Chapter 7

Lipopolysaccharide analogs improve efficacy of acellular pertussis vaccines and reduce type-I hypersensitivity

Jeroen Geurtsen, Sander A. Banus, Eric R. Gremmer, Henke Ferguson, Jolanda P. Vermeulen, Jan A. M. A. Dormans, Jan Tommassen, Peter van der Ley, Frits R. Mooi and Rob J. Vandebriel

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Abstract

Pertussis is an infectious disease caused by the Gram-negative bacterium *Bordetella pertussis*. Although mass vaccination has drastically reduced pertussis incidence, the disease has remained often the least well-controlled disease in childhood vaccination programs. Therefore, improved pertussis vaccines need to be developed. In this study, we compared three different adjuvants with respect to their potential to improve the efficacy and the immunogenicity of a combined Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccine. The adjuvants tested were aluminum, which is used in current aP vaccines, and two lipopolysaccharide (LPS) analogs, i.e., monophosphoryl lipid A and *Neisseria meningitidis* LpxL2 LPS. Mice were immunised, challenged intranasally with *B. pertussis*, and vaccine efficacy, antibody responses, histological alterations, and cytokine expression profiles were evaluated. The results showed that the LPS analogs were superior to aluminium when vaccine efficacy was evaluated. Interestingly, parameters for type-I hypersensitivity and T-helper responses indicated that the LPS analogs re-directed the immune response towards a Th1-type response and decreased type-I hypersensitivity. Combined with the observation that the LPS analogs, as compared to aluminum, only slightly increased vaccine reactogenicity, these results suggests that the use of LPS analogs as adjuvant forms a promising strategy for improving aP vaccines.

Introduction

Pertussis is a highly contagious disease of the respiratory tract that is caused by the Gram-negative bacterium *Bordetella pertussis*. Introduction of whole-cell pertussis (wP) vaccines in the 1940s and 1950s led to a rapid decline in pertussis incidence and reduced morbidity and mortality of the disease to low levels. However, soon after their introduction, it became clear that wP vaccines exhibited a relatively high reactogenicity, which was caused by the presence of strong immunogens, such as lipopolysaccharide (LPS) and pertussis toxin (PT). Therefore, less reactogenic acellular pertussis (aP) vaccines, consisting of purified and detoxified antigens, were developed and introduced in the 1980s and 1990s. Although pertussis mass vaccination has drastically reduced pertussis incidence, the disease remains often the least well-controlled disease in childhood vaccination programs, emphasising the importance of improving pertussis vaccines.

LPS or endotoxin is one of the major constituents of the Gram-negative bacterial outer membrane, where it is important for maintaining the membrane barrier function. LPS is an amphiphatic molecule that consists of three domains, i.e., the O-antigen, the core, and lipid A (Raetz and Whitfield, 2002). Besides endotoxic activity, LPS also has a powerful immune-stimulating potency. By engaging Toll-like receptor 4 (TLR4), LPS induces Th1 adaptive immunity (Dabbagh and Lewis, 2003; Dillon *et al.*, 2004; Kapsenberg, 2003; Medzhitov, 2001; Saito *et al.*, 2003). Since aP vaccines are devoid of LPS, concerns regarding their immunogenicity have been raised. In fact, this apprehension has been substantiated by an increase in invasive *Haemophilus influenzae* type B disease incidence in the UK that coincided with the distribution of combination vaccines that contain aP instead of wP (McVernon *et al.*, 2003). Furthermore, aP vaccination has been associated with a strong increase in parameters of type-I hypersensitivity, being total serum IgE, lung eosinophilia, and Th2 cytokine production by cells obtained from the lung-draining lymph nodes (LN) (Vandebriel *et al.*, 2007).

The non-toxic LPS derivative monophosphoryl lipid A (MPL) engages TLR4 (Evans *et al.*, 2003; Persing *et al.*, 2002), inducing Th1 adaptive immunity and deviating Th2-directed to Th1-directed responses (Baldridge *et al.*, 2000; Puggioni *et al.*, 2005; Reed *et al.*, 2003; Zhang *et al.*, 2005). MPL combined with aluminum (denoted AS04) is registered for clinical use as adjuvant in viral vaccines (hepatitis B virus (Boland *et al.*, 2004) and human papillomavirus (Giannini *et al.*, 2006)), while MPL combined with L-tyrosine is registered for clinical use as adjuvant in allergy therapy (Baldrick *et al.*, 2004; McCormack and Wagstaff, 2006). In mice, genetic mapping and functional studies have implicated a critical role for TLR4 in pertussis clearance and ensuing adaptive immunity

(Banus *et al.*, 2006). So, at least in mice, MPL engages a receptor critical for clearance of *B. pertussis* and immunity against this pathogen.

Besides naturally existing LPS forms, the possibilities of actively altering the LPS biosynthesis route has been explored in order to create novel LPS species with potentially useful properties. Recently, it was shown that a *Neisseria meningitidis* strain deficient for the late acyltransferase LpxL2 displays a dramatically decreased endotoxic activity when tested for its capability to stimulate human macrophages (van der Ley *et al.*, 2001). In addition, analysis of the adjuvant activity of the LPS from this strain showed that the mutant LPS exhibited only poor immune-stimulating activity (van der Ley *et al.*, 2001).

Here, we compared three different adjuvants with respect to their potential to improve the efficacy and the immunogenicity of a combined Diphtheria-Tetanus-acellular Pertussis (DTaP) vaccine. We first immunised mice with DTaP vaccines, adjuvated either with aluminum, MPL, or *N. meningitidis* LpxL2 LPS, and then, after challenge, analysed vaccine efficacy, antibody responses, histological alterations, and cytokine secretion profiles.

Materials and Methods

Bacterial strains and growth conditions

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

Vaccines

The acellular vaccine was a combined diphtheria, tetanus, acellular pertussis vaccine composed of > 30 International Units (IU) diphtheria toxoid, > 40 IU tetanus toxoid, 25 μg formaldehyde- and glutaraldehyde-detoxified pertussis toxin, 25 μg filamentous hemagglutinin, and 8 μg pertactin in 0.5 ml saline (Infanrix, GlaxoSmithKline). The vaccine contained aluminum hydroxide as an adjuvant and is equivalent to 1 human dose (HD) per 0.5 ml.

The whole-cell pertussis (wP) vaccine was prepared from *B. pertussis* strain B213 as described (chapter 5). In short, the bacteria were grown in synthetic THIJS medium (Thalen *et al*., 1999) for 68 h at 35°C while shaking (175 rpm). 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 phosphate-buffered saline (PBS) to an A_{590} of 2.5, i.e., 50 international opacity units per ml (~1.6 HD/ml). The suspensions were stored at 4°C.

Prior to immunisation, the DTaP and wP vaccines were further diluted in PBS to final concentrations of 1/5 or 1/10 of a HD per 0.5 ml (depending on the experiment), after which either 3 mg/ml aluminum phosphate (Brenntag) or 3 mg/ml aluminum hydroxide (Serva), 40 μg/ml MPL (Sigma-Aldrich), or 40 μg/ml *N. meningitidis* LpxL2 LPS (van der Ley *et al.*, 2001) was added as an adjuvant.

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 4- to 8-weeks old specific pathogen-free female BALB/cOlaHsd mice (Harlan, Horst, The Netherlands) were immunised subcutaneously with 0.5 ml vaccine (prepared as described above), or with 0.5 ml PBS/$AlPO_4$, PBS/$Al(OH)_3$, PBS/MPL, or PBS alone as controls, 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 medium as described (Willems *et al.*, 1998).

Autopsy and collection of bronchoalveolar lavage fluid cells

Mice were sacrificed at day 3, 5, or 7 after infection. Animals were anesthetised with ketamine, rompun, and atropine, and blood was collected from the orbital plexus. Perfusion was performed with 2 ml of PBS supplemented with 3.5% heat-inactivated Fetal Calf Serum (FCS; PAA, Linz, Austria) in the right heart ventricle. The lungs and spleen were excised, and used either to obtain bronchial LN, splenocytes, lung lobes for CFU determination, lung lobes for histological examinations, or bronchoalveolar lavage fluid (BALF) cells. BALF cells were obtained by placing a cannula intratracheally and fixing it using a suture. The lungs were placed in a 50-ml tube and one ml PBS was brought into the lungs 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-Grunwald and Giemsa.

CFU determination

Left lung lobes were collected in 1 ml of 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- and 100-fold for the

immunised mice and 1000-fold for the control mice, and 100-μl aliquots of the dilutions were plated on BG plates supplemented with 30 μg/ml 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} = 10log(mean CFU of PBS/$AlPO_4$-treated mice) - 10log(CFU of each individual immunised mouse).

Histological examination

Left lung lobes were fixed intratracheally with 4% formalin for 24 h. After overnight dehydration, they were embedded in paraffin. Five-μm sections were cut and stained with haematoxylin/eosin. Histological lesions were semi-quantitatively scored as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. This score incorporates the frequency as well as the severity of the lesions.

Pertussis Toxin-specific IgG

Total serum IgG antibody titers against PT were determined in an enzyme-linked immunosorbent assay (ELISA) as described (de Melker *et al.*, 2000). In short, flat-bottom 96-well microtiter plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated overnight at room temperature with 10 μg/ml PT (RIVM) in PBS. Antibody titers were measured for individual sera using horseradish peroxidase (HRP)-labelled goat anti-mouse secondary antibodies (SouthernBiotech). 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.

Total serum IgE

Blood was allowed to clot at 4°C overnight and centrifuged for 2 min at 16,100 x *g*. Total serum IgE was measured as previously described (Brewer *et al.*, 1996). Briefly, 96-well plates (Nunc-Immuno Plate) were coated with 2 μg/ml anti-mouse IgE (mAb R35-72; Pharmingen) in coating buffer (0.04 M carbonate buffer, pH 9.6). After overnight incubation at 4°C, the plates were washed (3 times with 0.05% Tween-20 in PBS), incubated in blocking buffer (10% FCS in PBS) for 1 h at 37°C, and washed. Normal mouse serum (Dako) was used as a standard. Standard and serial dilutions of sera were added. The plates were incubated for 2 h at 37°C and washed. Biotinylated anti-mouse IgE (0.1 μg/ml in blocking buffer; monoclonal antibody 23G3; Southern Biotechnology Associates) was added and incubated for 1 h at 37°C. The plates were washed and incubated for 20 min at room temperature with streptavidine-HRP (200 times diluted; R&D). To detect HRP, the

plates were washed and incubated for 3 min at room temperature in 10% sodium acetate, 1.66% tetramethylbenzidine, and 0.02% hydrogen peroxidase. Stop solution (R&D) was added and the plates were read at 450 nm.

Cell culture

The culture medium used was RPMI-1640 (Gibco) supplemented with 10% FCS, 100 μg/ml streptomycin, and 100 IU/ml penicillin. Cell suspensions were made by pressing the LN or spleens through a cell strainer (Falcon). Cells were counted using a Coulter Counter (Beckman Coulter). LN cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A (MP Biomedicals) in flat-bottom 12-well culture plates (Costar) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Spleen cell suspensions were cultured at 10^6 cells per ml culture medium with 5 μg/ml Concanavalin A or *B. pertussis* ($1.0x10^5$ heat-inactivated bacteria per well) in 96-well tissue culture plates (Nunc) at 37°C in a humidified atmosphere containing 5% CO_2 for 72 h. Bacteria were heat-inactivated at 56°C during 30 min.

Cytokine determination

Interleukin-6 (IL-6) concentrations in the sera were quantified with an ELISA against mouse IL-6 according to the manufacturer's instructions (eBioscience). To determine 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-□, TNFα, and GM-CSF (Bio-Rad) was used. 96-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/well was added. 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.

Statistics

One-way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test was performed (GraphPad). Alternatively, ANOVA followed by the T-Test was used (GraphPad). Histological data were analysed using the non-parametric Wilcoxon test (SPSS).

Results

Protection against *B. pertussis* challenge after immunisation with acellular pertussis vaccines

To evaluate the protection offered by the DTaP vaccines, groups of 9 BALB/c mice were subcutaneously immunised at day 0 and day 14 with 1/10 of a HD of the vaccines and subsequently challenged with *B. pertussis* strain B213. At day 5 after challenge, lung CFU numbers were determined. All three vaccines conferred significant protection against colonisation by the bacteria. When mutually compared, immunisation with the MPL-adjuvated vaccine (LOG_{Prot}~4.89 ±SD 1.28) and the LpxL2-adjuvated vaccine (LOG_{Prot}~4.78 ±SD 1.36) provided a significantly better protection, with *p* values of 0.014 and 0.028, respectively, than did immunisation with the $AlPO_4$-adjuvated vaccine (LOG_{Prot}~3.5 ±SD 0.63), indicating that the supplementation with LPS analogs improved vaccine efficacy.

PT-specific antibody responses

As PT-specific IgG antibody titers have been shown to correlate with protection (Cherry *et al.*, 1998), anti-PT IgG levels at days 3 and 7 after challenge were determined in groups immunised with 1/5 HD of the $Al(OH)_3$-adjuvated vaccine or the MPL-adjuvated vaccine using PT-specific ELISAs. High PT-specific serum IgG titers were detected in both groups (Fig. 1A). However, the titers were significantly higher in the mice immunised with the MPL-adjuvated vaccine both at day 3 (5.4-fold) and at day 7 (2.6-fold).

Total serum IgE levels

Traditionally, an increased serum IgE level forms one of the hallmarks of type-I hypersensitivity. Total serum IgE antibody titers were determined at day 3 after challenge in the groups immunised with 1/5 HD of the $Al(OH)_3$- or the MPL-adjuvated vaccine, or with the adjuvants alone as controls. As compared to aluminum or MPL alone, DTaP vaccination elicited high serum IgE titers (Fig. 1B). However, when mutually compared, no significant differences between the two DTaP-vaccinated groups were found.

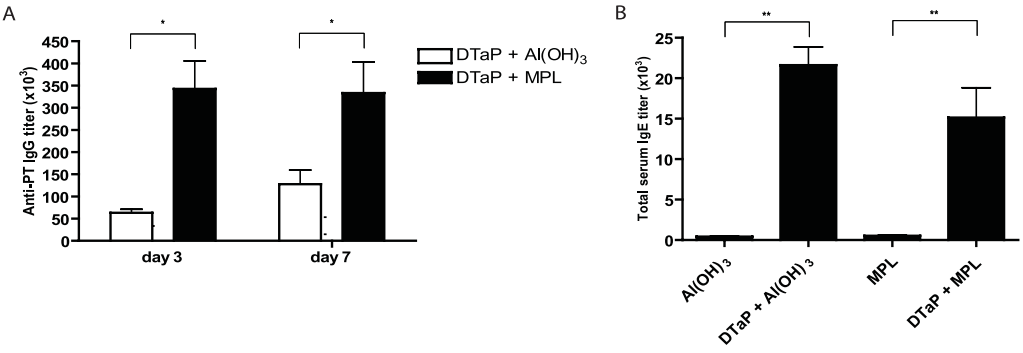

Fig. 1. Antibody responses. Anti-PT IgG (A) and total serum IgE (B) titers were measured in mice immunised with the $Al(OH)_3$- or MPL-adjuvated DTaP vaccines, or with $Al(OH)_3$ or MPL alone as controls using ELISA. Results are expressed as mean antibody titers (± SEM) from six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Evaluation of histological changes

A second parameter for type-I hypersensitivity is lung eosinophilia. Hence, lung eosinophilia was quantified by histological examination of the left lung lobes at day 3 after challenge in the groups immunised with 1/5 HD of the $Al(OH)_3$- or the MPL-adjuvated vaccine, or with the adjuvants alone as controls. The lungs of the mice immunised with the MPL-adjuvated vaccine showed significantly reduced eosinophilia, as compared to all other groups (Fig. 2). Further histological examination showed that immunisation with the $Al(OH)_3$- ($p=0.009$) or the MPL-adjuvated ($p=0.016$) vaccine induced significant perivasculitis. Immunisation with both DTaP vaccines also resulted in minor increases in peribronchiolitis, hypertrophy of the bronchiolar mucus cells, and alveolitis (data not shown).

Bronchoalveolar lavage fluid cells

To determine whether the various adjuvants affected cell-type distribution in BALF samples, BALF cells collected from mice immunised with 1/10 HD were gathered and visually differentiated at day 5 after challenge. The percentages and numbers of macrophages, neutrophils, and lymphocytes were similar in all groups, and also the total number of BALF cells was not differentially affected (data not shown). However, the group immunised with the $AlPO_4$-adjuvated vaccine showed a significantly higher eosinophil fraction than the groups immunised with the MPL-adjuvated vaccine, the LpxL2-adjuvated vaccine, or PBS alone (Fig. 3), again indicating that the vaccines adjuvated with the LPS analogs evoked lower type-I hypersensitivity than did the aluminum-adjuvated vaccine.

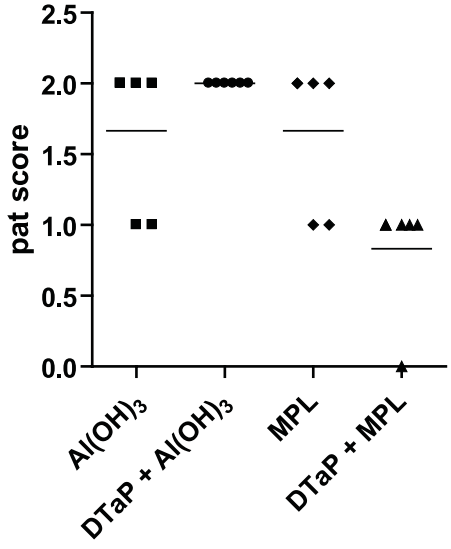

Fig. 2. Lung eosinophilia. Histological lesions in lungs from mice immunised with 1/5 HD of the $Al(OH)_3$- or MPL-adjuvated DTaP vaccine, or with adjuvants alone as controls were semi-quantitatively scored (pat-score) as absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5), respectively. Each symbol represents an individual mouse; horizontal lines represent the group average.

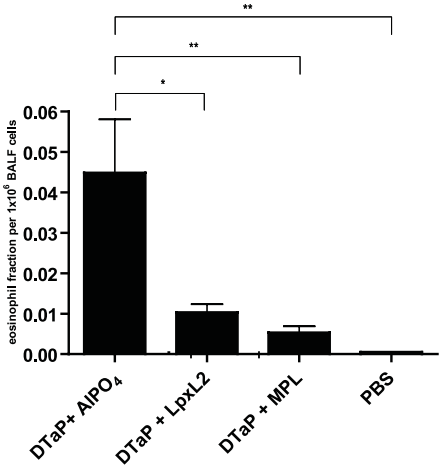

Fig. 3. BALF eosinophil numbers. Mice were immunised with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. At day 5 after challenge, the eosinophil fraction in the lungs was determined. Results are expressed as the mean fraction of eosinophils per $1x10^6$ BALF cells (± SEM). The data represent the averages of six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Cytokine production by splenocytes and bronchial LN cells

To evaluate whether the vaccines elicited a Th1- or Th2-type of response, splenocytes and bronchial LN cells from the groups immunised with 1/10 HD of the DTaP vaccines or with PBS as a control were isolated at day 5 after challenge and stimulated with heat-inactivated *B. pertussis* cells and Concanavalin A, respectively, after which their cytokine secretion profiles were analysed. Mutual vaccine comparison did not reveal major differences for most of the cytokines analysed, i.e., IL-1α, IL-1β, IL-2, IL-6, IL-10, IL-13, TNFα, IFN-γ, and GM-CSF. Yet, bronchial LN cells from the group immunised with the $AlPO_4$-adjuvated vaccine secreted higher amounts of IL-4 and IL-5 (both indicative of a Th2-type response) than did the bronchial LN cells from the groups immunised with the MPL- or LpxL2-adjuvated vaccines, or with PBS alone (Fig. 4A). For IL-4, these differences were not significant, but for IL-5 they were. Also the splenocytes from the group immunised with the $AlPO_4$-adjuvated vaccine secreted higher amounts of both IL-4 and IL-5 than did the splenocytes from mice of any of the other groups (Fig. 4B). However, most of the differences were not significant (Fig. 4B).

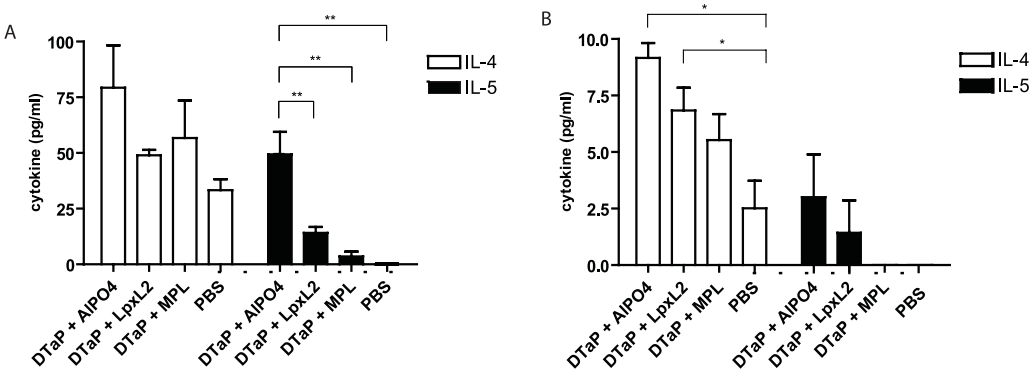

Fig. 4 Cytokine expression profiles. Mice were immunised with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. Bronchial LN cells (A) and splenocytes (B) were isolated at day 5 after challenge and stimulated with concanavalin A and heat-inactivated *B. pertussis*, respectively. IL-4 and IL-5 concentrations in the cell-culture supernatants were measured. Results are expressed as mean cytokine concentrations (± SEM) from six mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Serum pro-inflammatory cytokines

To address the *in vivo* reactogenicity of the DTaP vaccines, we analysed the concentration of the pro-inflammatory cytokine IL-6 in serum samples taken 4 h after the primary or booster immunisation (Fig. 5). As controls, serum IL-6 levels were also

measured in mice immunised with a wP vaccine or with PBS alone. Consistent with the relatively high reactogenicity of wP vaccines, immunisation with the wP vaccine elicited significantly higher serum IL-6 levels than did immunisation with the DTaP vaccines or with PBS alone. When compared to the PBS control group, primary immunisation with the $AlPO_4$-adjuvated DTaP vaccine evoked similarly low serum IL-6 levels. Yet, when this vaccine was used for booster immunisation, significantly higher IL-6 levels were found. As compared with the $AlPO_4$-adjuvated vaccine, the MPL- and LpxL2 LPS-adjuvated vaccines displayed higher reactogenicity at both the primary and the booster vaccination, as indicated by the significantly higher serum IL-6 levels in these groups (Fig. 5). Nonetheless, these IL-6 levels were clearly lower than those measured in the group immunised with the wP vaccine.

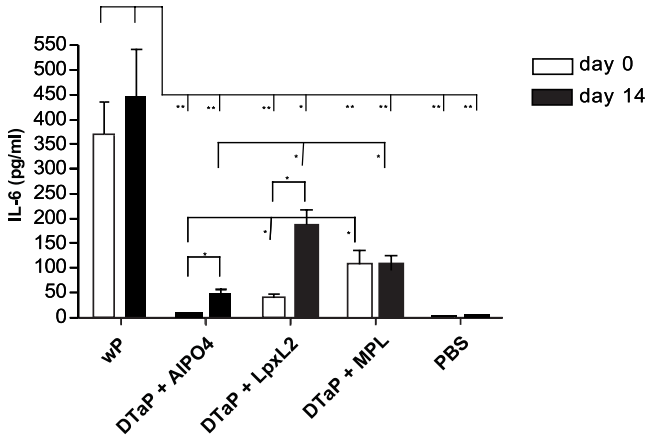

Fig. 5 Serum pro-inflammatory cytokines. Mice were immunised (at day 0 and day 14) with 1/10 HD of the DTaP vaccine adjuvated with either $AlPO_4$, LpxL2 LPS, or MPL, or with PBS alone as a control. Serum IL-6 concentrations were determined 4 h post immunisation. Results are expressed as mean cytokine concentrations (± SEM) from seven mice per group. Single and double asterisks mark significant ($p<0.05$) and highly significant ($p<0.001$) differences, respectively.

Discussion

This study was aimed at comparing the efficacy, immunogenicity, and reactogenicity of aluminum-, MPL-, or LpxL2 LPS-adjuvated DTaP vaccines. We showed that all three formulations tested conferred significant protection against a challenge with *B. pertussis*. Importantly, we found that the DTaP vaccines supplemented with the LPS-analogs provided a significantly better protection than did the vaccine adjuvated with aluminum. For LpxL2 LPS, this result was unexpected since it was previously shown that it has only poor immune-stimulating activity (van der Ley *et al.*, 2001). This discrepancy may be explained by the notion that van der Ley *et al.* (2001) measured adjuvant activity by analysing serum bactericidal antibody titers, whereas in our study, the exhibited adjuvant activity was based upon vaccine efficacy.

Cellular and humoral immunity have complementary roles in protective mechanisms against *B. pertussis* (Mills, 2001). To gain more insight into the consequences of the use of different adjuvants on immune effector mechanisms, we first analysed the PT-specific serum IgG levels. The results revealed that immunisation with the MPL-supplemented vaccine elicited significantly higher anti-PT antibody titers than did immunisation with the aluminum-adjuvated vaccine. This observation suggests that an increased vaccine efficacy may correlate with elevated anti-PT antibody titers. Consistently, it has been reported that PT forms an important protective antigen in both humans and mice (reviewed in Mattoo and Cherry, 2005; Mills, 2001).

It has been shown previously that especially aP vaccines induce or augment parameters of type-I hypersensitivity, such as increased total serum IgE levels, increased lung eosinophilia and BALF eosinophil numbers, and increased Th2 cytokine production by the bronchial LN cells (Vandebriel *et al.*, 2007). In the present study we showed that, as compared to vaccination with the aluminum-adjuvated vaccine, immunisation with the MPL- or LpxL2 LPS-adjuvated vaccine reduced lung eosinophilia (only determined for the MPL-adjuvated vaccine), BALF eosinophil numbers, and Th2 cytokine production by both splenocytes and bronchial LN cells. However, the total serum IgE levels were not influenced by the type of adjuvants used. Of note, total serum IgE levels differ from the other type-I hypersensitivity parameters, since it is systemic and not influenced by *B. pertussis* infection (Vandebriel *et al.*, 2007). Together our results demonstrate that the addition of LPS analogs, instead of aluminum, to the DTaP vaccine re-directed the immune response towards a more Th1-type response and generally reduced type-I hypersensitivity, except for the serum IgE levels.

Analysis of serum samples taken 4 h after primary and booster immunisation revealed that MPL or LpxL2 LPS in the vaccine significantly increased the levels of the pro-inflammatory cytokine IL-6. This result shows that these adjuvants increased the reactogenicity of the vaccine. However, as compared to the wP vaccine, the exhibited reactogenicity was still low. Unexpectedly, we found that the IL-6 levels, evoked by the aluminum-adjuvated vaccine, were significantly higher after booster immunisation than after primary immunisation. Since the vaccine composition was the same, this observation suggests that booster immunisation, also in the absence of strong immune-stimulatory molecules, such as LPS, may elicit a stronger pro-inflammatory cytokine response than primary immunisation. Similar results were observed after immunisation with the LpxL2-adjuvated vaccine, but not with the MPL-adjuvated vaccine.

The results presented here show that the use of MPL or LpxL2 LPS rather than aluminum as an adjuvant in DTaP vaccines reduces vaccine-induced type-

I hypersensitivity and re-directs the evoked immune response towards a Th1-type response. Furthermore, these adjuvants significantly increased vaccine efficacy. It is tempting to speculate that this increased efficacy is due to an improved balance between the cellular (Th1) and humoral (Th2) response. In any case, these results indicate that the use of LPS analogs as adjuvants may be a useful strategy to improve aP vaccines. Since MPL is registered for clinical use as an adjuvant already, clinical testing of combinations of MPL and current aP vaccines should be feasible within a reasonable time frame. Obviously, the data presented here are not only important in the field of pertussis vaccinology, but may also offer opportunities for the development of vaccines against other microorganisms.

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