Chapter 10

General and Summarising Discussion

Pertussis or whooping cough is a highly contagious respiratory tract disease that is caused by the Gram-negative bacterium *Bordetella pertussis*. Since the introduction of pertussis mass-vaccination in the mid 1900s, pertussis incidence, morbidity, and mortality have been reduced dramatically in most Western nations and to a lesser extent in many developing countries. However, the success of pertussis vaccination is not as complete as that of other vaccines and pertussis is frequently the least well-controlled disease in childhood vaccination programs. Furthermore, as of the 1980s, pertussis incidence is rising again in many countries and most importantly, whereas pertussis first was a typical disease of young children, it is nowadays predominantly found among adolescents, adults, and, of most concern, small infants. The exact reasons for this re-emergence and altered age distribution are currently not well understood and remain under debate.

Lipopolysaccharide (LPS) is one of the major constituents of the Gram-negative bacterial outer membrane and is also known as endotoxin due to its ability to induce endotoxic shock in higher organisms (Raetz and Whitfield, 2002). It is one of the major causes of the relatively high reactogenicity of whole-cell pertussis (wP) vaccines. At present, the majority of developing countries use wP vaccines because they are cheap, effective, and easy to produce, whereas most industrialised countries have switched to less reactogenic, but more expensive acellular pertussis (aP) vaccines. These latter vaccines do not contain LPS and were shown to be comparably effective. Yet, aP vaccines have, as compared to wP vaccines, some important drawbacks. An example of this is the increased cost/benefit ratio. Since the production costs of aP vaccines are high, administration in the developing world forms an important “economical” problem. In addition, the currently used aP vaccines are based upon a small number of purified antigens, which might potentially result in a rapid selection of escape mutants. Another important issue is that aP vaccines evoke an immunologically different response (Th2-type response) than do wP vaccines and natural infection, which elicit Th1-type immune responses (reviewed in Mills, 2001). Th2-type immune responses have been linked to type-1 hypersensitivity diseases, such as asthma, atopic dermatitis, and anaphylactic shock (Wills-Karp, 1999; Foster *et al.*, 2002). Especially the consequences of aP vaccination for young, immunologically immature infants are currently not well understood. These notions, together with the problems described above with respect to pertussis re-emergence and alterations in age distribution, emphasise the idea that the development of improved pertussis vaccines remains important. In this thesis, we explored the possibilities of improving pertussis vaccines by altering their LPS composition, either with the help of LPS-modifying enzymes (**chapters 2** to **5**), by adding non-toxic LPS

derivatives (**chapters 6** and **7**), or by changing LPS biosynthesis (**chapters 8** and **9**). The work was mainly focused on the development of a less-reactogenic wP vaccine; however, as demonstrated in **chapter 7**, LPS (derivatives) may also be useful for the improvement of aP vaccines.

LPS-modifying enzymes

Gram-negative bacteria are able to modify the structure of lipid A. These modifications can promote resistance to host cationic antimicrobial peptides and alter recognition by TLR4/MD-2 (Ernst *et al.*, 2001). Regulated lipid A modifications, and the enzymes responsible, were first characterised in *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium). Later on, they were also identified in other Gram-negative bacteria. It is hypothesised that selective pressure on both pathogen and host resulted in marked variations in lipid A structures and the host responses through TLR4, and this diversity is likely to have played a role in the evolution of infectious diseases. Modification of lipid A can be induced under various conditions, such as growth in the presence of antimicrobial peptides or by growing under cation depletion (Bader *et al.*, 2005). Other environmental conditions, such as temperature and anaerobiosis, promote modification of lipid A in *Yersinia pestis* and *Pseudomonas aeruginosa*, respectively (Miller *et al.*, 2005). In *S.* Typhimurium, lipid A modifications are regulated by the environmental sensor-kinase transcriptional regulatory system PhoP/PhoQ (Ernst *et al.*, 2001). It has been shown that modification of LPS often reduces its endotoxic activity (Guo *et al.*, 1997; Kawasaki *et al.*, 2005). Hence, we hypothesised that LPS-modifying enzymes may be useful tools for reducing the reactogenicity of *B. pertussis* LPS and, thereby, for the development of safer wP vaccines. One of the primary candidates for this application was an enzyme known as PagL.

PagL is a PhoP/PhoQ-regulated lipid A 3-*O*-deacylase that was initially identified in *S.* Typhimurium (Trent *et al.*, 2001). At that moment, no obvious *pagL* homologs could be found in the nonredundant or unfinished microbial databases, except in the closely related strains *S. enterica* serovars Typhi and Paratyphi (Trent *et al.*, 2001). Therefore, PagL was postulated to be a *Salmonella*-specific virulence factor (Trent *et al.*, 2001). However, it was then already known that also other Gram-negative bacteria contain 3-*O*-deacylated lipid A species. We started this project by searching the genomes of Gram-negative bacteria for homologs of *S.* Typhimurium *pagL*. As shown in **chapters 2** and **3**, we found that *pagL* homologs were much more widely disseminated among Gram-negative bacteria than initially described. Interestingly, we also identified *pagL*

homologs in several non-pathogenic (soil) bacteria, excluding its role as a dedicated virulence factor. Of note, this of course does not eliminate the option that PagL contributes to the virulence of some species. Nevertheless, there are still Gram-negative bacteria which are known to contain 3-*O*-deacylated lipid A species, but that do not seem to harbour a *pagL* homolog, e.g., *Rhizobium leguminosarum* and *Rhizobium etli*. In a previous study, it was shown that 3-*O*-deacylase activity in these latter species, unlike PagL (**chapter 3**), is dependent on divalent cations (Basu *et al.*, 1999). Possibly, the sequence homology of the *Rhizobium* enzymes to the genuine PagL enzymes is too low to be detected. Another possibility is that in *Rhizobium* species, but maybe also in other Gram-negative bacteria, structurally distinct lipid A 3-*O*-deacylases exist. From this last perspective, it would be tempting to speculate that in some bacteria, lipid A deacylation may be mediated by a homolog of the outer membrane phospholipase OMPLA. As we showed in **chapter 3**, the active-site architecture of PagL very much resembles that of OMPLA, suggesting that OMPLA, in theory, may be able to function as a lipid A deacylase. Hence, if OMPLA by spontaneous mutations has gained affinity for LPS in particular bacteria, such as *R. leguminosarum* and *R. etli*, this may explain the presence of 3-*O*-deacylated lipid A species in the absence of PagL. Consistently, OMPLA has been shown to be calcium-dependent (Ubarretxena-Belandia *et al.*, 1998), which could explain the cation-dependency of lipid A deacylation. Interestingly, we also found a bacterium that contains multiple *pagL* genes, i.e., *Pseudomonas putida*. Apart from the homolog encoded by locus PP_0737 identified in **chapter 2**, also locus PP_3154 on the chromosome of *P. putida* KT2440 encodes a PagL homolog (J.G., unpublished observation). The two *P. putida* homologs show a mutual sequence identity (based on the amino acid sequence) of 58%, which makes it unlikely that the two copies are the result of a recent gene duplication (J.G., unpublished observation). When tested in an *in vitro* activity assay similar to the one used for *P. aeruginosa* PagL (**chapter 3**), both *P. putida* homologs showed 3-*O*-deacylase activity (J.G., unpublished result), suggesting that the enzymes show redundancy. The reason why *P. putida* has two *pagL* homologs remains to be elucidated and may, for instance, be related to differential gene regulation or differences in affinity for particular subsets of LPS species.

When we screened the *Bordetella* genomes, we found that the *B. pertussis* genome also contains a *pagL* homolog; however, the open reading frame was disrupted by a frame-shift mutation. In contrast, in the closely related species *Bordetella bronchiseptica* and *Bordetella parapertussis*, the ORFs were found to be intact (**chapter 2**). The loss of *pagL* expression in *B. pertussis* may thus be an example of host adaptation. To gain more insight into the dissemination of this frame-shift mutation

among *B. pertussis* isolates, we screened twenty *B. pertussis* strains that were isolated in the period 1949-2000. All tested strains contained the frame-shift mutation (J.G., unpublished result), suggesting that the loss of *pagL* expression is a common feature among *B. pertussis* strains. We have recently developed a *B. pertussis* strain in which the chromosomal *pagL* ORF has been restored. Preliminary results show that this *B. pertussis* strain expresses *pagL* and contains fully deacylated LPS (J.G., unpublished result). It will be interesting to investigate how this strain behaves in a murine infection model. Furthermore, identification of the *Bordetella pagL* homologs enabled us to use *Bordetella* PagL, in stead of PagL from more distantly related species, for expression in *B. pertussis*, thereby greatly reducing the risk of problems concerning for instance awkward codon-usage and protein instability.

During our BLAST searches, we observed that the mutual sequence identity between the newly identified PagL homologs was very low. This made us wonder about what enzymatic mechanism underlies PagL activity. Hence, we decided, before continuing with the experiments in *B. pertussis*, to try to find out how the enzyme actually works. Elucidation of the PagL crystal structure revealed that monomeric PagL has all features needed for activity. As discussed in **chapter 3**, this raised the interesting question how PagL activity can be regulated. In a recent paper, it was shown that PagL activity was increased in environmental isolates of *P. aeruginosa* by growth in medium limited for magnesium and decreased by growth at low temperature in laboratory-adapted strains of *P. aeruginosa* (Ernst *et al.*, 2006). Although these results demonstrate that *pagL* expression can be regulated, they do not provide a possible mechanism for silencing of PagL, once produced. Therefore, this question remains to be elucidated and further experiments, which, for instance, address the relevance of dimerisation for PagL inactivation or the possible role of other LPS modifications, should be conducted. Another interesting observation was that after expression of PagL in *E. coli* BL21 Star, the LPS underwent PagP-mediated re-modification with palmitate (**chapter 2**). PagP is an outer-membrane localised lipid A palmitoyl transferase (Bishop *et al.*, 2000). It is known that PagP becomes activated upon disturbance of the outer membrane, for instance upon addition of EDTA (Jia *et al.*, 2004). Thus, this observation suggests that deacylation of LPS reduces the integrity of the outer membrane. This conclusion is consistent with the observation that deacylation increased LPS release from *B. pertussis* membranes (**chapter 4**). However, when *pagL* was heterologously expressed in *E. coli* XL1-Blue or *S.* Typhimurium, such re-modification with palmitate was not observed (Ernst *et al.*, 2006). Possibly, different strain backgrounds or expression levels used are responsible for the different results. An interesting hypothesis is that in *S.* Typhimurium and *E. coli*

XL1-Blue, which is an *E. coli* K-12 derivative, the LPS core domain compensates for the PagL-mediated decrease in outer membrane integrity, whereas this is not the case in *E. coli* BL21 Star, an *E. coli* B derivative, which has a substantially smaller LPS core domain. It has previously been shown that mutations in the LPS outer-core region can influence outer membrane integrity (Skurnik *et al.*, 1999). Interestingly, when we expressed enzymatically inactive PagL in *E. coli* BL21 Star and analysed the LPS profiles, bands with lower electrophoretic mobility than that of wild-type LPS were observed (**chapter 2**, Fig. 9B). It is tempting to speculate that these bands correspond to PagP-modified LPS. If this is indeed the case, also inactive PagL can apparently activate PagP and thus the presence of the PagL β-barrel alone would already be enough to disturb the outer membrane. Possibly, the tilted membrane-orientation of PagL (**chapter 3**) creates membrane disturbances by which phospholipids can flip to the outer leaflet of the outer membrane and become a substrate for PagP. Of note, besides PagL, PagP is the only known outer-membrane protein with a tilted orientation in the membrane (Ahn *et al.*, 2004). It would be very interesting to test whether PagL can induce phospholipid flip-flop across a lipid bilayer. Another question that needs to be answered is how the specificity of PagL for the lipid A 3 position is mediated. Since amino acid conservation is very low, it is unlikely that a dedicated LPS-binding motif is involved. However, it is conceivable that for instance positively charged residues in the extracellular loops, which can interact with the negatively charged lipid A backbone, play a role or that the barrel displays some sort of steric specificity.

The biological function of PagL remains unknown, given that *S.* Typhimurium and *P. aeruginosa pagL* mutants do not display obvious phenotypes (Trent *et al.*, 2001; **chapter 2**). Nevertheless, recent studies have provided some interesting insights that may help to resolve this question. It has, for instance, been shown that deacylation of LPS reduces its endotoxic activity, which may prevent recognition by the host innate immune system (Kawasaki *et al.*, 2005; **chapter 4**). In addition, it has been shown that *pagL* expression is increased in *P. aeruginosa* isolated from cystic fibrosis (CF) infants as compared to environmental isolates, whereas deacylated LPS species were not observed in some clinical *P. aeruginosa* isolates from patients with severe pulmonary disease, suggesting that loss of PagL function can occur during long-term adaptation to the CF airway (Ernst *et al*, 2006). These observations suggest that PagL may function in modulating the host immune response. However, the observation that *pagL* is also found in non-pathogenic (soil) bacteria suggests that besides a role in pathogenesis, PagL also must have a broader function. Since *pagL* expression influences the integrity of the outer membrane (**chapters 2** and **4**), it could well be that PagL has a function in

controlling, for instance, outer membrane fluidity and thereby adaptation to environmental conditions.

Role of LPS composition in the endotoxic activity of *B. pertussis* LPS and whole bacterial cells

One of the main targets of this thesis was to lower the reactogenicity of *B. pertussis* LPS and, thereby, of the bacterium itself. Less-reactogenic *B. pertussis* strains could then function as the basis for the development of safer wP vaccines. The endotoxic activity of LPS is based upon its recognition by the host innate immune system, activation of NF-κB, and subsequent up regulation of pro-inflammatory cytokines. During the late 1980s, experiments with LPS and LPS derivatives revealed that lipid A forms the bioactive component of LPS, responsible for the majority of IL-1 induction and immuno-regulation in human mononuclear cells (Loppnow *et al.*, 1989; Loppnow *et al.*, 1990). To date, a wide range of synthetic LPS structures has been tested for their biological activity and much is known regarding the contribution of specific elements to the overall toxicity of the molecule. It has become clear that the number, length, and positioning of the acyl chains and the phosphorylation status of the disaccharide backbone are important. For instance, dephosphorylated LPS shows a ~1000-fold lower endotoxic activity than wild-type *E. coli* lipid A (Rietschel *et al.*, 1987). However, not the phosphate groups themselves, but rather the negative charge they represent seems to be the determining factor, since lipid A substituted with a phosphono-oxyethyl group has an activity comparable to phosphate-substituted lipid A (Ulmer *et al.*, 1992). Furthermore, glucosamine monosaccharide preparations carrying phosphates and acyl chains at various positions in general lack biological activity (Aschauer *et al.*, 1990), suggesting that the disaccharide backbone is also important.

Like other amphiphatic molecules, LPS and lipid A form large supra-molecular aggregates in aqueous environments above the critical micellar concentration, which depends, amongst others, on the hydrophobicity (Brandenburg and Wiese, 2004). The structure of these aggregates is dependent on the chemical composition and influenced by pH, temperature, water content, and the presence of cations. LPS/lipid A aggregates can adopt two main phases, the gel phase and the liquid-crystalline phase. At a characteristic temperature, a reversible transition between these two phases takes place. Given the complex structure of LPS/lipid A (aggregates), the consequences of alterations in chemical composition on transition temperature are currently not fully understood.

Some striking correlations between the phase-transition temperature and

structure of the LPS/lipid A aggregates and exhibited endotoxic activity have been described. However, it is hard to distinguish whether the observed effects are due to alterations in the fluidity or structure of the aggregates, or a combination of both. For instance, it has been shown that deep rough LPS in the presence of Zn^{2+} has an enhanced capability to induce pro-inflammatory cytokine secretion at 37°C (Wellinghausen *et al.*, 1996). The authors found that an increased acyl chain order facilitated stronger interactions between LPS and LPS-binding protein and thereby enhanced the transport of LPS to the host TLR4/MD-2 complex. Thus, the phase transition temperature determines, for an important part, what kind of supramolecular structure LPS/lipid A aggregates adopt and, thereby, their endotoxic activity (Schromm *et al.*, 2000; Seydel *et al.*, 2000). So-called cylindrical-shaped lipid A species, such as those from *Rhodobacter capsulatus* and *Chromobacterium violaceum*, but also tetra- and penta-acyl species from *Enterobacteriacae*, adopt a lamellar structure and are poorly active. These lipid A species were found to work as efficient LPS antagonists if a sufficiently high negative backbone charge was present (Schromm *et al.*, 1998). In contrast, partially conical-shaped lipid A species that adopt a mixed lamellar/cubic structure, such as MPL and lipid A from *Campylobacter jejuni*, had an intermediate activity, whereas conical-shaped lipid A species, such as enterobacterial hexa-acyl lipid A, were highly active (Brandenburg and Wiese, 2004). Thus, in conclusion, the effect of one particular structural alteration on the toxicity of one particular LPS/lipid A species is relatively easy to predict. However, LPS preparations are often heterogeneous, meaning that the exhibited biological activity is the sum of all separate species including agonists, antagonists, and inactive forms. It becomes even more complicated when one realises that the interest is often in the toxicity of complex biological samples, such as wP vaccines, which contain besides LPS also additional (immune-modulating) components.

A clear example that changes in LPS composition can sometimes lead to unexpected results was provided in **chapter 4**. In that chapter, we explored the consequences of PagL and PagP expression on the *in vitro* toxicity of *B. pertussis* LPS and whole bacterial cells. Previously, it was shown that wild-type *B. pertussis* LPS already is a poor cytokine inducer in human monocytes, as can be expected from its cylindrical-shaped structure (Haeffner-Cavaillon *et al.*, 1989). We showed that deacylation by PagL further reduces the endotoxic activity of the LPS. This suggests that the number of acyl chains influences the endotoxic activity of *B. pertussis* LPS. Consistently, the PagP-mediated addition of an extra acyl chain led to an increased endotoxic activity of the LPS. Although modification of hexa-acylated *Salmonella* LPS by PagP was earlier reported to result in LPS with a reduced endotoxic activity (Janusch

et al., 2002; Kawasaki *et al.*, 2005), the increase in endotoxic activity of *B. pertussis* LPS we observed is not surprising, since *B. pertussis* LPS is a mixture of tetra- and penta-acylated forms, which, after modification by PagP, will be converted into more active penta- and hexa-acylated forms.

After having addressed the toxicity of purified *B. pertussis* LPS and its PagP- and PagL-modified derivatives, our next goal was to determine how these modifications influenced the toxicity of whole bacterial cells. To our surprise, we found that cells expressing either *B. bronchiseptica* or *P. aeruginosa* PagL, in contrast to the purified LPS preparations, had an increased potency to induce the production of IL-6 by human macrophages. It is well established that soluble LPS aggregates, on a molar basis, are biologically more active than LPS present in membrane structures such as in bacterial cells, outer membrane vesicles or liposomes (Post *et al.*, 2005). As deacylation of LPS may weaken LPS-LPS interactions, we hypothesised that the increased endotoxic activity of the PagL-expressing cells could potentially be due to an increased LPS release and subsequent formation of soluble aggregates. Such a mechanism could then counter-balance the effect of the reduced endotoxic activity of purified deacylated LPS. Consistent with this hypothesis, we observed that PagL expression increased the release of LPS from the bacterial cells into the medium. Furthermore, as anticipated, a treatment known to result in LPS release, i.e., incubation in the presence of EDTA, increased the IL-6 inducing capacity of a *B. pertussis* cell suspension. Therefore, increased release of deacylated LPS could eventually lead to a net increase in the endotoxic activity of PagL-expressing cells. However, it cannot be excluded that also other factors contribute to the observed increase in endotoxic activity, since it is conceivable that LPS deacylation also facilitates the release of other (immune-modulating) compounds. Remarkably, a similar discrepancy between the effects of an LPS modification on purified LPS and on whole bacterial cells was observed in **chapter 9**, in which we identified a new LPS glycosyltransferase operon in the *B. pertussis* genome. By insertionally inactivating the genes and studying the effects on LPS structure, we demonstrated that at least two of the genes in this operon encode active LPS glycosyltransferases. When we tested the purified wild-type and mutant LPSs and whole cells for their endotoxic activity, we found that the purified LPS from the ΔBP2329 mutant strain exhibited a reduced biological activity, whereas the mutant cells instead showed a slightly increased endotoxic activity. Thus, changes in LPS composition influence the endotoxic activity at different levels, i.e., not only directly through altering the LPS per se, but also indirectly through the effect on LPS accessibility. In the case of PagL and the ΔBP2329 mutant strain, these effects worked in opposite directions. Altogether, these results suggest that *B. pertussis*

has a relatively fragile outer membrane and that alterations in LPS composition can easily disturb its integrity, thereby promoting the release of LPS. This conclusion is consistent with the finding that also an *lpxL1*-deficient mutant of *B. pertussis* displays decreased outer membrane integrity. As demonstrated in **chapter 8**, LpxL1 mediates the addition of a previously unrecognised secondary 2-OH C12 moiety to *B. pertussis* lipid A. We showed that this moiety is present in wild-type *B. pertussis* LPS, yet, only at a low frequency (<10% of the total LPS species). Nevertheless, when we knocked out the *lpxL1* gene, we found that the mutant strain exhibited an increased sensitivity towards rifampicin, which is indicative for decreased outer membrane integrity. This result supports the conclusion that relatively small changes in LPS composition can have dramatic effects on the stability of the *B. pertussis* outer membrane and that this phenomenon can diminish the effect of reducing LPS toxicity. Perhaps, this may also be one of the reasons why *B. pertussis* is unable to survive outside the human host. Possible solutions to the problem are the addition of membrane-stabilising agents or the removal of soluble LPS aggregates. It would for instance be interesting to determine whether replacing aluminum phosphate by aluminum hydroxide in wP vaccines would beneficially influence vaccine reactogenicity since the binding affinity of soluble LPS aggregates for aluminum hydroxide is much higher than for aluminum phosphate (Shi *et al.*, 2001).

Role of LPS in pertussis vaccination

The role of LPS in *Bordetella* pathogenesis and immunity is unclear. It has been shown that *Bordetella* LPS is highly immunogenic, acts as an immunological adjuvant, and exhibits the properties that are expected of an endotoxin (Amano *et al.*, 1990; Chaby and Caroff, 1988; Watanabe *et al.*, 1990). Furthermore, *Bordetella* LPS and its recognition by the TLR4/MD-2 complex have been shown to be important determinants in the course of infection in mice (Mann *et al.*, 2004a; Mann *et al.*, 2004b). In addition, antibodies against *B. pertussis* LPS have been shown to play a role in the protection against *B. pertussis* infection (Granstrom and Granstrom, 1993; Shahin *et al.*, 1994), and they have also been shown to bind to *B. parapertussis* and inhibit their adherence to respiratory tract epithelial cells, suggesting cross-protection between the two *Bordetella* species (van den Berg *et al.*, 1999). Thus, apparently, *B. pertussis* LPS plays a dual role; on the one hand as an immune-stimulating compound and on the other hand as a protective antigen. In **chapter 4**, we showed that expression of the LPS-modifying enzymes PagP and PagL modulates the *in vitro* toxicity of *B. pertussis* LPS and whole bacterial cells. The next goal was to determine how these alterations in

LPS composition affect the efficacy and reactogenicity of wP vaccines (**chapter 5**). Mice were immunised with different wP vaccines and then challenged with *B. pertussis*. We found that expression of PagL, but not of PagP, significantly increased vaccine efficacy without altering vaccine reactogenicity. The latter conclusion deviates from that of the *in vitro* experiments which indicated that PagL-expressing *B. pertussis* cells exhibited an increased toxicity (**chapter 4**). Thus, apparently, *in vitro* toxicity data cannot predict the outcomes of *in vivo* experiments, emphasising the importance of both *in vitro* and *in vivo* analyses. Possible explanations for this discrepancy are differences between human (**chapter 4**) and murine (**chapter 5**) cells or the use of only a single cell type in the *in vitro* experiments, i.e., macrophages, whereas *in vivo* experiments comprise many different cell types simultaneously. Also, the availability of LBP and CD14 probably differs in the two settings. Since an increased vaccine potency probably allows for a reduction of the vaccine dosage, these data imply that PagL-expressing *B. pertussis* strains may form a good basis for the development of less reactogenic wP vaccines, as lower vaccine dosages will probably also reduce vaccine side-effects. Thus, in conclusion, we provided proof of principle that LPS-modifying enzymes may indeed form promising tools for the development of safer wP vaccines. Obviously, this observation is not only significant in the field of pertussis vaccinology, but may also be applicable for the development of vaccines against other Gram-negative bacteria. Furthermore, these data showed, for the first time, that LPS composition is an important determinant for the efficacy of wP vaccines.

In **chapter 6**, we took a somewhat different approach and explored the possibilities of improving wP efficacy and reducing reactogenicity by supplementing the vaccine with LPS analogs that are capable of modulating immune responses. We showed that addition of monophosphoryl lipid A (MPL) increased vaccine efficacy without changing the reactogenicity. Furthermore, we demonstrated that *Neisseria meningitidis* LpxL2 LPS functions as an LPS antagonist and that supplementation of the wP vaccine with this LPS not only decreased the reactogenicity, but also increased the efficacy of the vaccine. Thus, apparently, the LPS derivatives can influence both the efficacy and reactogenicity of the wP vaccines and their application may therefore be a useful strategy for vaccine improvement. However, more detailed studies using different dosages and different experimental backgrounds should be conducted before definite conclusions concerning the application of such an approach can be drawn. Furthermore, it may be worthwhile to test a broader range of LPS derivatives.

Interestingly, as shown in **chapter 7**, LPS analogs may not only be beneficial for improving wP vaccines, but also for improving aP vaccines. One of the main problems

concerning aP vaccination is that it drives the immune response towards a Th2-type response (Mills, 2001). Th2-type immune responses are correlated with type-1 hypersensitivity and probably provide only suboptimal protection, since it is known that in both mice and man cellular (Th1) and humoral (Th2) immunity have complementary roles in protection against *B. pertussis* infection (Mills, 2001). Since LPS has strong Th1-skewing properties, we hypothesised that supplementation of a DTaP vaccine with LPS analogs would probably alter the Th1/Th2 balance by tipping it towards more a Th1-type response, thereby decreasing type-1 hypersensitivity and simultaneously increasing vaccine efficacy. Indeed, we found in a mouse model that addition of LPS derivatives to the DTaP vaccine re-directed the immune response towards a Th1-type response. Furthermore, it decreased parameters, such as lung eosinophilia, indicative for type-1 hypersensitivity and, most importantly, it improved vaccine efficacy. These results support the hypothesis that LPS derivatives can modulate aP-induced immune responses. In addition, we analysed the effect of LPS supplementation on vaccine reactogenicity and found that MPL and LpxL2 LPS significantly increased the post-immunisation serum IL-6 levels as compared to an aluminum-adjuvated vaccine. However, as compared to a wP vaccine, the serum IL-6 levels evoked were still low. Thus, the application of LPS derivatives may form a useful strategy for improving aP vaccines, since they re-directed the immune response towards to a more Th1-type response, decreased type-1 hypersensitivity, and improved vaccine efficacy. Of course, such a strategy will not provide a solution for the problems concerning aP production-costs and escape-mutant selection.

The results described in this thesis demonstrate that efficacy and reactogenicity of both wP and aP vaccines can be changed in the right direction by modulating the LPS content. We have shown that alterations in LPS composition, i.e., in case of the PagL/expressing strain (**chapter 5**), or addition of LPS derivatives (**chapters 6** and **7**) can increase vaccine efficacy. Further analysis revealed that the increased vaccine efficacy observed did not correlate with higher anti-*B. pertussis* total IgG, IgG1, or IgG2a antibody titers (**chapters 5** and **6**). However, the increased efficacy of the DTaP vaccines supplemented with MPL or LpxL2 LPS did coincide with higher anti-pertussis toxin antibody titers, suggesting that these antibodies may be important for aP-mediated protection (**chapter 7**). Nevertheless, alterations in LPS composition can modulate antibody responses, as was revealed, for example, by the decreased IgG1/IgG2a ratio in the mice immunised with the PagP- and PagL-expressing strains, as compared to the mice immunised with the wild-type strain (**chapter 5**). Analysis of the cytokine concentrations in the lung homogenates revealed no major differences between

the immunised groups and the control groups, except for gamma-interferon (IFN-γ) (**chapters 5** and **6**). The importance of IFN-γ for protection against *Bordetella* infection has been shown repeatedly (Mills *et al.*, 1998; Mahon *et al.*, 1997; Barbic *et al.*, 1997; Pilione and Harvill, 2006). IFN-γ induces the transcription of several activation markers, various cell surface receptors, chemokine receptors, and co-stimulatory receptors involved in antigen presentation (Dalton *et al.*, 1993; Ellis *et al.*, 2004; Janeway, 2001). Furthermore, IFN-γ activates and recruits neutrophils (Ellis *et al.*, 2004; Burch *et al.*, 2006). Consistently, we observed that the wP-immunised mice exhibited an increased lung neutrophil-fraction. 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. Thus, analysis of these parameters may allow the reliable prediction of vaccine efficacy in mice. Whether this is also true for humans remains to be determined. Interestingly, in a recent study, it was shown that besides IFN-γ-producing cells, also IL-17-producing T cells are important for protective cellular immunity against *B. pertussis* (Higgins *et al.*, 2006). It would therefore be interesting to also study IL-17 responses in future experiments.

Prospects for pertussis vaccination

Pertussis causes nearly 300,000 deaths in children every year. Current pertussis vaccines have proved to be effective and relatively safe in large-scale clinical trials. At present, most developing countries use wP vaccines because they are cheap, effective, and easy to produce, whereas most industrialised countries have switched to less reactogenic, but more expensive aP vaccines. In general, pertussis vaccination comprises a three-dose immunisation series starting at 6 weeks to 3 months after birth and is usually completed by the age of 6 months. In addition, many schedules include a toddler booster, and a fourth or fifth dose at 4–6 years of age. Pertussis vaccination provides protection against death and admission to hospital, although cases of pertussis disease in recently vaccinated people are sporadically observed.

Despite high vaccination coverage, pertussis is still prevalent and increasing and is frequently the least well-controlled disease in childhood vaccination programs, possibly as a result of waning immunity. Addition of new antigens, such as adenylate cyclase, to aP vaccines might improve some aspects of the immune response to vaccination, but is unlikely to significantly increase the duration of protection. Potentially more interesting would be supplementation of aP vaccines with LPS analogs, which, as we showed, increases vaccine efficacy and drives the immune response towards a more protective

Th1-type response without substantially increasing vaccine reactogenicity (**chapter 7**). Since MPL has already been registrated for use in a human vaccine (hepatitis B), MPL-supplemented aP vaccines might be the least complicated to introduce from a regulatory perspective. However, this approach, of course, does not provide a solution for the problems concerning the high production costs of aP vaccines and their risk to select for escape mutants more rapidly. Today, many scientists from around the world are putting tremendous effort in trying to improve pertussis vaccines. These efforts include the development of live-attenuated vaccines (Mielcarek *et al.*, 2006), DNA vaccines (Kamachi *et al.*, 2003), and safer wP vaccines (**this thesis**). Here, we provided proof of principle that LPS engineering can be a useful strategy for improving pertussis vaccines. However, at this moment, it is too early to tell which approach will be most promising and should thus be pursued. Currently, I would say that the most promising candidate for an improved pertussis vaccine would be a wP vaccine supplemented with non-toxic LPS antagonists, such as LpxL2 LPS. We have shown that this approach led to a less-reactogenic vaccine with an increased efficacy. However, it is very well imaginable that such an approach will stumble on regulatory difficulties since it requires the addition of extra LPS to wP vaccines. Therefore, the use of the PagL-expressing *B. pertussis* strains may also be a good option. In addition, it would be worthwhile to proceed with testing other vaccine strains, such as the core OS mutants (**chapter 9**) or the *lpxL1*-deficient mutant (**chapter 8**), additional LPS-modifying enzymes, such as the lipid A 3'-*O*-deacylase LpxR and the lipid A 1- and 4'-phosphatases LpxE and LpxF, additional LPS mutations, and adjuvants, e.g., aluminum phosphate vs. aluminum hydroxide. In any case, introducing new pertussis vaccines will take a substantial amount of time, and thus, in the meanwhile, the potential of currently available pertussis vaccines should be maximised. As pertussis has persisted in a huge adult and adolescent reservoir during the past three decades (Mattoo and Cherry, 2005), rationalisation of pertussis boosters in childhood and introduction of widespread repeat booster vaccination in adolescents and adults can be expected to lessen disease prevalence and morbidity among susceptible infants. Complementary to this, it might be possible to protect the youngest infants, for instance by immunising pregnant mothers or by vaccinating directly after birth.

References

Ahn, V. E., Lo, E. I., Engel, C. K., Chen, L., Hwang, P. M., Kay, L. E., Bishop, R. E., and Prive, G. G. (2004) A hydrocarbon ruler measures palmitate in the enzymatic acylation of endotoxin. *EMBO J.* **23:** 2931-2941.

Amano, K., Fukushi, K., and Watanabe, M. (1990) Biochemical and immunological comparison of lipopolysaccharides from *Bordetella* species. *J. Gen. Microbiol.* **136:** 481-487.

Aschauer, H., Grob, A., Hildebrandt, J., Schuetze, E., and Stuetz, P. (1990) Highly purified lipid X is devoid of immunostimulatory activity. Isolation and characterization of immunostimulating contaminants in a batch of synthetic lipid X. *J. Biol. Chem.* **265:** 9159-9164.

Bader, M. W., Sanowar, S., Daley, M. E., Schneider, A. R., Cho, U., Xu, W., Klevit, R. E., Le Moual, H., and Miller, S. I. (2005) Recognition of antimicrobial peptides by a bacterial sensor kinase. *Cell* **122:** 320-322.

Barbic, J., Leef, M. F., Burns, D. L., and Shahin, R. D. (1997) Role of gamma interferon in natural clearance of *Bordetella pertussis* infection. *Infect. Immun.* **65:** 4904-4908.

Basu, S. S., White, K. A., Que, N. L., and Raetz, C. R. H. (1999) A deacylase in *Rhizobium leguminosarum* membranes that cleaves the 3-*O*-linked beta-hydroxymyristoyl moiety of lipid A precursors. *J. Biol. Chem.* **274:** 11150-11158.

Bishop, R. E., Gibbons, H. S., Guina, T., Trent, M. S., Miller, S. I., and Raetz, C. R. H. (2000) Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. *EMBO J.* **19:** 5071-5080.

Brandenburg, K., and Wiese, A. (2004) Endotoxins: relationships between structure, function, and activity. *Curr. Top. Med. Chem.* **4:** 1127-1146.

Burch, L. H., Yang, I. V., Whitehead, G. S., Chao, F. G., Berman, K. G., and Schwartz, D. A. (2006) The transcriptional response to lipopolysaccharide reveals a role for interferon-gamma in lung neutrophil recruitment. *Am. J. Physiol. Lung Cell Mol. Physiol.* **291:** L677-682.

Chaby, R., and Caroff, M. (1988) Lipopolysaccharide of *Bordetella pertussis* endotoxin. p. 247-271. In A. C. Wardlaw, and R. Parton (eds). Pathogenesis and immunity in pertussis. John Wiley, New York.

Dalton, D. K., Pitts-Meek, S., Keshav, S., Figari, L. S., Bradley, A., and Stewart, T. A. (1993) Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* **259:** 1739-1742.

Ellis, T. N., and Beaman, B. L. (2004) Interferon-gamma activation of polymorphonuclear neutrophil function. *Immunology* **112:** 2-12.

Ernst, R. K., Adams, K. N., Moskowitz, S. M., Kraig, G. M., Kawasaki, K., Stead, C. M., Trent, M. S., and Miller, S. I. (2006) The *Pseudomonas aeruginosa* lipid A deacylase: selection for expression and loss within the cystic fibrosis airway. *J. Bacteriol.* **188:** 191-201.

Ernst, R. K., Guina, T., and Miller, S. I. (2001) *Salmonella typhimurium* outer membrane remodeling: role in resistance to host innate immunity. *Microbes Infect.* 3: 1327-1334.

Foster, P. S., Martinez-Moczygemba, M., Huston, D. P., and Corry, D. B. (2002) Interleukins-4, -5, and -13: emerging therapeutic targets in allergic disease. *Pharmacol. Ther.* **94:** 253-264.

Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M., and Miller, S. I. (1997) Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes phoP-phoQ. *Science* **276:** 250-253.

Granstrom, M., and Granstrom, G. (1993) Serological correlates in whooping cough. *Vaccine* **11:** 445-448.

Haeffner-Cavaillon, N., Carroff, M., and Cavaillon, J-M. (1989) Interleukin-1 induction by lipopolysaccharides: structural requirements of the 3-deoxy-D-manno-2-octulosonic acid (KDO). *Mol. Immunol.* **26:** 485-494.

Higgins, S. C., Jarnicki, A. G., Lavelle, E. C., and Mills, K. H. (2006) TLR4 mediates vaccine-induced protective cellular immunity to *Bordetella pertussis*: role of IL-17-producing T cells. *J. Immunol.* **177:** 7980-7989.

Janeway, C. (2001) Immunobiology. Garland Publishing, New York, N.Y.

Janusch, H., Brecker, L., Lindner, B., Alexander, C., Gronow, S., Heine, H., Ulmer, A. J., Rietschel, E. T., and Zähringer, U. (2002) Structural and biological characterization of highly purified hepta-acyl lipid A present in the lipopolysaccharide of the *Salmonella enterica* sv. Minnesota Re deep rough mutant strain R595. *J. Endotoxin Res.* **8:** 343-356.

Jia, W., El Zoeiby, A. E., Petruzziello, T. N., Jayabalasingham, B., Seyedirashti, S., and Bishop, R. E. (2004) Lipid trafficking controls endotoxin acylation in outer membranes of *Escherichia coli*. *J. Biol. Chem.* **279:** 44966-44975.

Kamachi, K., Konda, T., and Arakawa, Y. (2003) DNA vaccine encoding pertussis toxin S1 subunit induces protection against *Bordetella pertussis* in mice. *Vaccine* **21:** 4609-4615.

Kawasaki, K., Ernst, R. K., and Miller, S. I. (2005) Purification and characterization of deacylated and/or palmitoylated lipid A species unique to *Salmonella enterica* serovar Typhimurium. *J. Endotoxin Res.* **11:** 57-61.

Kirimanjeswara, G. S., Mann, P. B., and Harvill, E. T. (2003) Role of antibodies in immunity to *Bordetella* infections. *Infect. Immun.* **71:** 1719-1724.

Kirimanjeswara, G. S., Mann, P. B., Pilione, M., Kennett, M. J., and Harvill, E. T. (2005) The complex mechanism of antibody-mediated clearance of *Bordetella* from the lungs requires TLR4. *J. Immunol.* **175:** 7504-7511.

Loppnow, H., Brade, H., Durrbaum, I., Dinarello, C. A., Kusumoto, S., Rietschel, E. T., and Flad, H. D. (1989) IL-1 induction-capacity of defined lipopolysaccharide partial structures. *J. Immunol.* **142:** 3229-3238.

Loppnow, H., Durrbaum, I., Brade, H., Dinarello, C. A., Kusumoto, S., Rietschel, E. T., and Flad, H. D. (1990) Lipid A, the immunostimulatory principle of lipopolysaccharides? *Adv. Exp. Med. Biol.* **256:** 561-566.

Mahon, B. P., Sheahan, B. J., Griffin, F., Murphy, G., and Mills, K. H. G. (1997) Atypical disease after *Bordetella pertussis* respiratory infection of mice with targeted disruptions of IFN-γ receptor or immunoglobulin μ chain genes. *J. Exp. Med.* **186:** 1843-1851.

Mann, P. B., Elder, K. D., Kennett, M. J., and Harvill, E. T. (2004a). Toll-like receptor 4-dependent early elicited tumor necrosis factor alpha expression is critical for innate host defense against *Bordetella bronchiseptica*. *Infect. Immun.* **72:** 6650-6658.

Mann, P. B., Kennett, M. J., and Harvill, E. T. (2004b) Toll-like receptor 4 is critical to innate host defense in a murine model of bordetellosis. *J. Infect. Dis.* **189:** 833-836.

Mattoo, S., and Cherry, J. D. (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. *Clin. Microbiol. Rev.* **18:** 326-382.

Mielcarek, N., Debrie, A. S., Raze, D., Bertout, J., Rouanet, C., Younes, A. B., Creusy, C., Engle, J., Goldman, W. E., and Locht, C. (2006) Live attenuated *B. pertussis* as a single-dose nasal vaccine against whooping cough. *PLoS Pathog.* **2:** e65.

Miller, S. I., Ernst, R. K., and Bader, M. W. (2005) LPS, TLR4 and infectious disease diversity. *Nat. Rev. Microbiol.* **3:** 36-46.

Mills, K. H. G. (2001) Immunity to *Bordetella pertussis*. *Microbes. Infect.* **3:** 655-677.

Mills, K. H. G., Ryan, M., Ryan, E., Mahon, B. P. (1998) A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. *Infect. Immun.* **66:** 594-602.

Pilione, M. R., and Harvill, E. T. (2006) The *Bordetella bronchiseptica* type III secretion system inhibits gamma interferon production that is required for efficient antibody-mediated bacterial clearance. *Infect. Immun.* **74:** 1043-1049.

Pishko, E. J., Kirimanjeswara, G. S., Pilione, M. R., Gopinathan, L., Kennett, M. J., and Harvill, E. T. (2004) Antibody-mediated bacterial clearance from the lower respiratory tract of mice requires complement component C3. *Eur. J. Immunol.* **34:** 184-193.

Post, D., Zhang, D., Eastvold, J., Teghanemt, A., Gibson, B. W., Weiss, J. P. (2005) Biochemical and functional characterization of membrane blebs purified from *Neisseria meningitidis* sergroup B. *J. Biol. Chem.* **280:** 38383-38394.

Raetz, C. R. H., and Whitfield, C. (2002) Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71:** 635-700.

Rietschel, E. T., Brade, H., Brade, L., Brandenburg, K., Schade, U., Seydel, U., Zahringer, U., Galanos, C., Luderitz, O., Westphal, O., Labischinski, H., Kusumoto, S., and Shiba, T. (1987) Lipid A, the endotoxic center of bacterial lipopolysaccharides: relation of chemical structure to biological activity. *Prog. Clin. Biol. Res.* **231:** 25-53.

Schromm, A. B., Brandenburg, K., Loppnow, H., Moran, A. P., Koch, M. H., Rietschel, E. T., and Seydel, U. (2000) Biological activities of lipopolysaccharides are determined by the shape of their lipid A portion. *Eur. J. Biochem.* **267:** 2008-2013.

Schromm, A. B., Brandenburg, K., Loppnow, H., Zahringer, U., Rietschel, E. T., Carroll, S. F., Koch, M. H., Kusumoto, S., and Seydel, U. (1998) The charge of endotoxin molecules influences their conformation and IL-6-inducing capacity. *J. Immunol.* **161:** 5464-5471.

Seydel, U., Oikawa, M., Fukase, K., Kusumoto, S., and Brandenburg, K. (2000) Intrinsic conformation of lipid A is responsible for agonistic and antagonistic activity. *Eur. J. Biochem.* **267:** 3032-3039.

Shahin RD, Hamel J, Leef MF, Brodeur BR. (1994) Analysis of protective and nonprotective monoclonal antibodies specific for *Bordetella pertussis* lipooligosaccharide. *Infect. Immun.* **62:** 722-725.

Shi, Y., HogenEsch, H., Regnier, F. E., and Hem, S. L. (2001) Detoxification of endotoxin by aluminum hydroxide adjuvant. *Vaccine* **19:** 1747-1752.

Skurnik, M., Venho, R., Bengoechea, J. A., and Moriyon, I. (1999) The lipopolysaccharide outer core of *Yersinia enterocolitica* serotype O:3 is required for virulence and plays a role in outer membrane integrity. *Mol. Microbiol.* **31:** 1443-1462.

Trent, M. S., Pabich, W., Raetz, C. R., and Miller, S. I. (2001) A PhoP/PhoQ-induced Lipase (PagL) that catalyzes 3-O-deacylation of lipid A precursors in membranes of Salmonella typhimurium. *J. Biol. Chem.* **276:** 9083-9092.

Ubarretxena-Belandia, I., Boots, J. W., Verheij, H. M., and Dekker, N. (1998) Role of the cofactor calcium in the activation of outer membrane phospholipase A. *Biochemistry* **37:** 16011-16018.

Ulmer, A. J., Heine, H., Feist, W., Kusumoto, S., Kusama, T., Brade, H., Schade, U., Rietschel, E. T., and Flad, H. D. (1992) Biological activity of synthetic phosphono-oxyethyl analogs of lipid A and lipid A partial structures. *Infect. Immun.* **60:** 3309-3314.

van den Berg, B. M., Beekhuizen, H., Willems, R. J., Mooi, F. R., and van Furth, R. (1999) Role of Bordetella pertussis virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. *Infect. Immun.* **67:** 1056-1062.

Watanabe, M., Takimoto, H., Kumazawa, Y., and Amano, K. (1990) Biological properties of lipopolysaccharides from *Bordetella* species. *J. Gen. Microbiol.* **136:** 489-493.

Wellinghausen, N., Schromm, A. B., Seydel, U., Brandenburg, K., Luhm, J., Kirchner, H., and Rink, L. (1996) Zinc enhances lipopolysaccharide-induced monokine secretion by alteration of fluidity state of lipopolysaccharide. *J. Immunol.* **157:** 3139-3145.

Wills-Karp, M. (1999) Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* **17:** 255-281.