were quantified with an ELISA for human IL-6 according to the manufacturers' instructions (PeliKine Compact™).

Vaccine preparation

Vaccines were prepared from 68-h cultures of *B. pertussis* strain B213. The bacterial cell suspensions were heat-inactivated for 10 min at 56°C in the presence of 8 mM formaldehyde, after which the cells were collected by centrifugation for 10 min at 16,100 x *g* and resuspended in PBS to an A_{590} of 2.5, i.e., 50 international opacity units (IOU). The suspensions were stored at 4°C. Prior to immunisation, the suspensions were further diluted in PBS to a final concentration of 3.2 IOU/ml, after which 0.3 mg/ml aluminum phosphate (final concentration), alone or in combination with 40 µg/ml MPL (Sigma; prepared from *Salmonella enterica* serotype Minnesota Re 595) or 40 µg/ml LpxL2 LPS, was added as an adjuvant. The resulting vaccine concentration corresponded to 1/10 human dose per 0.5 ml.

Immunisation and intranasal challenge

All animal experiments were performed at the Netherlands Vaccine Institute in accordance with the Dutch national guidelines for animal experimentation. Groups of nine 4- to 5-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml of the vaccine (prepared as described above), or with 0.5 ml of PBS/$AlPO_4$ as a control, at days 0 and 14. At day 28, the mice were challenged intranasally with $2x10^7$ colony-forming units (CFU) of *B. pertussis* strain B213 in 40 µl of Verwey medium (Willems *et al.*, 1998).

Autopsy, analysis of bronchoalveolar lavage fluid cells, and CFU counting

To obtain bronchoalveolar lavage fluid (BALF) from the lungs, a cannula was placed intratracheally and fixed using a suture. 0.5 ml of PBS was brought into the right lung lobes and sucked up. This was repeated twice. BALF cells were pelleted by centrifugation, resuspended in PBS, counted using a Coulter Counter Z2 (Beckman Coulter B.V), and cytospin preparations were made using a cytospin centrifuge (Shandon) and cells were visually differentiated after staining according to May-Grunewald and Giemsa. The left lung lobes were collected in 1 ml Verwey medium (Tritium) and homogenised using a tissue homogeniser (Pro-200, ProScientific, Monroe, CT, USA) at maximum speed for 10 s. The homogenates were diluted 10- or 100-fold for the immunised mice or 1000-fold for the PBS/$AlPO_4$ treated mice, and 100-μl aliquots of the dilutions were plated on BG plates (Tritium) supplemented with streptomycin and incubated at 35°C for 5 days. Results are reported as log protection (LOG_{Prot}) values, which allows for comparison of vaccine efficacies. The LOG_{Prot} was calculated using the following equation: LOG_{Prot} = log10(mean CFU of PBS/$AlPO_4$ treated mice) – log10(CFU of each individual immunised mouse). Blood was collected 4 h post immunisation at days 0 and 14 for cytokine analysis and at day 28 for the assessment of antibody titers.

Antibody titer determination

Total IgG, IgG1, and IgG2a antibody titers against whole cells of *B. pertussis* strain B213 were determined in an ELISA as previously described (Abdillahi and Poolman, 1987). In short, flat-bottom 96-well microtiter plates were coated overnight at room temperature with 100-μl sample of a whole-cell suspension (A_{590} = 0.05 in PBS). Antibody titers were measured for individual sera. A four-parameter curve fit was made for optical density values of 3-fold serial dilutions, and the antibody titers were calculated in reciprocal dilutions that gave 50% of the maximum absorbance.

Cytokine determination

IL-6 and TNFα concentrations in the sera were quantified in an ELISA for mouse IL-6 or TNFα according to the manufacturers' instructions (eBioscience). For determination of cytokine concentrations in the supernatant of lung homogenates, an 11-plex panel containing beads for mouse IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, TNFα, and GM-CSF (Bio-Rad, Hercules, CA) was used. Ninety-six-wells filter bottom plates (Bio-Rad) were pre-wet with 100 μl of Bio-Plex assay buffer (Bio-Rad). After each step, buffer was removed by vacuum. Beads were diluted in assay

buffer, and 50 μl of the solution was added per well. The plates were washed twice with 100 μl of Bio-Plex wash buffer (Bio-Rad). The cytokine standards were diluted in Verwey medium to a range of 32,000 to 0.18 pg/ml. Of standards and samples, 50 μl/well were added. The plates were incubated for 30 min: each incubation step consisted of vortexing the plates at 1100 rpm for 30 s, followed by incubation with shaking at 300 rpm. The plates were washed 3 times with 100 μl of wash buffer. Detection antibody was diluted in detection antibody diluent (Bio-Rad), and 25 μl of the solution was added per well. The plates were incubated for 30 min, and washed 3 times with 100 μl of assay buffer. Streptavidin-phycoerythrin was diluted in assay buffer, and 50 μl/well was added. The plates were incubated for 10 min, and washed 3 times with 100 μl of wash buffer. The beads were resuspended in 125 μl of assay buffer and analysed on a Bio-Plex (Bio-Rad). The results shown were obtained at low photo multiplier tube settings. All steps were performed at room temperature.

Statistical analysis

Data were statistically analysed using one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test (Graphpad). Alternatively, a Student's *t* test (two-tailed, two-sample unequal variance) was used. Differences were considered to be significant when $p < 0.05$.

Results

N. meningitidis LpxL2 LPS functions as an LPS antagonist *in vitro*

To test whether tetra-acylated *N. meningitidis* LpxL2 LPS functions as an antagonist for stimulation of macrophages, we stimulated the human macrophage cell line MM6 with serial dilutions of purified *B. pertussis* LPS (Fig. 1A) or a *B. pertussis* whole-cell suspension (Fig. 1B) in the absence or presence of 1 μg/ml of LpxL2 LPS. In the absence of LpxL2 LPS, *B. pertussis* LPS and whole-cell suspensions induced high levels of IL-6 in the culture supernatant. However, in the presence of LpxL2 LPS, IL-6 production was considerably lower (Fig. 1), indicating that LpxL2 LPS indeed functions as an antagonist for the stimulation of macrophages in these assays.

Protection against *B. pertussis* challenge

To evaluate whether supplementation of a wP vaccine with MPL or *N. meningitidis* LpxL2 LPS influences its efficacy, 4/5-wk-old BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a human dose of a standard wP vaccine

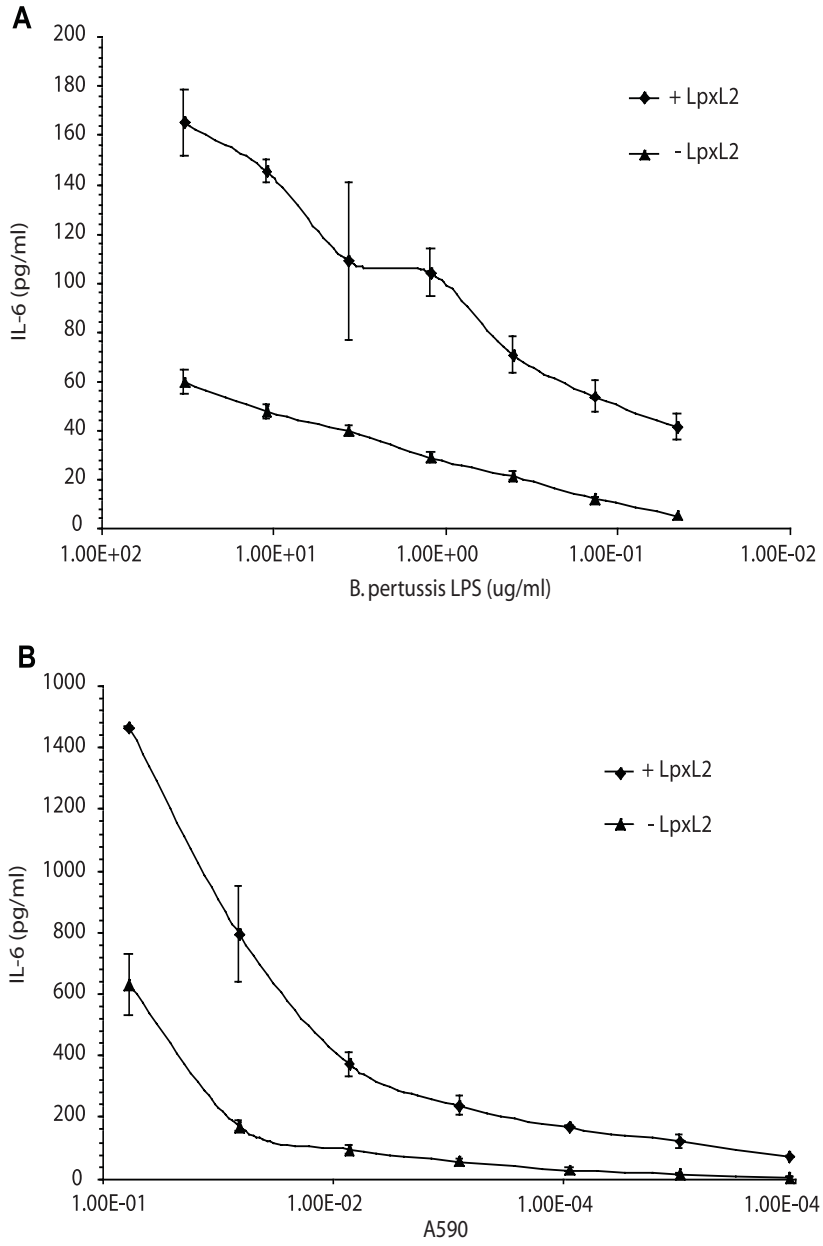

Fig. 1. IL-6 induction by purified *B. pertussis* LPS and whole cells in the absence or presence of *N. meningitidis* LpxL2 LPS. The production of IL-6 by the human macrophage cell line MM6 was stimulated with serial dilutions of (A) a stock solution of purified LPS from *B. pertussis*, (B) a whole-cell suspension of *B. pertussis*, both in the absence (-LpxL2) or presence (+LpxL2) of 1 μg/ml LpxL2 LPS. The IL-6 concentration in the culture supernatant was quantified using an ELISA for human IL-6. The data represent the averages of three individual experiments.

either supplemented or not with one of the LPS-analogs and subsequently challenged with *B. pertussis*. All three wP vaccines conferred significant protection against colonisation by the *B. pertussis* strain (Fig. 2). However, the MPL- and LpxL2 LPS-supplemented wP vaccines provided better protection (LOG_{Prot} ~2.5) than did the standard vaccine (LOG_{Prot}~1.5), indicating that both supplements increased the efficacy of the wP vaccine.

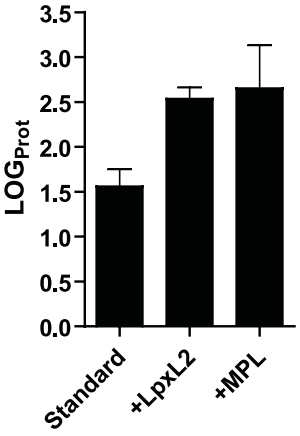

Fig. 2. Efficacy of whole cell pertussis vaccines in a mouse model. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL and challenged with *B. pertussis* at day 28. At day 5 after challenge, lungs were removed aseptically and CFU numbers were determined. Results are expressed as mean log protection values (± SEM) from nine mice per group and are representative of three separate experiments.

B. pertussis-specific antibody responses

B. pertussis-specific IgG serum antibody titers at day 28 after immunisation were determined in a whole-cell ELISA. *B. pertussis*-specific total serum IgG titers were similar between the different groups (Fig. 3A). Also the analysis of the IgG1/IgG2a subclass distribution did not reveal differences between the groups (Fig. 3B). Thus, supplementation of the wP vaccine with MPL or LpxL2 LPS did not alter the level of anti-*B. pertussis* serum IgG antibodies nor the IgG1/IgG2a subclass distribution.

Lung cytokine concentrations and cell-type distribution in BALF

Cytokine concentrations in the lung homogenate supernatant of mice sacrificed 5 days post-challenge were assessed in an 11-plex Bio-plex assay. Compared to the PBS/$AlPO_4$-treated control group, all groups of wP-immunised mice displayed increased levels of IFN-γ (Fig. 4A). This difference was significant for the group treated with the LpxL2-supplemented vaccine, but not for the groups treated with the standard or MPL-supplemented vaccine. Analysis of the other cytokines tested, i.e., IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, TNFα, and GM-CSF did not reveal further differences.

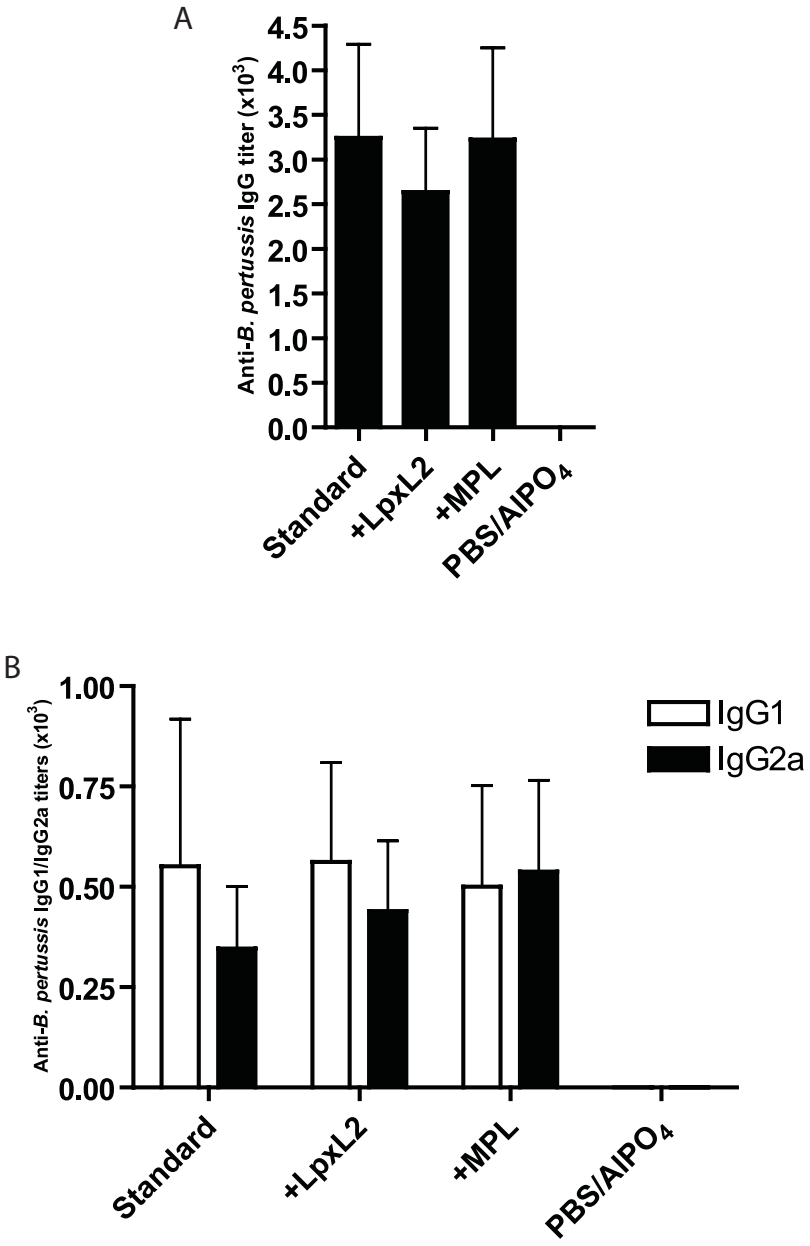

Fig. 3. *B. pertussis*-specific antibody responses. Anti-*B. pertussis* IgG (A) and IgG1/IgG2a (B) titers at day 28 post primary immunisation were measured in mice immunised with a standard wP vaccine, or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL using a whole-cell ELISA. Results are expressed as mean antibody titers (± SEM) from nine mice per group and are representative of three separate experiments.

To obtain more insight into possible variations in immune effector mechanisms within the lungs, we also analysed the cell-type distribution in BALF samples. As shown in Fig. 4B, the wP-immunised mice showed, as compared to the PBS/$AlPO_4$-treated control group, an increased neutrophil fraction, whereas the abundance of other cell types, such as macrophages, eosinophils, lymphocytes, and monocytes was similar (data not shown). This difference was significant for the group treated with the LpxL2-supplemented vaccine, but not for the other wP immunised groups. Overall, these data indicate that at day five after challenge the differences between the control group and the wP-immunised groups in the lungs are limited, with only higher IFN-γ and neutrophil levels found. Furthermore, these data show that the supplementation of the wP vaccine with LPS analogs did not markedly affect the lung cytokine concentrations or cell-type distribution.

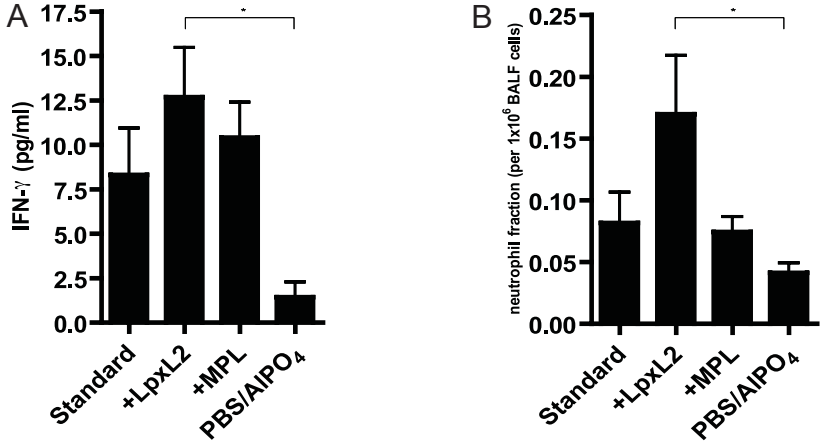

Fig. 4. Lung IFN-γ concentration and neutrophil fractions at day 5 post challenge. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL, or with PBS/$AlPO_4$ as a control and challenged with *B. pertussis*. At day 5 after challenge, IFN-γ concentration (A) and neutrophil fraction (B) in the lungs were determined. Results are expressed as mean cytokine concentrations and the fraction of neutrophils per $1x10^6$ BALF cells (± SEM), respectively, from six mice per group and are representative of two separate experiments. A single asterisk marks significant ($p<0.05$) differences.

Serum pro-inflammatory cytokine concentrations

Previously, it has been shown that an increased pro-inflammatory cytokine production is an important determinant for the reactogenicity of wP vaccines (Armstrong *et al.*, 2003; Loscher *et al.*, 1998). Hence, to determine whether the *in vivo* reactogenicity of the wP vaccines can be reduced by the addition of LPS analogs, we analysed the concentration of two pro-inflammatory cytokines, i.e., TNFα and IL-6, in serum samples

taken 4 h after immunisation. As shown in Fig. 5A, serum TNFα levels were similar in all groups. In contrast, compared to treatment with PBS/$AlPO_4$, wP immunisation elicited high serum IL-6 levels (Fig. 4B). Interestingly, the IL-6 levels in the group immunised with the LpxL2 LPS-supplemented vaccine were significantly lower than in the groups immunised with the standard or the MPL-supplemented vaccine. These results indicate that supplementation of the vaccine with LpxL2 LPS significantly decreased its reactogenicity, which shows that LpxL2 LPS works also *in vivo* as an LPS antagonist.

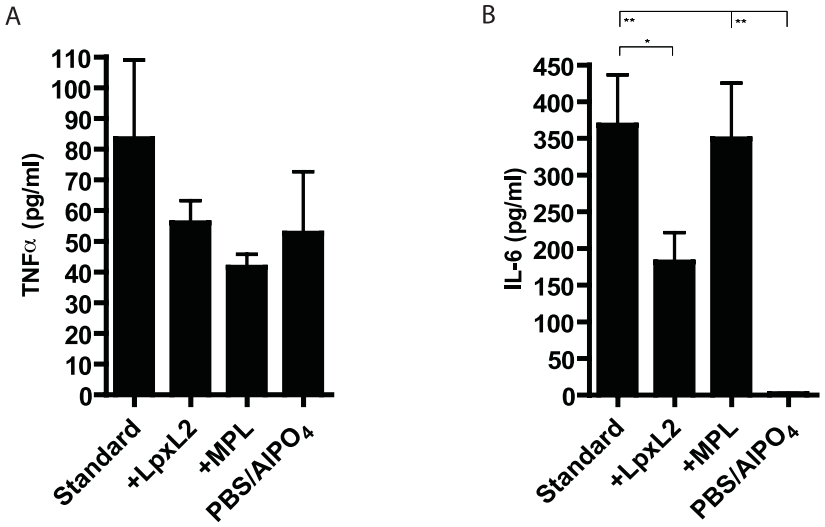

Fig. 5. Serum pro-inflammatory cytokine concentrations 4 h post immunisation. Mice were immunised with a standard wP vaccine or with wP vaccines supplemented with *N. meningitidis* LpxL2 LPS or MPL, or with PBS/$AlPO_4$ as a control. Serum TNFα (A) and IL-6 (B) concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from nine mice per group and are representative of three separate experiments. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Discussion

This study was aimed at determining whether the addition of non-toxic LPS derivatives may be a useful strategy to improve the efficacy and reduce the reactogenicity of wP vaccines. One of the LPS analogs studied, MPL, has been developed as an adjuvant for human vaccines. As is clear from data presented here, addition of MPL indeed increased the efficacy of a wP vaccine. Moreover, we showed that MPL did not lead to major differences in immunological responses, neither with respect to the antibody response nor to the cellular responses in the lung. Furthermore, addition of MPL did not increase vaccine reactogenicity, as assessed by measuring the levels of pro-inflammatory cytokines in serum after immunisation. Therefore, addition of MPL,

or compounds with similar properties, to wP vaccines seems to be a useful strategy by which the potency of wP vaccines can be improved. Such increased potency will possibly allow for the use of lower vaccine dosages and thereby reduce reactogenicity.

It has been shown previously that *P. gingivalis* LPS can act *in vivo* as an LPS antagonist in mice (Qureshi *et al.*, 1991). Such LPS-types are non-toxic and can antagonise the LPS-induced production of pro-inflammatory cytokines. We reasoned that addition of such a compound to a wP vaccine may reduce its *in vivo* reactogenicity. Here, we showed first that the tetra-acylated LpxL2 LPS of *N. meningitidis*, which was developed by making alterations in the lipid A biosynthetic route, also exhibits antagonistic properties. In the presence of LpxL2 LPS, the macrophages produced less IL-6 when stimulated with either a *B. pertussis* cell suspension or purified *B. pertussis* LPS. Interestingly, LpxL2 LPS did not completely inhibit stimulation of IL-6, suggesting that besides LPS, also other bacterial components induce the production of pro-inflammatory cytokines. This result is consistent with a previous study in which it was shown that LPS-deficient *N. meningitidis* cells still induce a substantial production of pro-inflammatory cytokines (Sprong *et al.*, 2001).

The addition of LpxL2 LPS to the wP vaccine resulted in a higher efficacy. This result was unexpected, since it has previously been shown that LpxL2 LPS has poor adjuvant properties (van der Ley *et al.*, 2001). Possibly, the different approaches by which this activity was measured form the reason for this discrepancy: whereas in the previous study adjuvant activity was correlated with the level of serum (bactericidal) antibody titers, in our study, adjuvant activity was assessed by determining lung CFU counts. Indeed, when we compared the antibody titers at day 28 after primary immunisation, we found that the LpxL2 LPS supplement, did neither induce higher *B. pertussis*-specific antibody titers in the serum, nor changes in the IgG subclass distribution. Apparently, the increased vaccine efficacy of the supplemented vaccines was not dependent on the induction of higher antibody titers, although it cannot be excluded that alterations in antibody levels against specific antigens play a role. This conclusion is consistent with a previous study in which we explored the effects of LPS modifications on the efficacy and reactogenicity of wP vaccines. Also in that study, a higher efficacy did not significantly correlate with higher antibody titers or changes in IgG subclass distributions (**chapter 5**). Furthermore, it is well-known that besides the antibody-mediated response, cellular immune responses play an important role in the protection against *B. pertussis* infection (Mills, 2001).

To gain more insight in the factors that may be responsible for the increased efficacy of the vaccines supplemented with LPS analogs, we analysed the levels of eleven

different cytokines in the lungs at day 5 after challenge. In most cases, no differences between the various wP-immunised and PBS/$AlPO_4$-treated control group were found. Only the levels of IFN-γ were 5- to 10-fold higher in the lungs of the three wP-immunised groups. The importance of IFN-γ for the protection against *Bordetella* infection has been shown in many experiments (Mills *et al.*, 1998; Mahon *et al.*, 1997; Barbic *et al.*, 1997; Pilione and Harvill, 2006; **chapter 5**. IFN-γ has been shown to induce the transcription of several activation markers, including CD14, proinflammatory cytokines, various cell surface receptors, chemokine receptors, and co-stimulatory receptors involved in antigen presentation, i.e., MHC-II (Dalton *et al.*, 1993; Ellis and Beaman, 2004; Janeway, 2001). Furthermore, IFN-γ has been shown to activate and recruit neutrophils (Ellis and Beaman, 2004; Burch *et al.*, 2006). Consistently, we observed that the wP-immunised mice exhibited increased lung neutrophil-counts. Neutrophils have been shown to play an important role in *Bordetella* clearance (Kirimanjeswara *et al.*, 2003; Kirimanjeswara *et al.*, 2005; Pishko *et al.*, 2004) Overall, our data suggest a central role for both IFN-γ and neutrophils in the clearance of *B. pertussis* from the lungs.

Analysis of pro-inflammatory cytokine levels in serum samples taken 4 h after primary immunisation showed that supplementation of LpxL2 LPS significantly reduced the induction of serum IL-6 levels, and, thus, probably decreased vaccine reactogenicity. Together with increased vaccine efficacy, this result suggests that addition of LpxL2 LPS may be a useful way to develop safer and improved wP vaccines.

Overall, this study shows that addition of LPS analogs may be a useful strategy to develop improved and safer wP vaccines. Moreover, such a strategy may also be applicable to other vaccines, including aP vaccines. In contrast to wP vaccines and natural *B. pertussis* infection, which evoke a Th1 response, these latter vaccines have been shown to skew towards a Th2 immune response (Ausiello *et al.*, 1997; Mills, 2001). Since LPS is potent Th1-skewing compound (Levy, 2005), addition of non-toxic LPS analogs may be a useful strategy to re-direct aP-mediated immune responses towards Th1 without increasing vaccine reactogenicity.

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