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

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Abstract

When given passively or elicited actively, antibodies induced by a detoxified *E. 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 immunoadjuvant to improve antibody responses. In preparation for a Phase 1 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. TLR2, 4, and 9 were each essential for generating robust cytokine and antibody responses. The signature cytokine of dendritic cells, IL-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

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 (GNB) 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 GNB.^{2,3,4,5}

An Rc chemotype mutant bacterial strain derived from *Escherichia coli* 0111: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 anti-serum against *E. coli* J5 showed protection in animal models of lethal sepsis.^{3,4} In a prospective clinical trial, Ziegler and colleagues demonstrated a significant reduction in mortality with J5 antiserum in hospitalized GNB septic patients, particularly for patients with profound septic shock (those with hypotension requiring vasopressor agents).⁵ However, subsequent clinical trials using antibodies against LPS, including those targeting the lipid A portion, failed to demonstrate efficacy^{6,7,8,9} and immune-based anti-sepsis 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 OMP (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 1 trial demonstrated only a 2–3-fold increase in anti-CGL IgG the addition of an adjuvant was considered.¹³ Synthetic immuno-stimulatory oligodeoxynucleotides (ODN) containing unmethylated deoxycytidyl-deoxyguanosine dinucleotide (CpG) motifs are in advanced clinical development as vaccine adjuvants^{14,15,16,17} 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 GNB pneumonia.¹⁹

The mechanisms by which the J5dLPS-OMP subunit vaccine and CpG ODN induces markedly improved IgG antibody responses have not been elucidated. We hypothesized that this vaccine regimen would utilize three TLRs: 1) the detoxified LPS would stimulate TLR4, 2) the OMP is believed to signal via TLR2,²⁰ and 3) 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 3 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.

Material 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 de-*O*-acylation; 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, Pam₃Cys, was purchased from EMC Microcollections (Tubingen, Germany). Purified LPS from *E. coli* 0111:B4 (both the smooth strain and the J5 mutant) was obtained from List Biological Laboratories (Campbell, CA). Purified (LOS-free) *N. meningitidis* group B OMP (GBOMP) was provided as a gift by Apurba Bhattacharjee (formerly of WRAIR).

Mice

Six to eight week old C3H/HeJ, C3H/HeN, and C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) 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 microisolator 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) 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) 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), 10 mM HEPES (Gibco-BRL), and 1% Pen/Strep. After resting 18 h in 37°C with 5% CO₂, 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% CO₂ 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 minutes 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% CO₂ 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- κ B 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). The transfection was carried out with SuperFect transfection reagent (Qiagen Inc., Valencia, CA). Cells were washed with PBS and rested at 37°C with 5% CO₂ in cDMEM for 18 h. The transfected cells were washed with PBS, placed in serum-free DMEM and stimulated with LPS, Pam₃Cys, or J5dLPS-OMP. Cells were lysed in 1x reporter assay lysis buffer (Promega Corporation, Madison, WI) and luciferase (Promega, Luciferase assay system) and renilla (Promega, Renilla luciferase assay system) activities were analyzed, using an LMaxTM II384 Microplate Reader Luminometer (Molecular Devices Corp., California). 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 (RLU).

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 (BD, New Jersey). Peripheral blood mononuclear cells (PBMC) were isolated by established methods. Briefly, heparinized blood was mixed with an equal volume of RPMI-1640, layered over Ficoll gradient (Cellgro, Virginia), centrifuged, and the cloudy interface was collected, washed, and counted. Resuspended PBMC 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 PBMC were stimulated 18 h with agonists at 37°C with 5% CO₂ 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 two weeks after the last immunization, mice were euthanized by CO₂ 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, Corning, NY) were pre-treated with 50 µg/mL of poly-L-lysine hydrobromide (Sigma, St. Louis, MO) prior to coating with 2 µg/mL of purified J5 LPS (List, Campbell, CA). After blocking each well with 1% casein in PBS, serum samples were plated in serial two-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) prior to developing with TMB peroxidase substrate (Bio-Rad, Hercules, CA). The antibody concentrations were calculated by multiplying the dilution factor at the linear portion of the A₄₅₀ curve against the concentration calculated from the standard curve equation, created from mouse monoclonal IgG antibody (Sigma, St. Louis, MO), using SoftPro (Molecular Dynamics, Sunnyvale, CA).

Cytokine measurement

Cytokine production was measured from culture supernatant and cell lysates by commercially available ELISA detection kits as recommended by the manufacturer. Human interleukin- (IL-) 12 p40 and tumor necrosis factor- (TNF-) and mouse IL-1, IL-6, IL-10, and IL-12 p40 detection kits were purchased from R&D Systems (Minneapolis, MN); a mouse TNF- kit was purchased from Biosource (Camarillo, CA). 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 version 4.0 (San Diego, CA) 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-d}, renders C3H/HeJ mice highly susceptible to infection by GNB and hyporesponsive to LPS.²¹ When three 10 µg doses of J5dLPS-OMP were administered intraperitoneally (i.p.), wildtype (WT) 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).

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. Wildtype (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 WT 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;^{22,23,24} 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 3 different inbred mouse strains (C57BL/6, C3H/HeN, and BALB/c) as well as outbred mice (CD-1). (Table 1)

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 WT mice vaccinated without CpG, but these responses did not exceed those of the WT mice. Therefore, the addition of CpG but 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: 1) differential cytokine responses by each TLR, 2) different cell populations enlisted by each TLR and/or 3) an additive (incremental) stimulation of NF- κ B 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 WT (C3H/HeN-derived) macrophages produced robust levels of pro-inflammatory cytokines IL-1, 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. Wildtype macrophages that were stimulated simultaneously with J5dLPS-OMP plus CpG demonstrated IL-1, 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 WT 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 WT macrophages. (Fig. 2B) Low cytokine responses were elicited by CpG alone from C3H/HeJ macrophages which were comparable to those of WT macrophages, except for a more prominent IL-12 response which was a mean of 140 ng/ml in comparison to 70 ng/ml (WT). Thus, the differences observed in the antibody responses of HeJ and WT mice were reflected in the differences in cytokine production. As expected, purified LPS from both the smooth strain (*E. coli* 0111: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-1, 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 stimulated with purified GBOMP at concentrations that were calculated to be equivalent to its content in the J5dLPS-OMP vaccine. (Fig. 2C and 2D) With both WT 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 WT (C57BL/6) primary macrophages were similarly examined. Wildtype macrophages produced high levels of pro-inflammatory cytokines IL-1, 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-1, 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 WT macrophage was exposed simultaneously to the J5dLPS-OMP vaccine plus CpG, the production of IL-1, 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 WT 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 Pam₃Cys. (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-1, IL-6, IL-12 and TNF- α ; however, none of the individual synergistically-induced cytokine responses exceeded those of the WT macrophage when stimulated with J5dLPS-OMP alone. This is consistent with the pattern of our *in vivo* antibody responses in WT 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 WT and TLR2^{-/-} macrophages demonstrated cytokine responses to purified smooth LPS (data not shown) and mutant J5 LPS (Fig. 3C and 3D) 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. 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-1 and produced IL-10, unlike the macrophage. CpG alone elicited high levels of IL-6, IL-12, and IL-10. Splenocytes did not express IFN- γ , IL-1, 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.

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- κ B reporter construct, containing the ELAM-1 luciferase, was used to measure downstream signaling. HEK293T cells that expressed TLR4 elicited NF- κ B-dependent reporter activity when stimulated with the J5dLPS-OMP vaccine. (Fig. 5A) The TLR4 agonist (purified WT LPS) also demonstrated NF- κ B-dependent reporter activity but a synthetic TLR2 agonist (Pam₃Cys) did not. TLR2 transfectants also demonstrated NF- κ B-dependent reporter activity with the J5dLPS-OMP vaccine. (Fig. 5B) As expected, Pam₃Cys but not LPS demonstrated NF- κ B-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 indicates that the human immune response to J5dLPS-OMP vaccine would require both TLR2 and TLR4 function.

Cytokine responses in human PBMC

Although there is a high degree of homology, the TLRs of mice are different from that 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 PBMC, from three separate volunteers, under the same doses as the mouse macrophages and splenocytes. After resting overnight, PBMC were stimulated with J5dLPS-OMP and/or CpG. (Fig. 6) As opposed to mouse splenocytes, human PBMC demonstrated robust IL-1, IL-6, IL-12, TNF-, and IL-10 responses to J5dLPS-OMP alone. Stimulation with CpG, however, consistently elicited high levels of IL-6 and varied levels of IL-1 and TNF-, according to different volunteers. The production of TGF- β in response to either J5dLPS-OMP or CpG was approximately that of unstimulated PBMC (medium alone, data not shown) and there was no IFN-, IL-2, or IL-4 production. When PBMC were co-stimulated with the J5dLPS-OMP vaccine and CpG, the production of IL-12 and TNF- was consistently synergistically increased; however, the production of IL-1, 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 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 GNB 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 vaccine²⁰ 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 ODN, recognized as efficient TLR9 agonists, have been shown to be an effective vaccine adjuvant²³ 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.^{19,18} 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 4, 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 4 defective mice, even with the addition of CpG as an adjuvant, never exceeded that of the WT 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: 1) differential cytokine responses, 2) recruit distinct cell populations, and/or 3) an additive (incremental) stimulation of NF- κ B 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 PBMC *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-12³⁰ and the activation of dendritic cells.^{31,32,33} 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 PBMC 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- κ B activation. However, NF- κ B 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- κ B 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 1 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 TLRs 2, 4 and likely 9 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 complexity 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.

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Fig. 1.

Mice were immunized i.p. with 10 μ g 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 wildtype mice (C3H/HeN, n=5) and (B) TLR2 knockout (TLR2^{-/-} on C57 background, n=3) and wildtype mice (C57BL/6, n=4). Two-sided p-values based on Mann-Whitney test.

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 μ g/ml CpG, 1 μ g/ml OMP, or 100 ng/ml Pam₃Cys as indicated. After 18 h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean $\pm$ SEM of cytokine production; two experiments were conducted using a total of 2 WT and 4 HeJ mice with each cytokine assessed in duplicate.

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 μ g/ml CpG, 1 μ g/ml OMP, or 100 ng/ml Pam₃Cys as indicated. After 18 h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean $\pm$ SEM of cytokine production; three experiments were conducted using a total of 3 WT and 4 knock out mice. ** IL-1 level was 12,100 $\pm$ 210 ng/ml.

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 μ g/ml CpG ODN10103, 1 μ g/ml OMP, or 100 ng/ml Pam₃Cys as indicated. After 18 h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean $\pm$ SEM of cytokine production; representative of two experiments each run in 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 μ g/ml of Pam₃Cys. NF- κ B dependent stimulation was measured by ELAM-1 luciferase promoter activity and reported as RLU $\pm$ SEM; wells run in triplicate.

Fig. 6.

Human PBMCs were freshly obtained and stimulated with: 100 ng/ml LPS, 100 ng/ml J5dLPS-OMP, 10 μ g/ml CpG ODN10103, 1 μ g/ml OMP, or 1 μ g/ml Pam₃Cys as indicated. After 18 h incubation, supernatants were harvested and cytokines were measured by ELISA. Data are expressed as the mean $\pm$ SEM of cytokine production; representative of three human volunteers, each run in duplicate or triplicate.

Antibody responses (µg/ml) of mice vaccinated with J5dLPS-OMP and the effect of CpG as an adjuvant

Table 1

Mouse Strain	J5dLPS-OMP			J5dLPS-OMP +CPG			Fold-rise ¹	p ²
	N	Mean IgG	±SE	N	Mean IgG	±SE		
C57BL/6	3	348	109	3	1223	269	3.5	0.05
TLR 2 ^{-/-}	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
CrI:CD-1(ICR)BR	7	1011	558	7	2259	467	22	0.019

¹ Calculated from mean anti-CGL IgG of adjuvanted group/non-adjuvanted group

² p-value, mann-Whitney test