Research article

Innate Immunity

16(5) (2010) 322–332
© SAGE Publications 2010
ISSN 1753-4259 (print)
10.1177/1753425909346973

Enhanced antibody responses to a detoxified lipopolysaccharide-group B meningococcal outer membrane protein vaccine are due to synergistic engagement of Toll-like receptors

Wilbur H. Chen[1], Subhendu Basu[1], Apurba K. Bhattacharjee[2], Alan S. Cross[1]

[1]*Center for Vaccine Development, University of Maryland School of Medicine, Baltimore, Maryland, USA*
[2]*Walter Reed Army Institute of Research, Silver Spring, Maryland, USA*

When given passively or elicited actively, antibodies induced by a detoxified *Escherichia coli* Rc chemotype (J5) mutant lipopolysaccharide (J5dLPS)–group B meningococcal outer membrane protein (OMP) complex vaccine protected animals from lethal sepsis. The protection from sepsis is believed to be dependent on high levels of antibodies against the core glycolipid (CGL), a region of LPS that is rather conserved among *Enterobacteriaceae*. The addition of unmethylated deoxycytidyl-deoxyguanosine dinucleotide (CpG)-containing oligodeoxynucleotides (ODN) was used as an immuno-adjuvant to improve antibody responses. In preparation for a Phase I human trial, we elucidated potential contributions by which the sepsis vaccine (J5dLPS–OMP) and CpG ODN might enhance the antibody response and provide evidence that the generation of immune responses is Toll-like receptor (TLR) dependent. Toll-like receptor 2, TLR4, and TLR9 were each essential for generating robust cytokine and antibody responses. The signature cytokine of dendritic cells, interleukin-12, was one of the cytokines that demonstrated synergy with the optimal TLR ligand/engagement combination. We conclude that the involvement of multiple TLRs upon immunization was critical for the generation of optimal antibody responses. These observations provide further evidence for the inclusion of innate immune-based adjuvants during the development of next-generation vaccines.

Keywords: antibody, cytokine, endotoxin vaccine, Toll-like receptor (TLR), synergy

INTRODUCTION

Toll-like receptor (TLR) agonists have been proposed as adjuvants to boost the response to poorly immunogenic antigens. However, relatively little is known regarding the immune response to vaccine candidates when concurrently adding TLR agonists. We describe a vaccine antigen, itself a TLR agonist, with the use of additional TLR agonists to bring about significantly improved antibody responses. In this study, we explored the individual contribution of each TLR in these immune responses.

Gram-negative bacterial infections are a clinical problem because effective antimicrobial treatment is limited by the continuing emergence of multiple-drug resistant pathogens.[1] Since the core oligosaccharides and lipid A portion of endotoxin (or lipopolysaccharide, LPS) are rather conserved among *Enterobacteriaceae*, immunotherapy with anti-endotoxin antibodies has been proposed for protection against a wide variety of pathogenic Gram-negative bacteria.[2–5]

An Rc chemotype mutant bacterial strain derived from *Escherichia coli* O111:B4 (designated J5) lacks the O-polysaccharide side chain on its LPS, thereby exposing a conserved core oligosaccharide region to the immune system.[2] Early studies using antiserum against *E. coli* J5 showed protection in animal models of lethal sepsis.[3,4] In a prospective clinical trial, Ziegler and colleagues[5]

Received 20 July 2009; Revised 18 June 2009; 20 July 2009; Accepted 3 August 2009

Correspondence to: Wilbur H. Chen MD, Center for Vaccine Development, 685 West Baltimore Street, HSF 1 Suite 480, Baltimore, MD 21201, USA. Tel: +1 410 706 5328; Fax: +1 410 706 6205; E-mail: wchen@medicine.umaryland.edu

demonstrated a significant reduction in mortality with J5 antiserum in hospitalized Gram-negative bacterial septic patients, particularly for patients with profound septic shock (those with hypotension requiring vasopressor agents). However, subsequent clinical trials using antibodies against LPS, including those targeting the lipid A portion, failed to demonstrate efficacy[6–9] and immune-based antisepsis therapies moved to a strategy of targeting cytokines and other inflammatory mediators.

Because of the striking initial results with anti-core LPS (J5), we revisited this approach and demonstrated protection in a neutropenic rat model of sepsis with passive administration of affinity-purified polyclonal anti-J5 IgG.[10] Since a whole bacterial vaccine was highly reactogenic, we prepared a subunit vaccine composed of detoxified J5 LPS non-covalently complexed with group B meningococcal outer membrane protein (J5dLPS–OMP, final subunit vaccine).[11] Passive and active administration of anti-core glycolipid (CGL) IgG, elicited by J5dLPS–OMP, protected animals from lethal sepsis in both neutropenic rat and murine cecal-ligation and puncture models.[11,12]

Protection from sepsis with anti-CGL antibody was dose-dependent;[10] therefore, when a human Phase I trial demonstrated only a 2–3-fold increase in anti-CGL IgG, the addition of an adjuvant was considered.[13] Synthetic immunostimulatory oligodeoxynucleotides (ODNs) containing unmethylated deoxycytidyl-deoxyguanosine dinucleotide (CpG) motifs are in advanced clinical development as vaccine adjuvants[14–17] and, in anticipation of another phase 1 study of the J5dLPS–OMP vaccine plus CpG, was added to improve the antibody response. Compared to the vaccine alone, intraperitoneal (i.p.) vaccination with J5dLPS–OMP plus CpG ODN resulted in 5-fold higher serum anti-CGL IgG, lower levels of pro-inflammatory cytokines, and less bacterial dissemination in a murine cecal ligation and puncture (CLP) model of lethal polymicrobial sepsis.[18] When the sepsis vaccine was administered intranasally, the addition of CpG ODN significantly improved the antibody response in serum and bronchial washings and provided protection in a murine model of heterologous Gram-negative bacterial pneumonia.[19]

The mechanisms by which the J5dLPS–OMP subunit vaccine and CpG ODN induce markedly improved IgG antibody responses have not been elucidated. We hypothesized that this vaccine regimen would utilize three TLRs: (i) the detoxified LPS would stimulate TLR4; (ii) the OMP is believed to signal via TLR2;[20] and (iii) the CpG is recognized by TLR9. In this study, we provide evidence that the generation of an optimal immune response was dependent on the contribution of each of the three TLRs and these antibody responses *in vivo* correlated with distinct cytokine responses *in vitro*. These data suggest that multiple TLR engagement optimize the vaccine-induced immune response.

Materials and Methods

Reagents

The J5dLPS–OMP vaccine was prepared under Good Manufacturing Practice (GMP) conditions as previously described.[13] Briefly, this subunit vaccine is composed of *E. coli* J5 LPS whose ester-linked fatty acids were removed by alkaline *O*-deacylation, resulting in ~100-fold less pyrogenicity.[11] The detoxified LPS was non-covalently complexed with the OMP of *Neisseria meningitidis* group B, derived from LPS-free and membrane-free proteosomes. The final concentrations of LPS and OMP in the vaccine were 100 μg/ml and 136 μg/ml, respectively.

Synthetic immunostimulatory CpG ODN 10103 was purchased from Coley Pharmaceutical Group (Ontario, Canada). These ODN sequences are optimized for primates but can be used in mice. A synthetic ligand for TLR2, Pam_3Cys, was purchased from EMC Microcollections (Tübingen, Germany). Purified LPS from *E. coli* O111:B4 (both the smooth strain and the J5 mutant) was obtained from List Biological Laboratories (Campbell, CA, USA). Purified (LOS-free) *N. meningitidis* group B OMP (GBOMP) was provided as a gift by Apurba Bhattarcharjee (formerly of WRAIR).

Mice

Six–eight-week-old C3H/HeJ, C3H/HeN, and C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME, USA) and age-matched TLR2 knockout ($TLR2^{-/-}$) mice, bred in our animal facility from mating pairs of B6.129-$Tlr2^{tm1Kir}$/J mice originally purchased from Jackson Laboratories, were housed in micro-isolator cages in a pathogen-free animal facility. The Institutional Animal Care and Use Committee of the University of Maryland School of Medicine approved the animal protocols that were used.

Peritoneal macrophages

Three days after intraperitoneal (i.p.) injection of 3 ml of 4% thioglycollate, 10 ml sterile PBS (Gibco-BRL, Frederick, MD, USA) was injected into the peritoneal cavity and lavage fluid containing exudate cells were withdrawn. Cells were centrifuged and resuspended in culture medium prior to counting. If overt red blood cell contamination was present in the pellet, the cells were incubated in ice-cold red cell lysis buffer and re-washed.

The remaining cells were plated to 24-well Costar plates (Corning, NY, USA) at a concentration of 1.0×10^6 cells/ml in complete medium, consisting of RPMI-1640 (Gibco-BRL) with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA, USA), 10 mM HEPES (Gibco-BRL), and 1% Pen/Strep. After resting 18 h at 37°C with 5% CO_2, plates were washed and the adherent cells (peritoneal macrophages) were provided fresh complete medium. Primary macrophages were stimulated 18 h with agonists at 37°C with 5% CO_2 and supernatants and cell lysates were collected and stored at –80°C until cytokine analysis could be performed.

Splenocytes

Mice were euthanized and spleens were obtained by sterile technique. After each spleen was homogenized in 10 ml of RPMI-1640, the cell suspension was allowed to rest for 10 min at 20–22°C to allow fibrous tissue to settle. The remaining single cell splenocyte suspension was centrifuged and the pellet was resuspended in ice-cold red cell lysis buffer. After washing and resuspending in fresh complete medium, splenocytes were counted, and plated at 1.0×10^6 cells/ml to 24-well Costar plates. Splenocyte cultures were stimulated 18 h with agonists at 37°C with 5% CO_2 and the supernatants were collected and stored at –80°C until cytokine analysis could be performed.

Transfections

Human embryonic kidney epithelial cells (HEK293T) were seeded into 12-well Costar plates (Corning, NY) at 2.0×10^5 cells per well and incubated 18 h. Cells were washed with PBS and co-transfected for 3 h with pcDNA3-huTLR4, pcDNA3-huCD14, pEFBOS-HA-huMD-2 or pcDNA3-huTLR2 together with a NF-κB reporter (ELAM-1 luciferase) and the transfection normalization control plasmid TK-renilla. The final DNA quantity was adjusted to 1.5 μg/well with the pcDNA3 empty vector (Invitrogen, Carlsbad, CA, USA). The transfection was carried out with SuperFect transfection reagent (Qiagen Inc., Valencia, CA, USA). Cells were washed with PBS and rested at 37°C with 5% CO_2 in cDMEM for 18 h. The transfected cells were washed with PBS, placed in serum-free DMEM and stimulated with LPS, Pam_3Cys, or J5dLPS–OMP. Cells were lysed in 1 × reporter assay lysis buffer (Promega Corporation, Madison, WI, USA) and luciferase (Promega, Luciferase assay system) and renilla (Promega, Renilla luciferase assay system) activities were analyzed, using an LMax™ II384 Microplate Reader Luminometer (Molecular Devices Corp., CA, USA). The ELAM-1 promoter activity was calculated by normalizing each sample's luciferase activity to the TK-renilla activity measured within the same sample, and represented as relative luciferase units (RLUs).

Peripheral blood mononuclear cells

Whole blood was freshly obtained from healthy young adults, under an IRB-approved protocol, via venipuncture and collected in heparinized Vacutainer tubes (Becton Dickenson, NJ, USA). Peripheral blood mononuclear cells (PBMCs) were isolated by established methods. Briefly, heparinized blood was mixed with an equal volume of RPMI-1640, layered over Ficoll gradient (Cellgro, VA, USA), centrifuged, and the cloudy interface was collected, washed, and counted. Resuspended PBMCs in complete medium at 1.5×10^6 cells/ml were plated to a 24-well Costar plate. After confirmation of ≥95% viability, by Trypan Blue dye exclusion, the PBMCs were stimulated 18 h with agonists at 37°C with 5% CO_2 and the supernatants were collected and stored at –80°C until cytokine analysis could be performed.

Immunizations and antibody measurement

Mice were immunized i.p. on weeks 0, 2, and 4 with a 10 μg dose of J5dLPS–OMP ± a 25 μg dose of CpG ODN delivered in a total volume of 500 μl; dilutions were performed with sterile PBS (Gibco-BRL). At least 2 weeks after the last immunization, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation prior to collecting heparinized blood via cardiac puncture. Serum was collected and stored at 4°C until analysis, usually within one week. Serum samples were measured for anti-CGL IgG antibody by a previously described ELISA method.[10] Briefly, 96-well microtiter plates (Costar) were pre-treated with 50 μg/ml of poly-L-lysine hydrobromide (Sigma, St Louis, MO, USA) prior to coating with 2 μg/ml of purified J5 LPS (List, Campbell, CA, USA). After blocking each well with 1% casein in PBS, serum samples were plated in serial 2-fold dilutions and allowed to incubate at 20–22°C for 2 h. After washing, the plates were incubated for 2 h with horseradish peroxidase labeled anti-mouse IgG (Roche, Indianapolis, IN, USA) prior to developing with TMB peroxidase substrate (Bio-Rad, Hercules, CA, USA). The antibody concentrations were calculated by multiplying the dilution factor at the linear portion of the A_{450} curve against the concentration calculated from the standard curve equation, created from mouse monoclonal IgG antibody (Sigma), using SoftPro (Molecular Dynamics, Sunnyvale, CA, USA).

Cytokine measurement

Cytokine production was measured from culture supernatants and cell lysates by commercially available ELISA detection kits as recommended by the manufacturer. Human interleukin (IL)-12 p40 and tumor necrosis factor-α (TNF-α) and mouse IL-1β, IL-6, IL-10, and IL-12 p40 detection kits were purchased from R&D Systems (Minneapolis, MN, USA); a mouse TNF-α kit was purchased from Biosource (Camarillo, CA, USA). Cytokine concentrations were calculated using the standard curve equation created by cytokine standards supplied by each respective kit and SoftPro (Molecular Dynamics). The cytokine production, from each cell type, was compared between the different stimuli. When an increase in cytokine concentration, by a combination of ligands, was found to approximate the simple addition of the contribution of each ligand separately, it was called an arithmetic increase in cytokine production. When the cytokine production exceeded the simple additional increase anticipated by the addition of the ligands independently, this was called a synergistic increase in cytokine production.

Statistical analysis

Antibody and cytokine ELISA results are expressed as arithmetic means with the 95% confidence intervals (CI). Differences were analyzed by the Mann–Whitney *t*-test for comparison of two groups. Differences were considered significant with a P-value < 0.05. GraphPad Prism v.4.0 (San Diego, CA, USA) was used to perform these statistical calculations.

RESULTS

Dependence of antibody response on TLR4 and TLR2

The principal signal-transducing molecule that responds to LPS is TLR4. Since detoxified J5 LPS is the primary component of the sepsis vaccine, we first determined the contribution of TLR4 function to antibody responses. A spontaneous, yet critical, point mutation in the LPS response locus, $Tlr4^{Lps\text{-}d}$, renders C3H/HeJ mice highly susceptible to infection by Gram-negative bacteria and hyporesponsive to LPS.[21] When three 10 μg doses of J5dLPS–OMP were administered intraperitoneally (i.p.), wild-type C3H/HeN mice were able to mount robust anti-CGL IgG responses (mean 480 ± 250 μg/ml); however, C3H/HeJ mice were significantly impaired ($P = 0.0079$) in their ability to produce anti-CGL IgG (mean 68 ± 19 μg/ml; Fig. 1A) Both mouse strains that received CpG alone (control) failed to mount detectable anti-CGL IgG (data not shown).

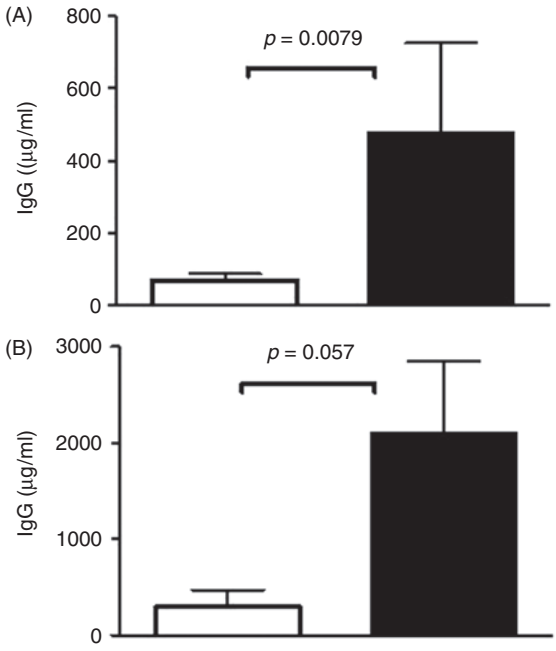


Fig. 1. Mice were immunized i.p. with 10 μg of J5dLPS–OMP (J5) at weeks 0, 2, and 4; serum was collected at week 6. ELISA anti-CGL IgG responses are illustrated in the bar graphs as mean and 95% CI for (A) LPS-hyporesponsive (C3H/HeJ; $n = 5$) and wild-type mice (C3H/HeN; $n = 5$) and (B) TLR2 knockout ($TLR2^{-/-}$ on C57 background; $n = 3$) and wild-type mice (C57BL/6; $n = 4$). Two-sided P-values based on Mann–Whitney test.

In early studies during the development of the J5 vaccine, detoxified LPS (J5dLPS) itself was poorly immunogenic, but it became highly immunogenic when non-covalently complexed with OMP.[11] Since OMP engages TLR2,[20] we determined the contribution of TLR2 to the antibody response of the subunit vaccine. Wild-type (C57BL/6) and $TLR2^{-/-}$ mice were vaccinated as in the previous experiments and serum anti-CGL IgG was measured. $TLR2^{-/-}$ mice trended toward impaired antibody responses (mean 300 ± 170 μg/ml) in comparison to wild-type mice (mean 2100 ± 750 μg/ml; $P = 0.057$; Fig. 1B).

The effect of CpG on the antibody response

CpG ODNs are efficient TLR9 agonists and have been studied as vaccine adjuvants;[22–24] we previously demonstrated a 5-fold or greater enhancement in antibody responses when CpG ODN was co-administered with the subunit vaccine.[18] These heightened antibody responses were observed with three different in-bred mouse strains (C57BL/6, C3H/HeN, and BALB/c) as well as out-bred mice (CD-1), as summarised in Table 1.

Table 1. Fold rises in anti-CGL IgG responses in mice that were vaccinated according to the three dose schedules with and without the addition of CpG as an adjuvant. Comparisons for significance are made between recipients of vaccine alone versus vaccine plus CpG

	Mouse strain	J5dLPS–OMP	J5dLPS–OMP + CpG			
	n	Mean IgG	*N*	Mean IgG	Fold-rise	*P*-value
C57BL/6	3	348 ± 109	3	1223 ± 269	3.5	0.05
TLR2$^{-/-}$	8	311 ± 75	8	501 ± 83	1.6	0.03
C3H/HeN	5	27 ± 10	5	521 ± 254	19.6	0.028
C3H/HeJ	5	13 ± 6.6	5	42 ± 17	3.2	0.075
BALB/c	6	11 ± 3	6	72 ± 30	6.5	< 0.01
Cr1:CD-1(ICR)BR	7	1011 ± 558	7	2259 ± 467	2.2	0.019

Fold-rise calculated from mean anti-CGL IgG of adjuvanted group/non-adjuvanted group.
P-value, Mann–Whitney test.

The ability of CpG ODN to enhance the antibody responses following vaccination with J5dLPS–OMP was investigated with C3H/HeJ, TLR2$^{-/-}$ mice, and their wild-type background strains (C3H/HeN and C57BL/6, respectively; Table 1). Significantly greater serum anti-CGL IgG antibody responses in TLR2$^{-/-}$ mice were observed when the vaccine was administered with CpG ODN ($P = 0.03$). In C3H/HeJ mice, there was a trend for higher antibody response with the addition of CpG ($P = 0.075$). In both the TLR2 deficient and the TLR4 mutant mice, the immunostimulatory effect of CpG ODN was able to restore the antibody response to the level of wild-type mice vaccinated without CpG, but these responses did not exceed those of the wild-type mice. Therefore, the addition of CpG was insufficient to fully overcome the absence of functional TLR4 or TLR2. However, the addition of a second and third TLR agonist to the vaccine regimen did enhance the adaptive immune response in an incremental manner.

Cytokine responses in primary macrophages

We hypothesized that the role of each additional TLR would act through any of three separate mechanisms: (i) differential cytokine responses by each TLR; (ii) different cell populations enlisted by each TLR; and/or (iii) an additive (incremental) stimulation of NF-κB activation. To evaluate whether each TLR agonist may induce the expression of characteristic cytokine profiles, we measured the cytokine responses of primary peritoneal macrophages when exposed to the J5dLPS–OMP vaccine. Primary wild-type (C3H/HeN-derived) macrophages produced robust levels of pro-inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α following overnight stimulation with J5dLPS–OMP, whereas CpG alone poorly elicited a cytokine response (Fig. 2A). Macrophages failed to elicit the production of the anti-inflammatory cytokine IL-10 following stimulation with J5dLPS–OMP or CpG. Since antibody responses to the vaccine were boosted with the addition of CpG *in vivo*, we measured the cytokine response of these macrophages when co-stimulated with J5dLPS–OMP and CpG. Wild-type macrophages that were stimulated simultaneously with J5dLPS–OMP plus CpG demonstrated IL-1β, IL-6, TNF-α, and particularly IL-12 cytokine responses which were higher than the arithmetic sums of each of the cytokines elicited from the two components alone. Therefore, a synergistic cytokine response was observed, which corresponds to the antibody boosting effect of CpG ODN when used as an adjuvant.

The antibody responses of LPS-hyporesponsive mice *in vivo* were lower compared to similarly vaccinated wild-type mice (Fig. 1A; note difference in scales). As anticipated, the magnitude of cytokine responses from LPS-hyporesponsive (C3H/HeJ) macrophages was lower than those of wild-type macrophages (Fig. 2B). Low cytokine responses were elicited by CpG alone from C3H/HeJ macrophages which were comparable to those of wild-type macrophages, except for a more prominent IL-12 response which was a mean of 140 ng/ml in comparison to 70 ng/ml (wild-type). Thus, the differences observed in the antibody responses of HeJ and wild-type mice were reflected in the differences in cytokine production. As expected, purified LPS from both the smooth strain (*E. coli* O111:B4) and the J5 mutant failed to elicit cytokines (such as TNF-α) in the C3H/HeJ macrophage (data not shown). The simultaneous stimulation of C3H/HeJ macrophages with J5dLPS–OMP plus CpG resulted in the striking synergistic production of TNF-α, while an increase in production of IL-1β, IL-6, IL-10, and IL-12 followed the pattern of an arithmetic sum of the two agonists.

Since it seemed likely that the LPS-hyporesponsive macrophages responded primarily to the GBOMP component of the subunit vaccine, macrophages were

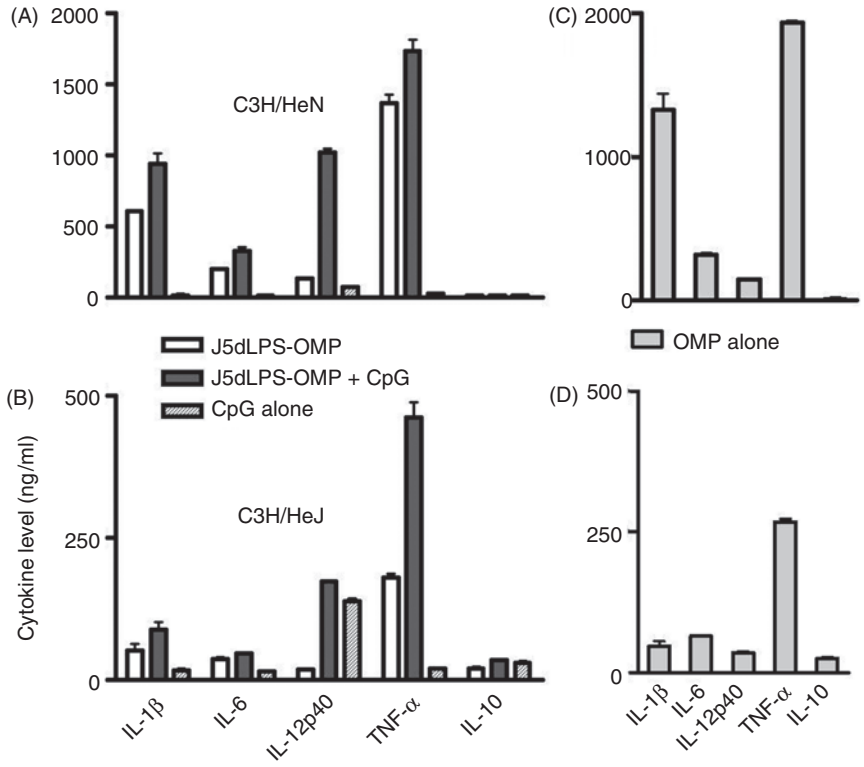


Fig. 2. Freshly harvested primary peritoneal macrophages were obtained from C3H/HeN (upper figures, A and C) and C3H/HeJ (lower figures, B and D) mice and after resting 18 h were stimulated with: 100 ng/ml LPS, 100 ng/ml J5dLPS–OMP, 10 μg/ml CpG, 1 μg/ml OMP, or 100 ng/ml Pam_3Cys as indicated. After 18-h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean ± SEM of cytokine production; two experiments were conducted using a total of two wild-type and four HeJ mice with each cytokine assessed in duplicate.

stimulated with purified GBOMP at concentrations that were calculated to be equivalent to its content in the J5dLPS–OMP vaccine (Fig. 2C,D). With both wild-type and LPS-hyporesponsive macrophages, the cytokine responses to purified GBOMP followed a similar pattern to those observed with J5dLPS–OMP; however, the cytokine responses were lower in HeJ mice.

The *in vitro* responses of $TLR2^{-/-}$ and wild-type (C57BL/6) primary macrophages were similarly examined. Wild-type macrophages produced high levels of pro-inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α when stimulated with J5dLPS–OMP alone (Fig. 3A). In comparison, CpG alone poorly stimulated the production of IL-1β, IL-6, and TNF-α; in contrast, the CpG-elicited levels of IL-10 and IL-12 were similar to those resulting from J5dLPS–OMP. When the wild-type macrophage was exposed simultaneously to the J5dLPS–OMP vaccine plus CpG, the production of IL-1β, IL-6, and TNF-α were synergistically boosted; the IL-12 response increased and the IL-10 response remained unchanged.

As projected, $TLR2^{-/-}$ macrophages produced lower quantities of cytokines in response to J5dLPS–OMP in comparison to wild-type macrophages (Fig. 3B). CpG-stimulated macrophages produced few or no cytokines. These macrophages also failed to produce cytokines in response to either purified OMP or Pam_3Cys. (data not shown). When $TLR2^{-/-}$ macrophages were exposed to both J5dLPS–OMP plus CpG, simultaneously, there was a synergistic increase in the production of IL-1β, IL-6, IL-12 and TNF-α; however, none of the individual synergistically-induced cytokine responses exceeded those of the wild-type macrophage when stimulated with J5dLPS–OMP alone. This is consistent with the pattern of our *in vivo* antibody responses in wild-type and $TLR2^{-/-}$ mice.

Since the $TLR2^{-/-}$ macrophage probably was responding to the LPS portion of the sepsis vaccine, *in vitro* stimulation with LPS alone was evaluated. Both wild-type and $TLR2^{-/-}$ macrophages demonstrated cytokine responses to purified smooth LPS (data not shown) and mutant J5 LPS (Fig. 3C,D) which followed the same pattern as that observed with the J5dLPS–OMP vaccine.

Cytokine responses in splenocytes

Because we hypothesized that the concurrent addition of TLR agonists might alternatively act through the recruitment of different cell subpopulations, we investigated the cytokine response of a mixed population of immune cells, such as those arising from the T- and B-cells, and antigen-presenting cells within the spleen.

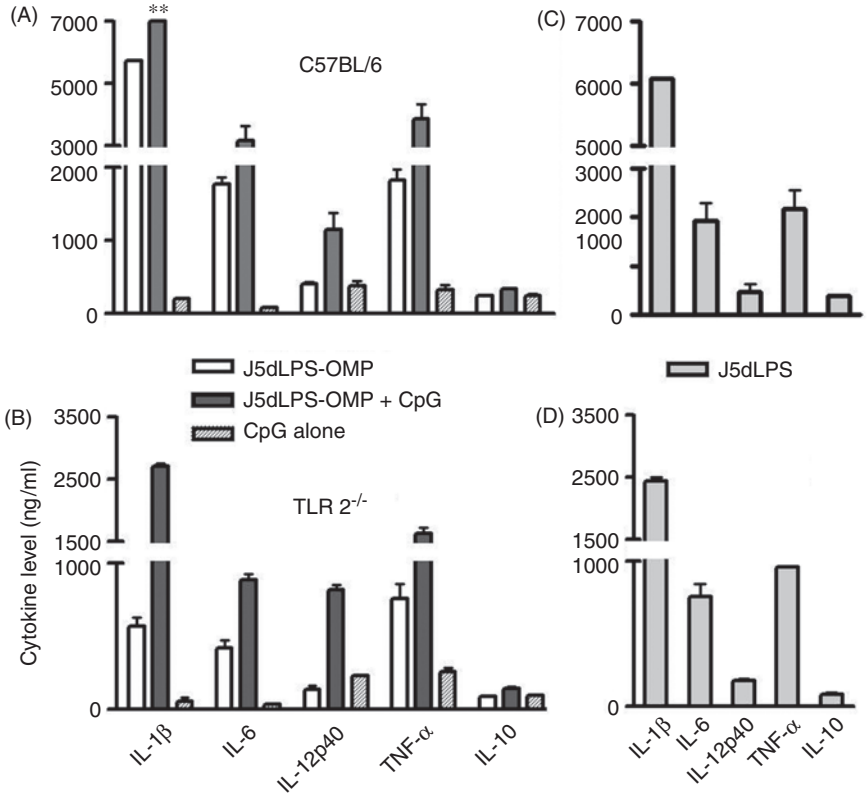


Fig. 3. Freshly harvested primary peritoneal macrophages were obtained from C57BL/6 (upper) and TLR2$^{-/-}$ (lower) mice and after resting 18 h were stimulated with: 100 ng/ml LPS, 100 ng/ml J5dLPS–OMP, 10 μg/ml CpG, 1 μg/ml OMP, or 100 ng/ml Pam_3Cys as indicated. After 18-h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean ± SEM of cytokine production; three experiments were conducted using a total of three wild-type and four knock out mice. **IL-1β level was 12,100 ± 210 ng/ml.

Freshly obtained splenocytes from naïve C3H/HeN mice were stimulated overnight with the same concentrations of J5dLPS–OMP and/or CpG as in the previous primary macrophage studies (Fig. 4). The J5dLPS–OMP vaccine stimulated small quantities of IL-6, IL-12, and IL-10 in a pattern distinctly different from the cytokine responses of primary macrophages. Splenocytes failed to make IL-1β and produced IL-10, unlike the macrophage. CpG alone elicited high levels of IL-6, IL-12, and IL-10. Splenocytes did not express interferon (IFN)-γ, IL-1β, IL-2, IL-4, or IL-5 in response to either J5dLPS–OMP or CpG. When splenocytes were stimulated simultaneously with J5dLPS–OMP plus CpG, the IL-6 production increased synergistically, the IL-12 response increased arithmetically, and the IL-10 response was no different from that of splenocytes stimulated with CpG alone. Thus, in the more complex setting of a mixed cellular population, these same TLR agonists induced another distinct pattern of cytokine secretion.

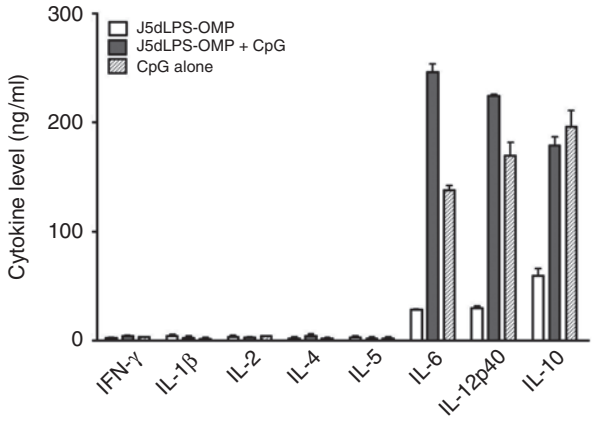


Fig. 4. Splenocytes were freshly obtained from a naïve C3H/HeN mice and stimulated with: 100 ng/ml LPS, 100 ng/ml J5dLPS–OMP, 10 μg/ml CpG ODN10103, 1 μg/ml OMP, or 100 ng/ml Pam_3Cys as indicated. After 18-h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean ± SEM of cytokine production; representative of two experiments each run in triplicate.

TLR2- and TLR4-transfected HEK cells

The preceding data implied that the anti-CGL IgG response elicited by the subunit vaccine *in vivo* and *in vitro* is dependent on both intact TLR2 and 4 function. To investigate whether this might also occur in a human system, HEK293T cells were transiently transfected with human TLR2 or TLR4 plus MD-2 and CD14

constructs, resulting in functional signaling molecules. An empty (blank) vector pcDNA3 was used as a control and an NF-κB reporter construct, containing the ELAM-1 luciferase, was used to measure downstream signaling. HEK293T cells that expressed TLR4 elicited NF-κB-dependent reporter activity when stimulated with the J5dLPS–OMP vaccine (Fig. 5A). The TLR4 agonist (purified wild-type LPS) also demonstrated NF-κB-dependent reporter activity but a synthetic TLR2 agonist (Pam_3Cys) did not. TLR2 transfectants also demonstrated NF-κB-dependent reporter activity with the J5dLPS–OMP vaccine (Fig. 5B). As expected, Pam_3Cys but not LPS demonstrated NF-κB-dependent reporter activity. We were unable to assess concurrent TLR2 and TLR4 activity using this system because the transfection was not stable. However, these data indicate that the human immune response to J5dLPS–OMP vaccine would require both TLR2 and TLR4 function.

Cytokine responses in human PBMCS

Although there is a high degree of homology, the TLRs of mice are different from those of humans. For example, there is specificity in the optimal sequence of CpG ODN recognized by human and mouse TLR9.[22] Therefore, we extended our *in vitro* observations by assessing the cytokine responses of freshly obtained human PBMCs, from three separate volunteers, under the same doses as the mouse macrophages and splenocytes. After resting overnight, PBMCs were stimulated with J5dLPS–OMP and/or CpG (Fig. 6). As opposed to mouse splenocytes, human PBMCs demonstrated robust IL-1β, IL-6, IL-12, TNF-α, and IL-10 responses to J5dLPS–OMP alone. Stimulation with CpG, however, consistently elicited high levels of IL-6 and varied levels of IL-1β and TNF-α, according to different volunteers. The production of TGF-β in response to either J5dLPS–OMP or CpG was approximately that of unstimulated PBMCs (medium alone, data not shown) and there was no IFN-γ, IL-2, or IL-4 production. When PBMCs were co-stimulated with the J5dLPS–OMP vaccine and CpG, the production of IL-12 and TNF-α was consistently synergistically increased; however, the production of IL-1β, IL-6, and IL-10 did not change appreciably.

DISCUSSION

While antigen recognition and innate immune instruction are required for the activation of most immune responses, there has been recent controversy on the role of TLRs in the induction of antibody responses.[25,26] Adaptive immune responses appear to be TLR-dependent when TLR ligands are the main innate immunogenic component of either adjuvants or pathogens. With some antigens, however, TLRs are less important.[26] In addition, B-cells express both TLRs and clonally rearranged antigen-specific B-cell receptors. The activation of B-cells via TLRs is poorly defined but it appears that B-cell-intrinsic TLR signals are not required for antibody production or maintenance, but they do amplify

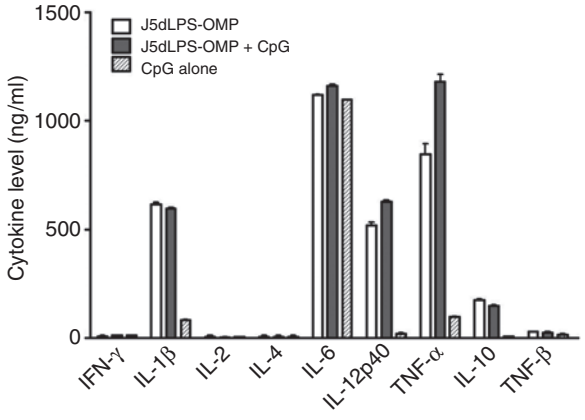


Fig. 6. Human PBMCs were freshly obtained and stimulated with: 100 ng/ml LPS, 100 ng/ml J5dLPS–OMP, 10 μg/ml CpG ODN10103, 1 μg/ml OMP, or 1 μg/ml Pam_3Cys as indicated. After 18-h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean ± SEM of cytokine production; representative of three human volunteers, each run in duplicate or triplicate.

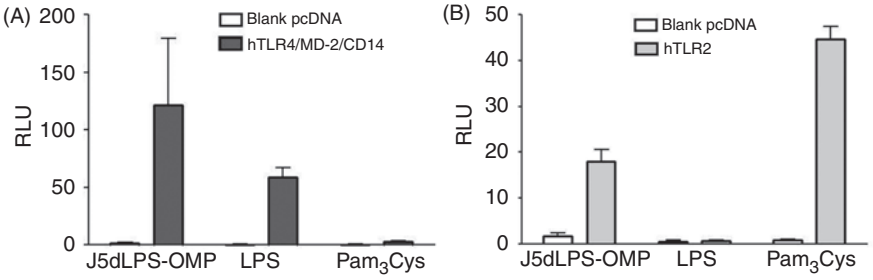


Fig. 5. HEK293T cells were transiently transfected to express TLR2, TLR4/MD-2/CD14, or given a blank vector (pCDNA3). Transfectants were incubated with 100 ng/ml of J5dLPS–OMP, 100 ng/ml of LPS, or 1 μg/ml of Pam_3Cys. NF-κB dependent stimulation was measured by ELAM-1 luciferase promoter activity and reported as RLUs ± SEM; wells run in triplicate.

the humoral immune response.[25] These and other studies, however, have used protein antigens. Thus we were interested in examining the TLR requirements for antibody responses in a vaccine whose immunogen is a glycolipid as well as a TLR agonist itself. Since the production and maintenance of high levels of anti-LPS antibodies is central to our therapeutic rationale for a broadly protective vaccine against infections caused by multiple Gram-negative pathogens, we were interested in exploring whether each of the multiple TLR agonists in our J5dLPS–OMP vaccine with CpG adjuvant contributed to the antibody response.

We previously speculated that the role of OMP in improving the antibody responses might arise from its ability to maintain critical conformational epitopes. We now demonstrate that the TLR2 agonist property of OMP also may be responsible for the improved antibody response. The engagement of TLR2 has been implicated as crucial for the optimal immunogenicity of the *Haemophilus influenzae* type b-OMP conjugate vaccine[20] and a pneumococcal polysaccharide vaccine.[20,27] In this study, we demonstrated that signaling through TLR2 and TLR4, with J5dLPS–OMP, resulted in pronounced *in vivo* specific polyclonal antibody responses (anti-CGL) and *in vitro* cytokine responses.

In addition, we investigated the basis by which CpG further boosted the antibody response to J5dLPS–OMP. In earlier studies, an attempt to use an aluminum-based adjuvant (alum, the only currently FDA-approved adjuvant) did not improve immunogenicity, yet CpG ODN significantly improved antibody responses.[18] When J5dLPS–OMP + alum + CpG was administered, the antibody response was lower than that with J5dLPS–OMP alone. In contrast to those results, alum adjuvant successfully improved the antibody response to hepatitis B vaccination with CpG compared to adjuvanting with CpG alone.[24] Perhaps in the instance of J5dLPS–OMP, alum masked important epitopes from the immune system.[11]

CpG ODNs, recognized as efficient TLR9 agonists, have been shown to be an effective vaccine adjuvant[23] and are also known to non-specifically stimulate protective innate immune responses.[28,29] We previously published that J5dLPS–OMP was well-tolerated and immunogenic in mice, when administered intraperitoneally, intramuscularly or intranasally; the addition of CpG boosted the antibody response and did not result in detectable clinical toxicity.[18,19] Interestingly, in our *in vitro* system, CpG alone poorly elicited pro-inflammatory cytokines from primary murine macrophages, yet J5dLPS–OMP plus CpG *in vitro* resulted in significantly higher (and sometimes synergistic) cytokine responses in both pure macrophage and mixed lymphocyte (*i.e.* splenocyte) cultures. The antibody and cytokine responses were boosted with the addition of a third TLR agonist (CpG). When there was an absence of functional TLR2 or TLR4, the addition of CpG as the second functional TLR agonist also increased the antibody and cytokine response. So, the addition of multiple TLR agonists resulted in better immune responses. Another important observation was that the *in vitro* cytokine and the *in vivo* antibody responses of the TLR2 or TLR4 defective mice, even with the addition of CpG as an adjuvant, never exceeded that of the wild-type mice, further suggesting that signaling via all three TLRs is required for optimal antibody responses.

We hypothesized that each additional TLR engagement might act in concert to induce: (i) differential cytokine responses; (ii) recruit distinct cell populations; and/or (iii) an additive (incremental) stimulation of NF-κB activation. We demonstrated distinct, and sometimes synergistic, cytokine responses with the combined engagement of the three TLRs. The synergistically increased cytokine production, including IL-12, from macrophages and splenocytes correlated with the heightened antibody responses *in vivo*. We also observed a synergistic increase in IL-12 production from human PBMCs *in vitro*, with the stimulation of three TLRs. Synergy with different combinations of TLR engagement have been previously described and the common pathway for the induction of TLR synergy might be the induction of IL-12[30] and the activation of dendritic cells.[31–33] However, we caution that we specifically measured the IL-12p40 dimer in these experiments and cannot exclude whether IL-23,[34] in fact, played a role in these immune responses.

We show evidence for the possibility of distinct cell population activation. The cytokine pattern of murine splenocytes, composed of a mixed population of immune cells, was different from that of macrophages; the cytokines elicited from human PBMCs were different from those of mice. Some of these differences were likely due to differences in the cell populations of these *in vitro* cultures. However, we did not specifically analyze the cytokine production of separate subpopulations of cells independently (*i.e.* $CD4^+$ or $CD8^+$ T-cells, *etc.*), so we can only conclude that the data imply the activation of distinct cell subpopulations. We cannot answer whether the improved antibody responses were generated from a larger pool of activated T- and B-cells or a fixed pool of higher quality T- and B-cells, as implied by Seder and colleagues.[35]

In this study, we did not directly evaluate NF-κB activation. However, NF-κB activation and translocation to the nucleus is a key transcriptional regulator of the immune system and is a common downstream effect of all TLR induction pathways. Therefore, the stimulation of each additional TLR might give rise to an additive (incremental) stimulation of NF-κB activation; this event may further support the synergistic increase in

IL-12 and other pro-inflammatory cytokines, the induction of distinct immune cell populations, and eventually pronounced antibody responses.

A Phase I human clinical trial is planned to test the safety and immunogenicity of the subunit vaccine with the addition of CpG as an adjuvant. Since we showed that TLR2, TLR4 and likely TLR9 each play a role in the murine antibody response to the vaccine, we would predict that there will be a similar boosting of antibody levels with CpG as an adjuvant. However, given the complex participation of multiple cell populations in the adaptive immune response and our own observations of the differing cytokine patterns expressed by these component cell populations, we are reluctant to attach too much significance to the role of any cytokines as surrogate predictors of vaccine efficacy. Furthermore, although animal studies are informative and provide guidance for our understanding of the complexity of the human immune response, we caution against over-interpreting these animal data. Indeed, we have repeatedly observed experimental vaccine constructs that worked well in animals, including non-human primates, but have not performed well in human trials. Nonetheless, when evaluating vaccine candidates, it is probable that other innate immune receptors will be critical for creating the optimal adaptive immune response. We conclude that multiple TLR agonists may optimize the immune response to future vaccine candidates.

ACKNOWLEDGEMENTS

This research was supported by National Institute of Allergy and Infectious Diseases grants RO1AI 42181 and T32AI07524 and National Center for Research Resources grant K12RR023250 (WHC).

REFERENCES

1. Jones RN. Resistance patterns among nosocomial pathogens: trends over the past few years. *Chest* 2001; **119**: 397S–404S.
2. Elbein AD, Heath EC. The biosynthesis of cell wall lipopolysaccharide in *Escherichia coli*: the biochemical properties of a uridine diphosphate galactose 4-epimeraseless mutant. *J Biol Chem* 1965; **240**: 1919–1925.
3. Ziegler EJ, Douglas H, Sherman JE, Davis CE, Braude AI. Treatment of *E. coli* and *Klebsiella* bacteremia in agranulocytic animals with antiserum to a UDP-gal epimerase-deficient mutant. *J Immunol* 1973; **111**: 433–438.
4. Ziegler EJ, McCutchan JA, Douglas H, Braude AI. Prevention of lethal pseudomonas bacteremia with epimerase-deficient *E. coli* antiserum. *Trans Assoc Am Phys* 1975; **88**: 101–108.
5. Ziegler EJ, McCutchan JA, Fierer J *et al*. Treatment of Gram-negative bacteremia and shock with human antiserum to a mutant *Escherichia coli*. *N Engl J Med* 1982; **307**: 1225–1230.
6. The Intravenous Immunoglobulin Collaborative Study Group. Prophylactic intravenous administration of standard immune globulin as compared with core-lipopolysaccharide immune globulin in patients at high risk of postsurgical infection. *N Engl J Med* 1992; **327**: 234–240.
7. Calandra T, Glauser MP, Schellekens J, Verhoef J. Treatment of Gram-negative septic shock with human IgG antibody to *Escherichia coli* J5: a prospective, double-blind, randomized trial. *J Infect Dis* 1988; **158**: 312–319.
8. Greenman RL, Schein RM, Martin MA *et al*. A controlled clinical trial of E5 murine monoclonal IgM antibody to endotoxin in the treatment of Gram-negative sepsis. The XOMA Sepsis Study Group. *JAMA* 1991; **266**: 1097–1102.
9. Ziegler EJ, Fisher Jr CJ, Sprung CL *et al*. Treatment of Gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. *N Engl J Med* 1991; **324**: 429–436.
10. Bhattacharjee AK, Opal SM, Palardy JE *et al*. Affinity-purified *Escherichia coli* J5 lipopolysaccharide-specific IgG protects neutropenic rats against Gram-negative bacterial sepsis. *J Infect Dis* 1994; **170**: 622–629.
11. Bhattacharjee AK, Opal SM, Taylor R *et al*. A noncovalent complex vaccine prepared with detoxified *Escherichia coli* J5 (Rc chemotype) lipopolysaccharide and *Neisseria meningitidis* group B outer membrane protein produces protective antibodies against Gram-negative bacteremia. *J Infect Dis* 1996; **173**: 1157–1163.
12. Cross AS, Opal SM, Warren HS *et al*. Active immunization with a detoxified *Escherichia coli* J5 lipopolysaccharide group B meningococcal outer membrane protein complex vaccine protects animals from experimental sepsis. *J Infect Dis* 2001; **183**: 1079–1086.
13. Cross AS, Opal SM, Palardy JE *et al*. Phase I study of detoxified *Escherichia coli* J5 lipopolysaccharide (J5dLPS)/group B meningococcal outer membrane protein (OMP) complex vaccine in human subjects. *Vaccine* 2003; **21**: 4576–4587.
14. Halperin SA, Van NG, Smith B, Abtahi S, Whiley H, Eiden JJ. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. *Vaccine* 2003; **21**: 2461–2467.
15. McCluskie MJ, Weeratna RD, Davis HL. The potential of oligodeoxynucleotides as mucosal and parenteral adjuvants. *Vaccine* 2001; **19**: 2657–2660.
16. Rothenfusser S, Tuma E, Wagner M, Endres S, Hartmann G. Recent advances in immunostimulatory CpG oligonucleotides. *Curr Opin Mol Ther* 2003; **5**: 98–106.
17. Cooper CL, Davis HL, Morris ML *et al*. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine* 2004; **22**: 3136–3143.
18. Opal SM, Palardy JE, Chen WH, Parejo NA, Bhattacharjee AK, Cross AS. Active immunization with a detoxified endotoxin vaccine protects against lethal polymicrobial sepsis: its use with CpG adjuvant and potential mechanisms. *J Infect Dis* 2005; **192**: 2074–2080.
19. Chen WH, Kang TJ, Bhattacharjee AK, Cross AS. Intranasal administration of a detoxified endotoxin vaccine protects mice against heterologous Gram-negative bacterial pneumonia. *Innate Immun* 2008; **14**: 269–278.
20. Latz E, Franko J, Golenbock DT, Schreiber JR. *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. *J Immunol* 2004; **172**: 2431–2438.

21. Poltorak A, He X, Smirnova I *et al*. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science* 1998; **282**: 2085–2088.
22. Bauer S, Kirschning CJ, Hacker H *et al*. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci USA* 2001; **98**: 9237–9242.
23. Klinman DM. CpG DNA as a vaccine adjuvant. *Expert Rev Vaccines* 2003; **2**: 305–315.
24. Weeratna RD, McCluskie MJ, Xu Y, Davis HL. CpG DNA induces stronger immune responses with less toxicity than other adjuvants. *Vaccine* 2000; **18**: 1755–1762.
25. Meyer-Bahlburg A, Khim S, Rawlings DJ. B cell intrinsic TLR signals amplify but are not required for humoral immunity. *J Exp Med* 2007; **204**: 3095–3101.
26. Palm NW, Medzhitov R. Immunostimulatory activity of haptenated proteins. *Proc Natl Acad Sci USA* 2009; **106**: 4782–4787.
27. Sen G, Khan AQ, Chen Q, Snapper CM. *In vivo* humoral immune responses to isolated pneumococcal polysaccharides are dependent on the presence of associated TLR ligands. *J Immunol* 2005; **175**: 3084–3091.
28. Elkins KL, Rhinehart-Jones TR, Stibitz S, Conover JS, Klinman DM. Bacterial DNA containing CpG motifs stimulates lymphocyte-dependent protection of mice against lethal infection with intracellular bacteria. *J Immunol* 1999; **162**: 2291–2298.
29. Klinman DM, Conover J, Coban C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. *Infect Immun* 1999; **67**: 5658–5663.
30. Ouyang X, Negishi H, Takeda R, Fujita Y, Taniguchi T, Honda K. Cooperation between MyD88 and TRIF pathways in TLR synergy via IRF5 activation. *Biochem Biophys Res Commun* 2007; **354**: 1045–1051.
31. Hirata N, Yanagawa Y, Ebihara T *et al*. Selective synergy in anti-inflammatory cytokine production upon cooperated signaling via TLR4 and TLR2 in murine conventional dendritic cells. *Mol Immunol* 2008; **45**: 2734–2742.
32. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 2005; **6**: 769–776.
33. Theiner G, Rossner S, Dalpke A *et al*. TLR9 cooperates with TLR4 to increase IL-12 release by murine dendritic cells. *Mol Immunol* 2008; **45**: 244–252.
34. Oppmann B, Lesley R, Blom B *et al*. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000; **13**: 715–725.
35. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008; **8**: 247–258.