Haemophilus influenzae Type b-Outer Membrane Protein Complex Glycoconjugate Vaccine Induces Cytokine Production by Engaging Human Toll-Like Receptor 2 (TLR2) and Requires the Presence of TLR2 for Optimal Immunogenicity

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Haemophilus influenzae Type b-Outer Membrane Protein Complex Glycoconjugate Vaccine Induces Cytokine Production by Engaging Human Toll-Like Receptor 2 (TLR2) and Requires the Presence of TLR2 for Optimal Immunogenicity

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Conjugate vaccines consisting of the capsular polysaccharide (PS) of Haemophilus influenzae type b (Hib) covalently linked to carrier proteins, unlike pure PS, are immunogenic in infants and have significantly reduced Hib infections in the United States, but require multiple doses to induce protective anti-PS Ab titers. Hib-meningococcal outer membrane protein complex (OMPC) conjugate vaccine, however, elicits protective anti-PS Ab titers after one dose. We found that OMPC and Hib-OMPC engaged human Toll-like receptor 2 (TLR2) expressed in human embryonic kidney (HEK) cells, inducing IL-8 production, and engaged mouse TLR2 on bone marrow-derived dendritic cells, inducing TNF release. Hib conjugated to the carrier proteins CRM197 and tetanus toxoid did not engage TLR2 on HEK or dendritic cells. Engagement of TLR2 by Hib-OMPC was MyD88 dependent, as Hib-OMPC-induced TNF production was ablated in MyD88 knockout (KO) mice. Hib-OMPC was significantly less immunogenic in TLR2 KO mice, inducing lower Hib PS IgG and IgM titers compared with those in wild-type mice. Splenocytes from OMPC-immunized TLR2 KO mice also produced significantly less IL-6 and TNF-α than those from wild-type mice. Hib-OMPC is unique among glycoconjugate vaccines by engaging TLR2, and the ability of Hib-OMPC to elicit protective levels of Abs after one dose may be related to TLR2-mediated induction and regulation of cytokines produced by T cells and macrophages in addition to the peptide/MHC II-dependent recruitment of T cell help commonly afforded by carrier proteins. TLR2 engagement by an adjuvant or carrier protein may be a useful strategy for augmentation of the anti-PS Ab response induced by glycoconjugate vaccines. The Journal of Immunology, 2004, 172: 2431–2438.

Bacterial polysaccharides (PS) are T cell-independent Ags, do not induce immunological memory or yield a booster response with repeated immunization, and are poorly immunogenic in children <24 mo of age (1–6). Conjugation of capsular PS such as those from Haemophilus influenzae type b (Hib) and Streptococcus pneumoniae to a carrier protein renders them immunogenic in infants and capable of eliciting memory, booster responses, and isotype switching of anti-PS Abs to IgG1 (7–9). These glycoconjugate vaccines, using a variety of carrier proteins, have had a significant role in the virtual eradication of Hib disease from the United States and in producing a marked decline in invasive pneumococcal infections in small children (7–9).

Pure PS are not processed by APC nor presented in context of MHC II to T cells. The enhanced immunogenicity of glycoconjugate vaccines compared with native bacterial PS is thought to be due to processing of carrier protein by the APC and presentation of carrier protein-derived peptides in the context of MHC class II molecules to Th cells, followed by induction of cytokine production. The cytokines produced by activated CD4+ T cells are presumed to stimulate PS-specific B cells to undergo clonal expansion, differentiation to memory cells, isotype switching to predominantly IgG1, and increased production of PS-specific Abs (2, 10). Unfortunately, glycoconjugate vaccines require multiple doses to induce long-lasting protective levels of anti-PS Abs in infants, greatly increasing the expense and making these vaccines difficult to use in the developing world (11).

Despite the requirement for multiple doses of glycoconjugate vaccines to induce protective Ab levels, the Hib vaccine in which the PS is conjugated to a complex of outer membrane proteins from meningococcus (OMPC) often induces protective levels (i.e., ≥1.0 μg/ml) of anti-Hib PS Abs after a single dose (12, 13). This characteristic of the Hib-OMPC conjugate vaccine is clinically relevant, as breakthrough infections in infants were recently observed when other conjugate vaccines were substituted for Hib-OMP as the first dose in a heavily Hib-colonized Native American population (14). Different immunogenicities of glycoconjugate vaccines that use various carrier proteins are presumed to be a result of processing of the different carrier proteins, yielding divergence in peptides and subsequent epitope specificity of CD4+ T cells that are recruited, although the precise mechanism explaining the early immunogenicity of Hib-OMPC compared with other Hib conjugate vaccines is unclear. Previous data have suggested that OMPC has adjuvant-like activity due to activation of macrophages and to

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Abbreviations used in this paper: PS, polysaccharide; Hib, Haemophilus influenzae type b; KO, knockout; OMPC, outer membrane protein complex; TLR, Toll-like receptor; WT, wild type.
a mitogenic effect on B cells (15). In addition, it has been previously reported that porin proteins from the surface of Neisseria meningitidis, one of the major constituents of Neisseria outer membrane proteins, increase the expression of the B cell costimulatory molecule B7-2 and require Toll-like receptor 2 (TLR2) expression for this effect (16, 17). In the current report we show that uniquely among the currently used glycoconjugate vaccine carrier proteins, OMPC and OMPC conjugated to Hib PS engage human and mouse TLR2 in a MyD88-dependent manner and induce cytokine production in vitro, and that the absence of TLR2 significantly reduces the immunogenicity of Hib-OMPC vaccine.

Materials and Methods

OMPC and other Hib vaccines

Purified OMPC from Neisseria meningitidis was supplied by Dr. A. Shaw (5.0 mg/ml 0.9% sodium chloride; Merck, West Point, PA) and was prepared as previously described (12). Hib-OMPC vaccine (Merck) was purchased commercially and contained 7.5 μg of Hib capsular PS, 125 μg of OMPC randomly cross-linked to Hib PS, and 225 μg of aluminum hydroxyposphate sulfate in 0.5 ml of 0.9% sodium chloride. Hib-CRM197 vaccine (Wyeth-Lederle, West Henrietta, NY) containing 10 μg of Hib PS covalently linked to 25 μg of CRM197 (Cross Reactive Material; a nontoxic, single amino acid mutant of diphtheria toxin) in 0.5 ml of 0.9% saline used in vitro and in mouse immunizations was obtained commercially. Hib-tetanus vaccine (ActHIB; Aventis Pasteur, Swiftwater PA), which contained 10 μg of purified Hib PS conjugated to 24 μg of inactivated tetanus toxoid in 0.5 ml of 0.9% saline, was also obtained commercially. Experimental grade (not for vaccine use) unconjugated Hib PS was supplied by Dr. R. Eby (Wyeth).

TLR-specific stimuli and controls

LPS from Escherichia coli 0111:B4 was purchased from Sigma-Aldrich (St. Louis, MO) and purified to eliminate contaminating TLR2 activation as previously described (18). Synthetic Mycoplasma-associated lipoprotein of 2 kDa, a TLR2-engaging microbial molecule, was purchased from EMC Microcollections (Tuebingen, Germany). Heat-killed Listeria monocytogenes was provided by Dr. G. Teti (Università degli Studi Messina, Messina, Italy) and prepared as previously described (19). Porin purified from Neisseria meningitidis was a gift from Dr. P. Massari (Boston University, Boston, MA). Human TNF-α was obtained from PeproTech (Rocky Hill, NJ). The LPS antagonist compound B1287 (also called e5564), which competitively binds to a control, TLR2-silenced HEK cells responsive to the respective TLR ligands. We have previously reported that porin proteins from the surface of H. influenzae TYPE B OMPC GLYCOCONEJUGATE VACCINE ENGAGES TLR2

Stable cell lines expressing TLR2 engagement in HEK and mouse dendritic cells

Stable cell lines expressing fluorescent versions of TLR2, TLR4/MD-2, or HEK-pcDNA were transplanted and seeded into 96-well plates at a density of 4 × 10^4 cells/well in triplicate. The cells were stimulated the following day with the various day with the day various vaccines, carrier proteins, and controls, and IL-8 release was measured in the supernatants using a commercially available ELISA (DuoSet; R&D Systems, Minneapolis, MN). The experiment was repeated three times with similar results.

Mouse dendritic cells were provided by culturing bone marrow cells from C57BL/6 mice or age- and sex-matched MyD88-deficient mice in RPMI 1640 medium supplemented with 10% FCS, 10 μg/ml ciprofloxacin (Mediatech, Herndon, VA), 10 ng/ml GM-CSF (PeproTech), and 50 μM 2-3′-Me (Sigma-Aldrich) for 7 days. Dendritic cells were seeded into 96-well plates (5 × 10^5 cells/well) 1 day before stimulation in triplicate. Cell culture supernatants were removed and analyzed for cytokines by ELISA (R&D Systems). These experiments were repeated twice with similar results.

Mouse strains

TLR2−/− and MyD88−/− mice were generated as described previously (23, 24) and were originally supplied by Dr. S. Akira (Osaka University, Osaka, Japan). TLR2 knockout (KO) mice were originally a 129sv strain and were four (TLR2+/− or five (MyD88−/−) generations backcrossed into C57BL/6 mice before use. C57BL/6 mice were used as the control wild-type (WT) mice (The Jackson Laboratory, Bar Harbor, ME). Mice were housed in microisolator cages in a pathogen-free facility of the Animal Resource Center of Case Western Reserve University. The animal care and use committee of Case Western Reserve University approved the animal protocols that were used. Animals were fed an ad libitum diet of autoclaved Teklad mouse chow (Harlan, Madison, WI).

Immunization of mice with glycoconjugate vaccines and OMPC

WT mice and TLR2 KO mice, aged 8–13 wk, were injected i.p. with Hib-OMPC (5.0 μg Hib capsular PS, 83.5 μg OMPC in 100 μl), OMPC (83.5 μg in 100 μl) or PBS (100 μl) on day 0. These doses were chosen based on dose-response experiments yielding the amount of conjugated Hib PS that generated optimal Hib PS Ab levels (data not shown). The WT group was treated seven mice, and the KO group contained 13 mice. Mice were reimmunized on dose-1, dose-2, and dose-3 with the same doses of Hib-OMPC, OMPC, or PBS previously administered to mimic the multiple dosing that is used in human infant immunization with Hib-OMPC. Tail bleeds were performed each week to obtain serum samples to measure Hib PS-specific and OMPC-specific Ab levels by ELISA. Two days after the fourth injection, a subset of seven WT and six KO mice immunized with OMPC were euthanized by CO2, and their spleens were removed. Splenocytes from both WT and TLR2 KO mice were isolated, seeded at a density of 1 × 10^6 cells/well in 12-well polystyrene tissue culture plates (BD Biosciences, Franklin Lakes, NJ), and stimulated for 48 h with Hib-OMPC (50 μl; 0.5 mg/ml Hib PS, 25 μg OMPC), OMPC (25 μg OMPC, 5.0 μg/ml purified Hib PS, 5.0 μg/ml Hib PS conjugated to CRM197 (Wyeth-Lederle, Pearl River, NY), or 5.0 μg/ml Hib PS conjugated to inactivated tetanus toxoid (ActHIB; Aventis Pasteur, Swiftwater, PA). Cytokines produced by mouse splenocytes after Ag stimulation were measured by ELISA on cell supernatants as described below.

ELISA measurement of Hib PS and OMPC Abs titers

Hib PS-specific capsular polysaccharide Abs were measured by ELISA as previously described (25, 26). Hib oligosaccharide-human serum albumin conjugate-coating Ag (provided by Dr. M. Nahm, University of Alabama, Birmingham, AL) was diluted in PBS to 4 μg/ml and 100 μl/well was adsorbed onto the wells of polystyrene microtiter plates (Nunc, Naperville, IL). Dilutions of the test serum were made in PBS containing 0.05% Tween and 1% BSA, and 100 μl of a 1/50 diluted serum from each mouse at each time point postimmunization was added to coated wells in duplicate after washing, then incubated overnight. After washing with PBS-Tween (0.05%), goat anti-mouse, class-specific Abs conjugated to alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL) diluted in PBS-Tween (0.05% Tween and 1% BSA) were added to each well. Plates were washed again after 2 h, and p-nitrophenyl phosphate (disodium; Sigma-Aldrich) was then added. Absorbance was read at 415 nm on a Microplate ELISA reader (model 550; Bio-Rad, Hercules, CA). Plates were normalized by reading each plate at the time the control sera were obtained (Hib-PS hyperimmunized mice) were at identical OD. Hib PS-specific Abs in mouse sera before and after immunization were detected using a similar ELISA in which microtiter plates were coated with 1.0 μg/ml purified OMPC (Merck, West Point, PA) on sera from a subset of five WT and nine immunized KO mice. After washing, 1/50 diluted mouse sera from each mouse at each time point were added in duplicate, followed by goat anti-mouse, class-specific Abs conjugated to alkaline phosphatase (Southern Biotechnology Associates). The assay plates were developed and read as described for the Hib PS ELISA above.
Detection of cytokine production by mouse splenocytes

WT and TLR2 KO mouse splenocytes obtained after four immunizations (wk 0, 2, 4, and 6) with 83.5 μg of OMPC (seven WT and six KO mice) were stimulated for 24 h with Hib-OMPC (5 μg/ml Hib PS, 83.5 μg/ml OMPC), 83.5 μg/ml OMPC, 5 μg/ml unconjugated Hib PS, 5 μg/ml Hib PS conjugated to CRM197 (HibTITER; Wyeth Lederle Laboratories), or 5 μg/ml Hib PS conjugated to inactivated tetanus toxoid (ActHIB; Aventis Pasteur, Swiftwater, PA). Supernatants were collected, and cytokine production by the stimulated cells was measured using ELISA, as previously described, in duplicate wells (26, 27). IL-1, IL-2, IL-4, IL-5, IL-6, IFN-γ, and TNF-α production was measured using OptEIA Mouse Cytokine Sets (BD PharMingen, San Diego, CA), and IL-10 and IL-12 were measured using Duo ELISA Development Sets (R&D Systems). Polystyrene microtiter plates (MaxiSorp; Nunc) were coated with 100 μl of the desired cytokine-specific capture Ab diluted according to the manufacturer’s protocol.

Statistics

Due to the skewness of the OMPC and Hib PS Ab levels, all analyses were performed on the natural logarithmic (loge) scale. Measures of IgG, IgM, and total Ig levels (absorbance) were analyzed and compared between the KO and WT mice groups by a repeated measures analysis for unbalanced data using maximum likelihood implemented with SAS Proc Mixed (version 8.2; SAS Institute, Cary, NJ). For each outcome, a model assuming separate geometric means at each time point was fit to the data. Under this model, a different geometric mean level is estimated at each time point from wk 1 through wk 6 for each of the two groups. The covariance structure of the repeated measures was modeled using an unstructured model that allows the variances and correlations of the levels to differ over time. For each model, the baseline (wk 0) value was controlled for in the analysis by including the term in the model. A test between the groups was performed at each week postbaseline, and comparison between the groups at baseline was performed using the two-sample t test.

Results

OMPC and Hib-OMPC, but not other Hib glycoconjugate, vaccines engage human TLR2 expressed on HEK cells

We first compared the effect of OMPC and other glycoconjugates on HEK-TLR2 transfected cells with control cells stably expressing the pcDNA3 plasmid (empty vector). The cells were incubated

![FIGURE 1. Hib-OMPC and OMPC engage human TLR2. HEK cells stably transfected with TLR2-YFP and MD-2 or with empty vector pcDNA3 were incubated with increasing concentrations of heat-killed Listeria monocytogenes (HKLM) or three different Hib conjugate vaccines (A). Cell supernatants were analyzed for IL-8 secretion by ELISA in triplicate wells after 16 h of stimulation. R. The stimulatory activity of the nonconjugated molecules (OMPC or Hib PS) was compared with that of porin prepared from N. meningitidis. These experiments were repeated three times with similar results, and the data shown are from one representative experiment.](http://www.jimmunol.org/)

![FIGURE 1.](http://www.jimmunol.org/)
with the three different glycoconjugate vaccines, Hib-OMPC, Hib-tetanus, and Hib-CRM197, and as a positive control with heat-killed *Listeria monocytogenes*, a potent TLR2-stimulating bacteria. Hib-OMPC induced vigorous production of IL-8 by the TLR2-transfected HEK cells as did heat-killed *Listeria monocytogenes* (Fig. 1A). Neither preparation was able to elicit an IL-8 response in the control cells. Hib-CRM197 and Hib-tetanus vaccines did not engage TLR2 (Fig. 1A). We then used the single components of the conjugate vaccine Hib-OMPC to decipher which part of the conjugate was responsible for the TLR2 activation. As a further positive control and to compare the TLR2 activity, we used a porin preparation derived from *Neisseria meningitidis*, which is known to strongly engage TLR2 (17). The TLR2-stimulating activity could be attributed to the protein (OMPC) part of the conjugate vaccine, as unconjugated Hib PS did not significantly engage TLR2 and was found to be equally potent to the TLR2-stimulating activity of neisserial porin (Fig. 1B).

We next tested whether the different conjugate vaccine preparations had TLR4/MD-2 stimulatory activity. Due to the ubiquitous presence and stability of bacterial LPS, contamination of the reagents and/or reaction mixtures was possible. We thus used the LPS antagonist compound B1287 to block the LPS activity present in the various vaccine preparations or ligands. The compound B1287 did not significantly influence the TLR2-stimulating activity of either OMPC or the Hib-OMPC conjugate vaccine (Fig. 2). By contrast, TLR4/MD2-expressing HEK cells responded vigorously to LPS, and the addition of the compound Hib PS did not significantly engage TLR2 and was found to be equally potent to the TLR2-stimulating activity of neisserial porin (Fig. 1B).

**OMPC and Hib-OMPC stimulate TNF release from murine bone marrow-derived dendritic cells that is MyD88 dependent**

We tested the ability of murine bone marrow-derived dendritic cells to respond to the different vaccine preparations as Ag-processing cells may be some of the first cells to interact with glycoconjugate vaccines after immunization. To assess the necessity of the TLR adapter molecule MyD88 for dendritic cell stimulation by OMPC we also included dendritic cells prepared from MyD88−/− mice in these experiments. Both OMPC and Hib-OPMC robustly stimulated murine DCs to release TNF-α in a MyD88-dependent fashion (Fig. 3A). As seen before with the LPS-sensitive HEK-TLR4/MD-2 cell line (Fig. 2), Hib PS and Hib-tetanus vaccine led to TNF-α production at higher doses. However, these stimulatory activities could be completely abrogated by addition of the LPS antagonist B1287, indicating that the activity seen is probably due to LPS contamination of these preparations (Fig. 3B). From these data we conclude that murine cells are stimulated by OMPC in a similar manner as human cells, and that the responses are dependent on the TLR adapter molecule MyD88.

**The ability to make Hib capsular polysaccharide Abs after Hib-OMPC immunization is reduced in TLR2 KO mice**

WT and TLR2 KO mice were immunized with three doses of Hib-OPMC vaccine in a dose previously found to yield optimal Hib PS Ab levels in the WT mice and in a regimen designed to mimic the multiple doses administered to human infants and were bled weekly to measure serum anti-Hib PS Ab levels. WT mice had significantly higher IgM anti-Hib PS Ab levels 6 wk after the first immunization with Hib-OMPC than TLR2 KO mice (*p* = 0.038; Fig. 4A). In addition, WT mice had significantly more IgG anti-Hib PS Abs at wk 4, 5, and 6 after the first immunization (*p* = 0.012, 0.025, and 0.012, respectively; Fig. 4B). Finally, total anti-Hib PS Ab Ig levels at wk 4, 5, and 6 after the first immunization in WT mice were also higher compared with those in TLR2 KO mice (*p* = 0.011, 0.012, and 0.003, respectively; Fig. 4C). No detectable IgA anti-Hib PS Abs were found in either WT or TLR2 KO mice, and control mice injected with PBS did not make Hib PS Abs (data not shown).

**Abs against the carrier protein OMPC are also reduced in TLR2 KO mice**

Serum OMPC Ab levels after WT and TLR2 KO mouse immunization with three doses of Hib-OMPC were also measured by ELISA in a subset of WT and KO mice. WT mice had significantly higher IgM OMPC Ab levels at wk 1, 4, and 5 postimmunization (*p* = 0.002, 0.047, and 0.009, respectively; Fig. 5A). WT mice had significantly higher IgG OMPC Ab levels wk 1 postimmunization (*p* = 0.001; Fig. 5B), but IgG levels were similar in both groups after wk 1. Finally, total Ig levels of OMPC Abs were also higher in WT mice wk 2 postimmunization (*p* = 0.012; Fig. 5C). Control mice injected with PBS did not make OMPC Abs (data not shown).

**Splenocytes from TLR2 KO mice make significantly less IL-6 and TNF-α than WT mice after immunization with OMPC**

A subset of WT and TLR2 KO mice receiving immunization with OMPC were sacrificed, and their splenocytes were stimulated with OMPC, Hib-OMPC, and a variety of other glycoconjugate vaccines to determine the cytokines produced after exposure to OMPC. Splenocytes obtained from OMPC-immunized TLR2 KO mice made significantly less IL-6 and TNF-α than WT mice after stimulation with OMPC ex vivo (Fig. 6, A and B, respectively). There were no differences in the production of IL-1, IL-2, IL-4,
IL-5, IL-10, or IFN-γ production by the stimulated splenocytes from TLR2 KO or WT mice (data not shown).

**Discussion**

Glycoconjugate vaccines consisting of the capsular PS of Hib have been effective in greatly reducing invasive infections caused by this pathogen. Unfortunately, most of the available glycoconjugate vaccines require three or four doses to elicit long-lasting immunity in small infants most susceptible to infection. Interestingly, one glycoconjugate vaccine that uses a carrier protein derived from the OMPC of *N. meningitidis* is often capable of inducing protective levels of anti-capsular Hib Abs after only one dose. We set out to determine whether this carrier protein interacted with the immune system in ways other than providing carrier protein-derived T cell help, as has been hypothesized to be the major contribution of carrier proteins to enhanced PS-specific Ab responses. We found that OMPC, either unconjugated or chemically linked to Hib PS, vigorously and specifically engaged TLR2 in cell lines that stably express human TLR2 and produce IL-8 after TLR2 engagement. The OMPC was specific for TLR2 and was not contaminated with LPS, as cell lines stably transfected with TLR4 and MD-2 (but not TLR2) were not stimulated by OMPC or Hib-OMPC. In addition,
the engagement of TLR2 was MyD88 dependent, as MyD88−/− cells could not be stimulated by OMPC. Finally, Hib conjugated to two other carrier proteins, tetanus toxoid and CRM 197, a nontoxic diphtheria toxin mutant, as well as unconjugated Hib PS did not engage TLR2. These vaccines did slightly engage TLR4, but only due to minor LPS contamination, as the addition of the LPS antagonist B1287 abrogated TLR4 engagement. The unconjugated Hib PS engaged TLR4 as expected, as this was an experimental lot of PS that was not vaccine grade and contained traces of LPS.

The OMPC used in the Hib-OMPC vaccine consists of the OMPC of *N. meningitidis* that is randomly cross-linked to the Hib capsular polysaccharide. Previous studies have demonstrated that the PorB porin protein from the outer membrane of *N. meningitidis* stimulates B cells and up-regulates surface expression of B7-2 via engagement with TLR2 in a MyD88-dependent fashion (16, 17). OMPC contains various outer membrane proteins, including porins; it thus seems likely that the TLR2 engagement mediated by Hib-OMPC vaccine is due to the presence of porins, as one of the carrier protein components linked to the Hib capsular PS.

As the Hib-OMPC glycoconjugate vaccine is known to be immunogenic after fewer doses than other Hib glycoconjugate vaccines in infants, we wanted to determine whether the presence of the TLR2 ligand was crucial to the enhanced immunogenicity in an animal model. TLR2 KO mice made significantly less IgM and IgG anti-Hib PS Abs after immunization with Hib-OMPC than WT mice. These data suggest that much of the enhanced immunogenicity of the Hib-OMPC vaccine may be related to the presence of TLR2 ligands and that the use of such ligands may be a useful method to improve the immunogenicity of bacterial PS and glycoconjugates (28).

We next determined whether the reduced immunogenicity of the PS component of Hib-OMPC in TLR2 KO mice might be related to differences in the induction of cytokines after exposure to OMPC. Interestingly, splenocytes obtained from OMPC-immunized WT mice made significantly more IL-6 after ex vivo stimulation with OMPC than splenocytes from OMPC-immunized KO mice. IL-6 was originally described as the B cell differentiation factor, which encourages terminal differentiation and maturation of Ab-producing plasma cells (29). In addition, IL-6 is known to induce IL-4 production and promote the differentiation of Th2 CD4+ T cells, the T cells that appear to be most important to the enhanced immunogenicity of bacterial glycoconjugate vaccines.
immunogenicity of Hib-OMPC glycoconjugate may also be related to induction of TNF-α after TLR2 engagement.

Interestingly, the TLR2 KO mice also made less IgM and IgG OMPC Abs than WT mice, although these differences were much less pronounced than those observed with Hib PS Abs. These data are consistent with a recent report showing decreased outer surface protein A Abs made in TLR2 KO mice after immunization with outer surface protein A from Borrelia burgdorferi, in which the response to a T cell-dependent protein Ag was blunted in the KO mice. Macrophages from TLR2 KO mice were also found to produce less IL-6 than those from WT mice after immunization. Cooperation between engagement of TLR1 and TLR2 was also observed, as was altered surface expression of TLR1 in low responder humans (33). TLR2 receptor activation is believed to occur after association with other TLRs, such as TLR1 and TLR6, and other recent experiments in mice with targeted lesions in TLR1 or TLR6 revealed that coengagement of TLR2 with TLR1 or TLR6 results in the recognition of subtle differences in the molecular structure of the ligand. For example, TLR1/TLR2 cooperatively recognize synthetic triacylated lipoproteins, and TLR2/TLR6 coengagement is needed for the recognition of synthetic diacylated lipoproteins (34, 35). As the HEK cells we used are known to express TLR1 and TLR6, we assume that coengagement occurred as part of the response we observed to TLR2. The role of TLR2 interactions with other TLR in the OMPC-mediated enhancement of Hib PS Ab levels remains to be investigated.

Dendritic cells presumably initiate T cell help that is necessary for the development of the enhanced immunogenicity and memory responses observed after immunization with glycoconjugate vaccines via uptake of the vaccine and processing and presentation of carrier protein-derived peptides to specific T cells in the context of MHC II molecules. We therefore were interested to determine whether Hib-OMPC directly stimulated dendritic cells. We found that Hib-OMPC and OMPC vigorously induced production of TNF-α by bone marrow-derived dendritic cells in a MyD88-dependent fashion. As TNF-α is known to induce MHC expression, it seems possible that TLR2 engagement by the glycoconjugate in these cells may yield improved Ag processing efficiency of the carrier protein, resulting in enhanced carrier protein-specific T cell help. Others have also found that use of an Ag that directly ligates APC TLR2 improved the efficiency of Ag presentation and subsequent CD4+ T cell responses (36). Furthermore, dendritic cell activation leads to up-regulation of costimulatory molecules, which also enhances the activation of peptide-specific T cells. It will be important to determine whether the OMPC component of the glycoconjugate vaccine yields enhanced carrier protein-specific CD4+ T cell stimulation that represents another mechanism for the PS-specific immunogenicity of this vaccine after fewer doses than other Hib glycoconjugate vaccines. As OMPC contains multiple proteins, isolation of each component may be required in future experiments to confirm which protein mediates TLR2 engagement and/or enhanced CD4+ T cell stimulation.

We conclude that Hib-OMPC glycoconjugate vaccine engages human TLR2, which enhances the immunogenicity of Hib PS. The mechanism of enhanced PS-specific immunogenicity of the Hib-OMPC may be related to increased induction of IL-6 and TNF-α. As a glycoconjugate vaccine containing this carrier protein is already in extensive use as a licensed vaccine for the prevention of Hib disease in infants, further use of TLR2 agonists to enhance vaccine immunogenicity in humans may be readily feasible. These data may be useful in the design of second-generation glycoconjugate vaccines that require fewer doses to achieve protective Ab levels against the capsular PS of bacterial pathogens.
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