In Vivo Humoral Immune Responses to Isolated Pneumococcal Polysaccharides Are Dependent on the Presence of Associated TLR Ligands[1]

Goutam Sen,[2] Abdul Q. Khan,[2] Quanyi Chen, and Clifford M. Snapper[3]

We determined whether T cell-independent Ig isotype responses to isolated pneumococcal polysaccharides (PPS) required TLR signaling in vivo. IgG anti-PPS responses to PPS3, PPS14, and C-polysaccharide (C-PS) were virtually undetectable in TLR2$^{-/-}$ mice, whereas specific IgM induction was variably reduced compared with wild-type mice. All PPS-containing preparations induced IL-6 and TNF-α from wild-type, but not TLR2$^{-/-}$, macrophages. TLR2 activity was distinct from that of PPS, in that it was phenol extractable. Immunization of wild-type mice with phenol-extracted PPS14 also resulted in a marked reduction in the IgG, although not the IgM-anti-PPS14, response compared with untreated PPS14. The commercial 23-valent PPS vaccine, Pneumovax-23 also contained TLR ligands (TLR2 and TLR4), which were absolutely critical for the IgG-inducing activity of the vaccine in mice. Finally, the commercial pneumococcal conjugate vaccine, Prevnar, contained a TLR2 ligand(s) that substantially enhanced both the primary and secondary anti-PPS responses in mice, especially the type 1 IgG isotypes. These data strongly suggest the absolute need for a distinct, TLR-dependent second signal for inducing in vivo IgG T cell-independent humoral immune responses to isolated pneumococcal polysaccharide Ags and highlight the potential importance of previously unappreciated copurified and/or contaminating TLR ligands in PPS vaccine preparations. ***The Journal of Immunology*****, 2005, 175: 3084–3091.**

The mechanism by which isolated polysaccharide (PS;[4] T cell-independent type 2 (TI-2)) Ags induce Ig responses in vivo is poorly understood. Previous studies using an in vitro polyclonal model for multivalent membrane Ig (mIg) cross-linking in response to PS Ags (i.e., multiple anti-IgD Abs conjugated to dextran ($\alpha\delta$-dex)) (1) indicated that multivalent mIg cross-linking alone or in concert with CD40-mediated activation induced vigorous proliferation, but no Ig secretion or isotype switching by highly purified B cells (2). However, addition of various TLR ligands, such as bacterial lipoprotein (TLR2), Neisserial porins (TLR2), unmethylated CpG-containing oligodeoxynucleotides (TLR9), or LPS (TLR4) to $\alpha\delta$-dex-activated B cell cultures induced substantial Ig secretion and isotype switching (2). The requirement for TLR ligands could be replaced by addition of various cytokines, such as IL-5 or IL-2 in combination with IL-3, GM-CSF, or IFN-γ. Of interest, some of these cytokines can be rapidly induced in vivo in response to TLR ligands. Collectively, these in vitro data suggested that in vivo Ig responses to PS Ags required at least two distinct signals, but the nature of the differentiative signal (signal 2) in vivo in response to isolated PS Ags or PS Ags expressed by intact bacteria has remained obscure.

Several studies have demonstrated the ability of isolated bacterial capsular PS to induce cytokine release in vitro (3–6). However, the nature of this stimulatory activity and its cognate receptor as well as its potential role in modulating anti-PS responses in vivo have not been elucidated. The recent discovery of TLRs and the creation of mice genetically deficient in either specific TLR or TLR adaptor proteins (7, 8) has provided a unique opportunity to test the hypothesis that TLR signaling is a critical costimulus for anti-PS Ig responses in vivo. Distinct TLR have been described, each with a characteristic specificity for one or more microbial ligands. Ligand engagement of TLR on the surface or within the cytoplasm of immune cells typically results in cytokine secretion and/or cellular maturation, thereby promoting innate immunity, as well as shaping the adaptive immune response. Innate immunity to intact *Streptococcus pneumoniae* (Pn) has recently been shown to depend on TLR signaling. In particular, a role for TLR2 has been demonstrated, probably due to the expression by Pn of various TLR2 ligands, such as lipoteichoic acid (LTA), peptidoglycan, and lipoprotein (9–11). However, additional TLR have been implicated in Pn-mediated immunity, including TLR4 in response to pneumolysin (12). A potential role for pneumococcal DNA (TLR9) or RNA (TLR7/8) in Pn-induced immunity has not, as yet, been demonstrated. Recently, we reported that TLR2 plays a critical role in promoting an in vivo type 1 humoral immune response to intact heat-killed Pn (13).

Using various preparations of isolated pneumococcal polysaccharides, including the commercial pneumococcal PS (PPS) vaccine, Pneumovax-23 (14), we now demonstrate that the polysaccharide Ag itself is ineffective at inducing detectable IgG and, to a lesser extent, IgM responses in vivo in the absence of a distinct

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[4] Abbreviations used in this paper: PS, polysaccharide; $\alpha\delta$-dex, multiple anti-IgD Abs conjugated to dextran; DOC, deoxycholate; KLH, keyhole limpet hemocyanin; LTA, lipoteichoic acid; mIg, membrane Ig; PBA, polyclonal B cell activator; PC, phosphorylcholine; Pn, *Streptococcus pneumoniae*; PPS, capsular PS; SN, supernatant; TEA, triethanolamine; TI-2, T cell independent type 2.

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second signal, in this case provided by the concomitant and hitherto unappreciated presence of TLR ligands within the PPS preparations. We also show that the presence of a TLR2 ligand(s) in the commercial PPS conjugate vaccine, Prevnar (15), up-regulates the induced IgG anti-PPS response. These data provide a mechanism underlying the induction of specific IgG anti-PS responses in vivo that is consistent with earlier predictions made using the in vitro polyclonal $\alpha\delta$-dex model system (2) and highlight the potential importance of previously unappreciated, copurified and/or contaminating TLR ligands in PPS vaccine preparations.

Materials and Methods

Mice

TLR2$^{-/-}$ (16) and MyD88$^{-/-}$ mice (17) were obtained originally from Dr. S. Akira (Osaka University, Osaka, Japan) and were bred in our facility. Control B6129SF2/J (for TLR2$^{-/-}$) and C57BL/6 (for MyD88$^{-/-}$) mice were obtained from The Jackson Laboratory and the National Cancer Institute, respectively. TLR2$^{-/-}$ (C3H/HeN background) and TLR2$^{-/-}$ $\times$ C3H/HeJ mice were obtained from Dr. C. J. Kirschning (Technical University of Munich, Munich, Germany). C3H/HeJ mice (18) were obtained from The Jackson Laboratory, and C3H/HeN mice were obtained from the National Cancer Institute. Mice were used between 6 and 10 wk of age. These studies were conducted in accordance with the principles set forth in the Guide for Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, revised 1996, and were approved by the Uniformed Services University of the Health Sciences institutional animal use and care committee.

Mouse genotyping

All TLR2$^{-/-}$ mice (background B6129SF2) and MyD88$^{-/-}$ mice (background C57BL/6) used in these experiments were first genotyped in our laboratory as previously described (13). The following mice were also genotyped in our laboratory: 1) TLR2$^{-/-}$ (background C3H/HeN): primer A, 5′-GGG CCA GCT CAT TCC TCC CAC TCA T-3′; B, 5′-CTT CCT GAA TTT GTC CAG TAC AGG G-3′; C, 5′-TCG ACC TCG ATC AAC AGG AGA AGG G-3′; PCR, 200 ng of genomic DNA; 94°C for 5 min, 94°C for 1 min, 59°C for 30 s, 70°C for 1 min (30 cycles), then 72°C for 2 min; wide-type product, 499 bp; TLR2$^{-/-}$ product, 334 bp; and 2) C3H/HeJ (TLR4-defective, background C3H/HeN): primer A, 5′-TGT CAG TGG TCA GTG TGA TTG-3′; B, 5′-TCA GGT CCA AGT TGC CGT TTC-3′; PCR, 200 ng of genomic DNA; 94°C for 5 min, 94°C for 1 min, 59°C for 30 s, 70°C for 1 min (30 cycles), then 72°C for 2 min; PCR products, *Bst*NI digest for 4 h at 60°C; separate products on 3% NuSieve GTG agarose gel; HeN products, two bands between 300 and 350 bp; HeJ products, two bands between 300 and 400 bp.

Reagents

Phosphorylcholine (PC)-keyhole limpet hemocyanin (KLH) was synthesized as described previously (19). The resulting conjugate had a substitution degree of 19 PC-KLH molecules. Purified *S. pneumoniae* capsular polysaccharides (PPS) types 3, 4, 6B, 9V, 14, 18C, 19F, 22F, and 23F were all vaccine grade, produced by Merck, and purchased from American Type Culture Collection. Purified *S. pneumoniae* cell wall C-PS was purchased from Statens Serum Institute. Pneumovax-23 was purchased from Merck and consisted of a mixture of highly purified capsular polysaccharides from the 23 most prevalent or invasive pneumococcal types of *S. pneumoniae* (14). Each 0.5-ml dose of vaccine contained 25 μg of each PS type dissolved in isotonic saline solution containing 0.25% phenol as a preservative. Prevnar was purchased from Wyeth Pharmaceuticals and consisted of seven PPS serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) conjugated to diphtheria CRM197 protein (15). Each 0.5 ml of Prevnar contained 0.125 mg of aluminum phosphate adjuvant, a total of 20 μg of CRM197, and 2 μg of each PPS, except for PPS6B (4 μg). The dose of Prevnar stated in the text referred to the total amount of PPS. All PPS-containing preparations were tested for the presence of endotoxin by the *Limulus* amebocyte lysate assay (LAL test; BioWhittaker) and were found to be $<$0.13 U/10 μg PPS/ml.

Phenol extraction of PPS14

Phenol extraction of PPS14 was conducted as described by Hirschfeld et al. (20) with minor modifications. Specifically, triethanolamine (TEA) was added to 0.5 ml of PPS14 (5 mg/ml) to make a 0.2% final concentration of TEA. Deoxycholate (DOC) was added to make a final concentration of 0.5% DOC. Water-saturated phenol (0.5 ml) was added and vortexed intermittently for 5 min. The solution remained undisturbed for 5 min at room temperature for phase separation, then was placed on ice for an additional 5 min. The material underwent centrifugation for 2 min at 10,000 $\times$ *g* at 4°C. The upper, aqueous layer was transferred to another tube. The phenol layer was re-extracted with 0.5 ml of water-saturated phenol containing 0.2% TEA and 0.5% DOC. The aqueous layers were pooled and re-extracted with 1 ml of water-saturated phenol containing 0.2% TEA and 0.5% DOC. Pooled aqueous layers were adjusted to 75% ethanol containing 30 mM sodium acetate and stored for 1 h at $-$20°C for precipitation. The material then underwent centrifugation for 10 min at 10,000 $\times$ *g* at 4°C. The precipitate was washed with 1 ml of ice-cold absolute ethanol and then air-dried. The PS precipitate was later dissolved in 0.5 ml of saline.

Isolation and activation of peritoneal macrophages

RPMI 1640 (10 ml) was injected into the peritoneal cavity through the fat pad using a 23-gauge needle. After 3 min, lavage fluid containing the peritoneal cells was withdrawn using an 18-gauge needle. Cells were pelleted by centrifugation at 400 $\times$ *g* for 10 min at room temperature. The cell pellet was suspended in culture medium (RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 0.05 mM 2-ME, 50 μg/ml penicillin, and 50 μg/ml streptomycin) and adjusted to 1 $\times$ 10^6 total cells/ml, and 1 ml of cell suspension was added to each well of a 24-well plate and incubated for 3 h at 37°C in a 5% CO_2-containing incubator. Plates were then washed to remove unbound cells. Fresh medium (0.5 ml) containing isolated PS was added to the cultures of bound cells and incubated for 48 h at 37°C, followed by plate centrifugation and collection of culture supernatant (SN) for determination of IL-6 and TNF-α concentrations by ELISA.

Measurement of cytokine concentrations in peritoneal macrophage culture SN

The concentrations of IL-6 and TNF-α released into the media of stimulated peritoneal macrophage cultures were measured using optimized standard sandwich ELISA. Recombinant cytokines used as standards as well as the capture mAbs, biotinylated mAbs used for detection, and avidin-HRP were purchased from BD Pharmingen. After incubation with avidin-HRP, plates were washed and developed using SureBlue tetramethylbenzidine microwell peroxidase substrate (Kirkegaard & Perry Laboratories). The reaction was stopped using tetramethylbenzidine stop solution, and plates were read using a 450-nm ELISA reader.

Immunization of mice and determination of serum titers of IgM and IgG isotype anti-PPS titers

Mice were immunized i.p. with 1 μg of PPS3, PPS14, or C-PS dissolved in sterile PBS. Sera were prepared from blood obtained from the tail vein on day 0 (before bleeding), day 7, and/or day 14. Serum titers of IgM and IgG anti-PPS or anti-PC (for C-PS) Abs were determined by ELISA as previously described (13). For the anti-PPS ELISAs, 5 μg/ml C-PS and 10 μg/ml PPS22F were added to the serum dilution buffer to block PPS-nonspecific binding. For measurement of serum titers of IgM and IgG anti-PPS induced after Pneumovax-23 immunizations, ELISA plates were coated with 23 μg/ml Pneumovax-23, representing 1 μg of each PPS/ml (50 μl/well). For measurement of serum titers of IgM and IgG isotypes induced after Prevnar immunizations, ELISA plates were coated with 5 μg/ml each of PPS4, 6B, 9V, 14, 18C, 19F, and 23F (50 μl/well). Serum titers of IgM and IgG anti-PPS Abs were calculated as reported previously (21).

Statistics

Serum titers of Ig were expressed as the arithmetic mean of individual serum samples from five to seven mice $\pm$ SEM. Concentrations of cytokines in peritoneal macrophage culture SN were expressed as the arithmetic mean of duplicate cultures from macrophages pooled from five mice $\pm$ SD. Levels of significance of the differences between groups were determined by Student's *t* test. A value of $p \leq 0.05$ was considered statistically significant.

Results

IgG responses to isolated PPS are abrogated in TLR2$^{-/-}$ mice

PPSs are T cell-independent type 2 (TI-2) Ags capable of stimulating specific IgM and IgG (mostly IgG3) responses in vivo, but the mechanism by which they promote differentiation and switching of PPS-specific B cells is poorly understood. In light of the important role of TLR2 in innate and adaptive immunity to intact

S. pneumoniae (13), we wanted to determine whether TLR2 was also important for promoting PPS-specific IgM and IgG responses to various isolated PPS preparations. Thus, wild-type and TLR2$^{-/-}$ mice were immunized with isolated PPS3, PPS14, or C-PS. All PPS preparations induced serum titers of PPS-specific IgM and IgG in wild-type mice, with peak titers observed by day 7 after immunization (Fig. 1). In contrast, IgG anti-PPS3, anti-PPS14, and anti-PC (C-PS) responses were essentially abrogated in TLR2$^{-/-}$ mice (Fig. 1). The PPS-specific IgG consisted mostly of IgG3, with barely detectable and variable amounts of IgG1 (data not shown). IgM anti-PPS3, but not IgM anti-PPS14 or IgM anti-PC, was also significantly reduced, but not eliminated in TLR2$^{-/-}$ mice (Fig. 1). No further increases in serum titers of PPS-specific IgM or IgG were observed from days 7 to 14 in either wild-type or TLR2$^{-/-}$ mice (data not shown).

The cytokine-inducing activity of PPS3, PPS14, and C-PS is due to the presence of a TLR2 ligand(s)

The data presented above suggested that the Ig-inducing capacity of the PPSs in vivo might be critically dependent on the presence of a TLR2 ligand(s). To determine this, we isolated macrophages from both wild-type and TLR2$^{-/-}$ mice and measured IL-6 and TNF-α secretion 48 h after initiation of culture with PPS3, PPS14, or C-PS (Fig. 2). Wild-type, but not TLR2$^{-/-}$, macrophages secreted IL-6 (Fig. 2) and TNF-α (data not shown) in response to PPS3, PPS14, or C-PS, indicating that the cytokine-inducing activities in these preparations were indeed secondary to the presence of a TLR2 ligand(s). TLR2$^{-/-}$ macrophages failed to secrete IL-6 (Fig. 2) and TNF-α (data not shown) in response to the TLR2 ligand, LTA, whereas LPS (a TLR4 ligand) induced comparable cytokine responses in wild-type and TLR2$^{-/-}$ macrophages (Fig. 2). These data demonstrate a critical role for a TLR2 ligand(s) in stimulating IgG and, to a lesser and more variable extent, IgM anti-PS Ig in vivo in response to isolated PS preparations. Recently, we observed that PPS4 and PPS23F also induced IL-6 and TNF-α from murine peritoneal macrophages in vitro, whereas no detectable induction of IL-6 or TNF-α was observed in response to PPS2, 6B, 7F, 9V, 18C, 19F, 22F, or 33F (our unpublished observations). All PPSs were obtained from American Type Culture Collection. The implications of these latter findings for Ig induction in vivo are currently under investigation.

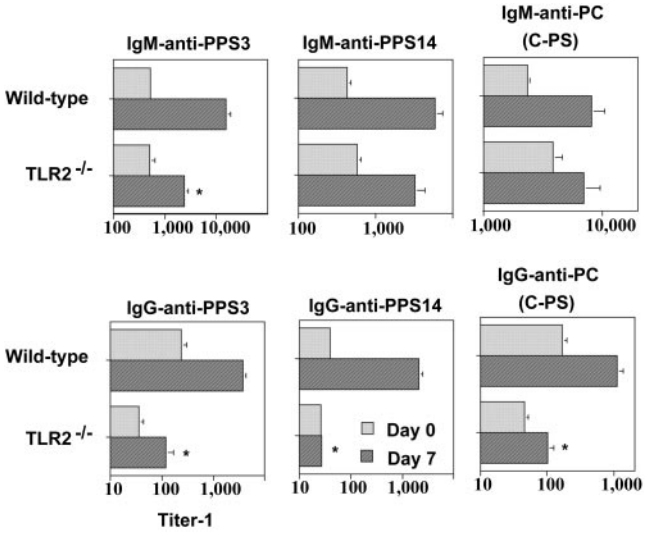

FIGURE 1. In vivo IgG anti-PPS responses to isolated PPS preparations are dependent on TLR signaling. Wild-type (B6129SF2/J) and TLR2$^{-/-}$ mice (five per group) were immunized i.p. with PPS3, PPS14, and C-PS dissolved in sterile PBS. In each case, 1 μg of each PPS was injected. Sera were prepared from blood obtained from the tail vein on day 0 (before immunization), day 7, and/or day 14 (data not shown). Serum titers of IgM and IgG anti-PPS were determined by ELISA. This experiment is representative of two independent experiments performed.

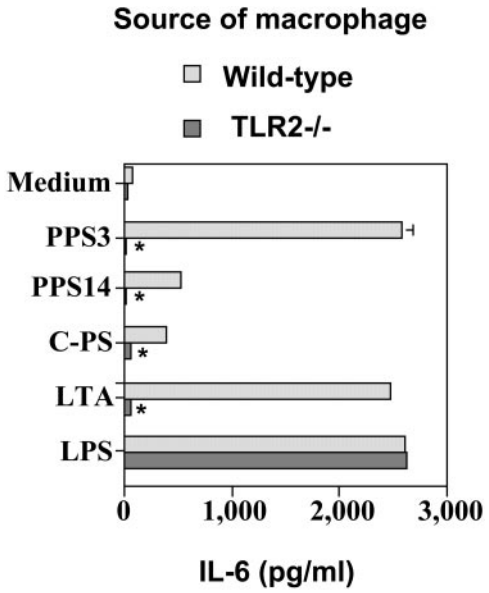

FIGURE 2. Isolated PPS preparations contain a TLR2 ligand(s). Peritoneal macrophages from wild-type and TLR2$^{-/-}$ mice were incubated at 37°C for 48 h with PPS3, PPS14, C-PS, (each at 10 μg/ml), LTA (2 μg/ml), or LPS (1 μg/ml), and SNs were collected for measurement of cytokine concentrations by ELISA. This experiment is representative of two independent experiments performed.

TLR2 activity in PPS preparations is distinct from PPS3, PPS14, or C-PS

Although isolated PPS preparations are known to contain ≤5% of various non-PPS, pneumococcal contaminants, the precise nature and functional relevance of these are largely unknown. In this regard, it was unclear whether the reported cytokine-inducing activities of some PPSs (3–6) were due to these non-PPS contaminants or were an intrinsic property of the PPS itself. To determine this, we phenol-extracted all PPSs in an attempt to remove these potentially distinct cytokine-inducing activities. Peritoneal macrophages from wild-type mice were isolated and cultured in the presence of varying amounts of stock or phenol-extracted PPS3, PPS14, or C-PS for 48 h, followed by measurement of concentrations of secreted IL-6 and TNF-α. Stock PPS3, PPS14, and C-PS all induced both IL-6 (Fig. 3*A*) and TNF-α (data not shown) from cultured peritoneal macrophages in a very similar dose-dependent manner. This activity could be mostly abrogated, in each case, by prior phenol extraction. Previous incubation of PPS14 and C-PS, although not PPS3, at 80°C for 1 h also led to the loss of cytokine-inducing activity (data not shown).

To further determine whether the removal of this cytokine-inducing activity affected the subsequent Ig response to the PPS in vivo, we immunized wild-type mice with stock or phenol-extracted PPS14. Phenol-extracted PPS14 induced a significantly reduced IgG, although not IgM-anti-PPS14, response compared with stock PPS14 (Fig. 3*B*), suggesting that this cytokine-inducing activity was important for IgG induction in vivo. A similar reduction in serum IgG, but not IgM, anti-PPS14 titers was observed upon immunization with heat-inactivated (80°C for 1 h) vs stock PPS14

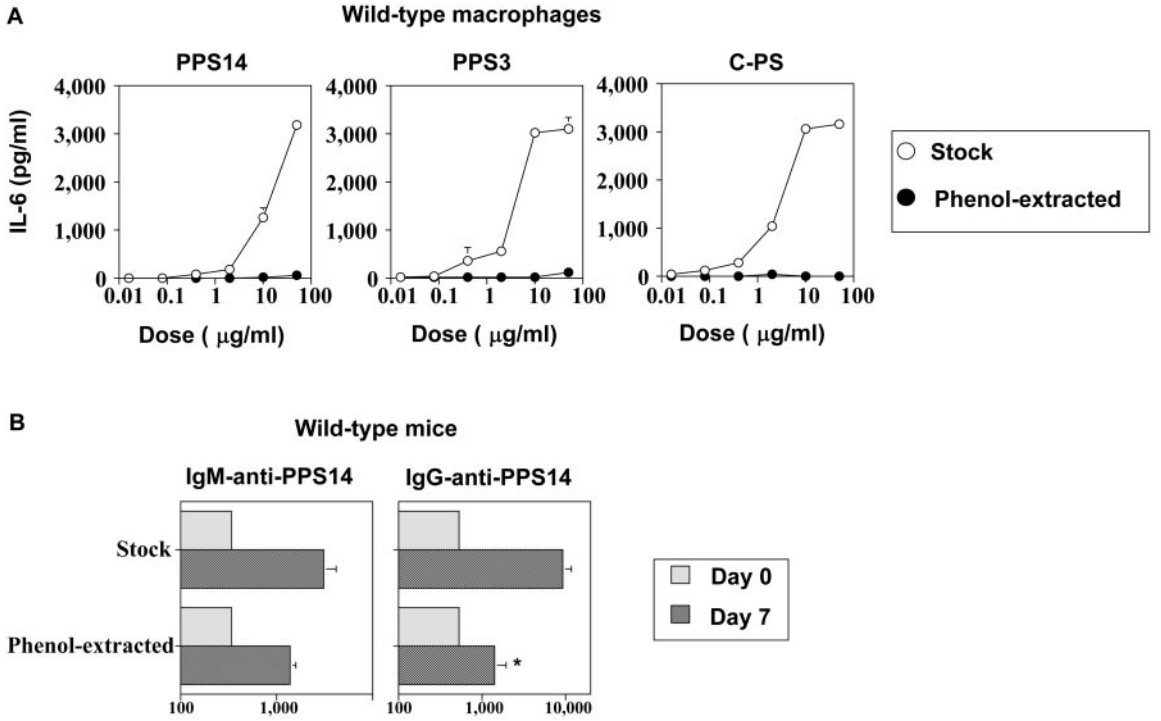

FIGURE 3. The TLR2 ligand(s) is distinct from PPS3, PPS14, or C-PS. *A*, Peritoneal macrophages from wild-type mice were incubated at 37°C for 48 h with the indicated concentrations of either untreated (stock) or phenol-extracted PPS3, PPS14, or C-PS. Supernatants were collected 48 h later for measurement of cytokine concentrations by ELISA. This experiment is representative of two independent experiments performed. *B*, Wild-type mice (seven per group) were immunized i.p. with 1 μg/mouse of either stock or phenol-extracted PPS14. Sera were prepared from blood obtained from the tail vein on day 0 (before immunization) and day 7. Serum titers of IgM and IgG anti-PPS14 were determined by ELISA. This experiment is representative of two independent experiments performed.

(data not shown). Coating the ELISA plates with stock, phenol-extracted, or heat-inactivated PPS14 had no significant effect on the serum IgG anti-PPS14 titers in any of the immunization groups (data not shown), strongly suggesting that neither phenol extraction nor heat inactivation significantly altered PPS14 epitopes.

Pneumovax-23 contains both TLR2 and TLR4 ligands that are collectively critical for induction of IgG and, to a lesser extent, IgM anti-PPS responses in vivo

We next determined the potential clinical relevance of possible contamination of commercial PPS vaccines with TLR ligands. Pneumovax-23 is a commercial vaccine consisting of a mixture of highly purified capsular polysaccharides in isotonic saline from the 23 most prevalent or invasive pneumococcal types of *Streptococcus pneumoniae* (14). This vaccine is administered to adults, especially the elderly, who are at increased risk for pneumococcal infections, and has shown moderate success in host protection via induction of IgG anti-PPS Ab. We first determined a potential role for TLR signaling in the ability of Pneumovax-23 to induce IgM and IgG anti-PPS Ab in vivo. TLR2$^{-/-}$ or MyD88$^{-/-}$ mice were immunized i.p. with Pneumovax-23, and sera were obtained 7 and 14 days later for measurement of IgM and IgG titers specific for the whole PPS vaccine. TLR2$^{-/-}$ mice showed a significant, although partial, reduction in both elicited IgM and IgG anti-PPS Ab titers compared with wild-type mice on both day 7 (Fig. 4*A*), when the peak of the response occurred, and day 14 (data not shown) after immunization. More strikingly, MyD88$^{-/-}$ mice failed to make any detectable IgG anti-PPS Ab after Pneumovax-23 immunization compared with wild-type mice (Fig. 4*A*). Similar to TLR2$^{-/-}$ mice, MyD88$^{-/-}$ mice exhibited a significant, although partial, reduction in IgM anti-PPS Ab titers. The difference in the elicited serum titers of IgG anti-PPS Ab between TLR2$^{-/-}$ and MyD88$^{-/-}$ mice compared with their wild-type controls suggested the presence in the Pneumovax-23 preparation of a ligand(s) for a TLR(s) in addition to TLR2.

Pneumovax-23 induced IL-6 (Fig. 4*B*) and TNF-α (data not shown) secretion in wild-type peritoneal macrophages in a dose-dependent manner. Macrophages from wild-type and TLR2$^{-/-}$ mice showed equivalent cytokine induction in response to Pneumovax-23 (Fig. 4*C*). In contrast, C3H/HeJ (TLR4-defective) macrophages were largely, although not completely, defective, whereas no detectable cytokine release was observed in macrophages from TLR2$^{-/-}$ $\times$ HeJ mice. Although heat pretreatment had no effect on Pneumovax-23-mediated cytokine induction in wild-type and TLR2$^{-/-}$ macrophages, it eliminated the residual cytokine secretion observed in macrophages from C3H/HeJ mice (Fig. 4*C*). These data strongly suggest the presence of both a heat-sensitive TLR2 and a heat-resistant TLR4 ligand(s) in the Pneumovax-23 preparation that appears to be critical for IgG and, to a lesser extent, IgM anti-PPS responses in vivo. Analysis of endotoxin content in Pneumovax-23 strongly suggests that this TLR4 ligand(s) is not LPS (see *Materials and Methods*).

Prevnar contains a TLR2 ligand(s) that enhances elicited IgG anti-PPS responses in vivo

Prevnar is a commercial pneumococcal conjugate vaccine consisting of seven PPS serotypes conjugated to diphtheria CRM197 protein and suspended in aluminum phosphate adjuvant (15). The linkage of the CRM197 protein to PPS converts the vaccine from a TI-2 to a TD Ag with resultant higher PPS-specific Ig titers and a broader IgG isotype profile. More importantly, although infants make poor anti-PPS responses to isolated PPS Ags, they elicit a

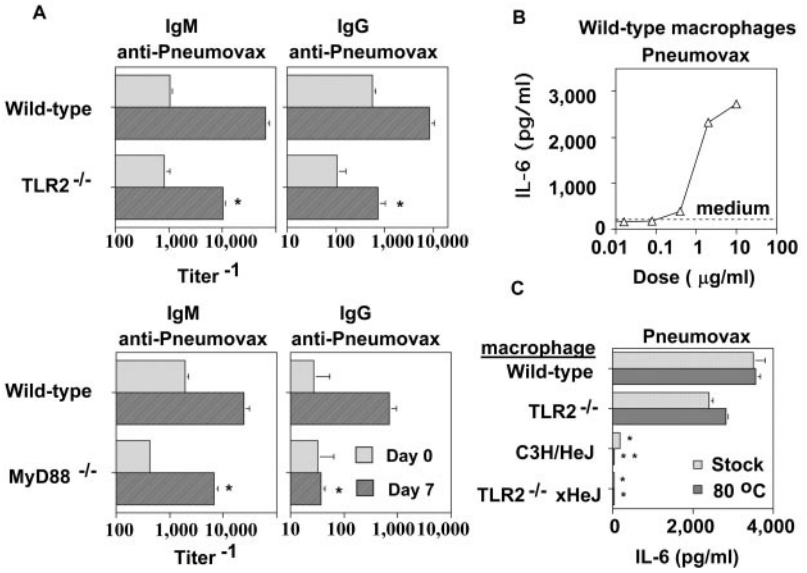

FIGURE 4. Pneumovax-23 contains TLR2 and TLR4 ligands critical for IgG induction in vivo. *A*, Wild-type (B6129SF2/J) and TLR2$^{-/-}$ mice (seven per group) or wild-type (C57BL/6) and MyD88$^{-/-}$ mice (seven per group) were immunized i.p. with Pneumovax-23 (1 μg of each PPS). Sera were obtained on days 0 (before immunization) and day 7. Serum titers of IgM and IgG specific for all PPSs contained within Pneumovax-23 were determined by ELISA. This experiment is representative of two independent experiments performed. *B*, Peritoneal macrophages from wild-type (B6129SF2/J) mice were incubated at 37°C for 48 h with the indicated concentrations of Pneumovax-23, and SNs were collected for measurement of cytokine concentrations by ELISA. This experiment is representative of two independent experiments performed. *C*, Peritoneal macrophages from wild-type (C3H/HeN), TLR2$^{-/-}$ (C3H/HeN background), C3H/HeJ, and TLR2$^{-/-}$ $\times$ C3H/HeJ mice were incubated at 37°C for 48 h with 10 μg/ml Pneumovax-23 (with or without previous incubation at 80°C for 1 h), and SNs were collected for measurement of cytokine concentrations by ELISA. One experiment was performed.

robust, protective anti-PPS response to Prevnar. As with Pneumovax-23, we wanted to determine whether Prevnar contains a TLR ligand(s), and whether this influences the elicited IgM and/or IgG anti-PPS responses in vivo. Prevnar induced both IL-6 (Fig. 5*A*) and TNF-α (data not shown) production in wild-type peritoneal macrophages in a dose-dependent manner. In contrast, this cytokine response was markedly attenuated in macrophages from TLR2$^{-/-}$ mice (Fig. 5*A*). On the basis of these data, wild-type and TLR2$^{-/-}$ mice were immunized i.p. with either 1.0 or 4.0 μg of Prevnar and boosted i.p. on day 42 with the same corresponding dose. Sera were collected on the days indicated for measurement of serum titers of IgM and IgG isotypes specific for all seven PPSs contained within the vaccine (see *Materials and Methods*). Prevnar elicited IgM and IgG of all isotypes in wild-type mice, with substantial boosting of IgG, although not IgM, upon secondary immunization (Fig. 5*B*). IgM and IgG anti-PPS titers were higher (4.0- to 7.5-fold for IgM and 5.5- to 21-fold for IgG) in mice receiving 4.0 μg of Prevnar compared with those given 1.0 μg (Fig. 5*B*). Wild-type and TLR2$^{-/-}$ mice elicited very similar IgM anti-PPS responses to either dose of Prevnar. In contrast, both primary and secondary IgG anti-PPS responses to 4.0 μg of Prevnar were substantially higher in wild-type compared with TLR2$^{-/-}$ mice (day 7, 4.4-fold; day 14, 3.4-fold; day 42, 1.9-fold (primary); day 49, 4.0-fold (secondary)). A significantly higher secondary IgG anti-PPS response in wild-type compared with TLR2$^{-/-}$ mice was also observed using 1.0 μg of Prevnar (2.9-fold; Fig. 5*B*). Analysis of the IgG isotype profiles of anti-PPS Abs elicited in the primary response demonstrated that the reduction in IgG in TLR2$^{-/-}$ mice was largely due to decreases in IgG3 and IgG2a. In contrast, all IgG isotypes in TLR2$^{-/-}$ mice were significantly reduced after secondary immunization compared with wild-type mice, especially with the higher, 4.0-μg dose of Prevnar (Fig. 5*B*). Collectively, these data indicate that the unexpected presence of a TLR2 ligand(s) in Prevnar exerts a significant enhancing effect on the elicitation of both primary and secondary IgG anti-PPS Abs.

Discussion

We previously demonstrated, using a polyclonal in vitro model for multivalent mIg cross-linking in response to PS Ags ($\alpha\delta$-dex), that multivalent mIg cross-linking alone induces vigorous B cell proliferation, but no differentiation or Ig class switching unless various TLR ligands or certain cytokines are also present (1, 2). It has long been known that isolated PS Ag preparations alone induce specific IgM and IgG responses in vivo, although the underlying mechanism for this induction has remained obscure. The reported ability of isolated capsular PS preparations to induce cytokines in vitro (3–6) suggested the presence of an activity that might costimulate PS-specific B cells to secrete Ig in vivo, but the nature of this activity has not been elucidated. Although specific amplifier and suppressor T cells can regulate Ig responses to isolated PS Ags (22), such Ig responses can still occur normally in the complete absence of T cells, suggesting the presence of a non-T cell second signal for Ig induction (23). Recently, a role for endogenous CD40-CD40L interactions in promoting anti-PS responses to isolated PS Ags has been demonstrated, although the cellular basis for this remains unknown (24). In this regard, previous in vitro studies have demonstrated that combined mIg plus CD40 signaling is markedly synergistic for B cell proliferation (25), but does not lead to differentiation or Ig class switching in the absence of additional stimuli (26). PS Ags may also activate complement in vivo (27), resulting in cocross-linking of mIg and the type 2 complement receptor (CD21). Although coligation of mIg and CD21 is synergistic for B cell activation (28), and linkage of C3d to isolated PS can enhance anti-PS responses in vivo (29), it is unclear whether B cell differentiation and Ig class switching will occur solely on the basis of mIg/CD21 cocross-linking. However, complement can activate numerous immune cell types, potentially leading to the release of mediators that promote B cell differentiation. Thus, the nature of the differentiative signal(s) for in vivo Ig responses to isolated PS Ags has remained of interest.

Our observations that specific in vivo IgG and, to a lesser and more variable degree, IgM responses to isolated PPS preparations critically depend on the presence of a distinct, copurified TLR2 ligand(s), suggest that purified PS Ags by themselves are ineffective immunogens for IgG induction in the absence of a separate TLR/cytokine costimulus. In this study we measured macrophage production of IL-6 and TNF-α in response to various PPS preparations to assess the presence of TLR ligands. However, this does not specifically imply a role for these particular cytokines in mediating the IgG anti-PPS response. Collectively our early studies using $\alpha\delta$-dex (2), our more recent study using intact Pn14 (13), and the current study support the idea that physiologic anti-PS responses to intact bacteria are probably mediated at least in part by PS-induced mIg cross-linking of specific B cells combined with

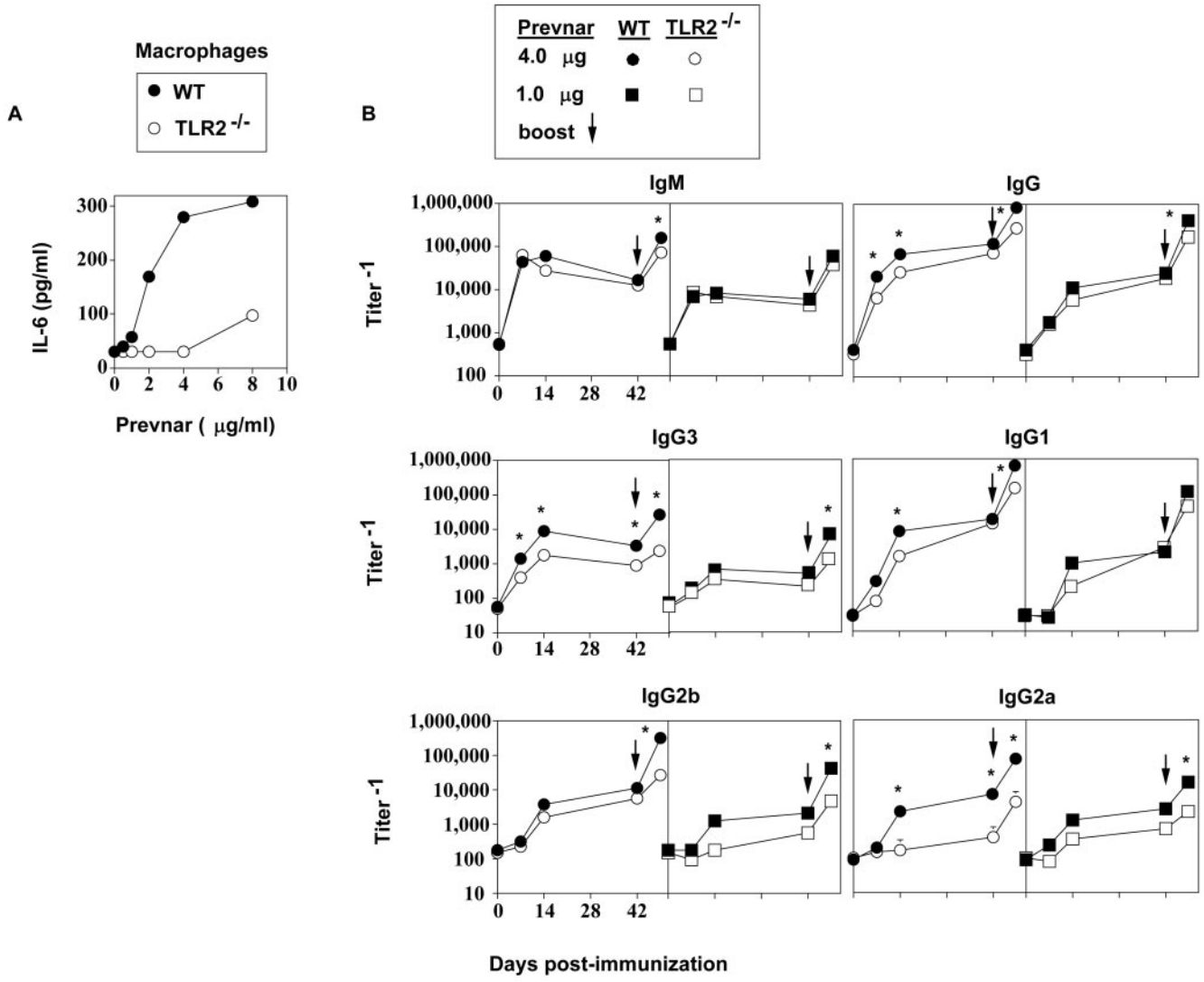

FIGURE 5. Prevnar contains a TLR2 ligand(s) that stimulates anti-PPS responses in vivo. *A*, Peritoneal macrophages from wild-type (B6129SF2/J) or TLR2$^{-/-}$ mice were incubated at 37°C for 48 h with the indicated concentrations of Prevnar, and SNs were collected for measurement of cytokine concentrations by ELISA. *B*, Wild-type (B6129SF2/J) and TLR2$^{-/-}$ mice (seven per group) were immunized i.p. with either Prevnar (containing either 0.12 or 0.5 μg (PPS) of each individual conjugate except PPS6B (0.25 or 1.0 μg); total of 1.0 or 4.0 μg, respectively) and boosted i.p. 42 days later with the same dose of Prevnar. Sera were collected on the indicated days for measurement of PPS-specific IgM and IgG isotype titers by ELISA. This experiment is representative of three independent experiments performed.

costimulation by associated bacterial TLR ligands. Additional activation signals mediated by CD40/CD40L interactions and CD4$^+$ T cells appear to amplify this response (19). The mechanism underlying the residual IgM anti-PS response remains unresolved. The ability of NK cells to directly induce mIg-activated B cells to secrete IgM (30) provides one possible TLR-independent mechanism, but this remains to be demonstrated in vivo.

These data also suggest that the effectiveness of PS-containing vaccines may currently depend on the unintended copurification of bacterial TLR ligands during the PS purification process. Thus, detectable IgG anti-PPS responses to Pneumovax-23 were totally dependent on the presence of associated TLR2 and TLR4 ligands. This latter point may have substantial relevance with regard to the specific procedures by which these PS Ags are purified, including the immunogenic consistency of different vaccine lots, because IgG is considered the main protective Ig isotype. The nature of the TLR ligands present within the PPS preparations is currently unknown. Of note, none of the preparations had significant endotoxin contamination (see *Materials and Methods*). Although the pneumococcal protein, pneumolysin, like endotoxin, is a TLR4 ligand (12), a heat-resistant TLR4 activity was observed in Pneumovax-23, suggesting the presence of an additional, nonprotein, TLR4 ligand. TLR2 ligands expressed by Pn include LTA, peptidoglycan, and lipoprotein (9–11).

This study is of interest in light of recent data indicating that commercial enterobacterial LPS preparations have modest TLR2-stimulating activity in addition to strong TLR4-activating properties. This TLR2 activity was shown to be the result of a lipoprotein contaminant that could be removed by phenol extraction (20, 31). More recently, it was demonstrated that an LPS preparation from *Porphyromonas gingivalis*, previously shown to activate immune cells through TLR2, but not TLR4, also had a contaminating lipoprotein as the principal component of the TLR2 activity (32). In contrast, *P. gingivalis* lipid A exhibited relatively weak TLR4 activity. Finally, it has been suggested that the presence of contaminating LPS and an undefined TLR2 ligand(s) may be important for the TI immunostimulatory potential of recombinant hepatitis B nucleocapsid preparations produced in *Escherichia coli* (33).

The ability of an associated TLR2 ligand in the pneumococcal conjugate vaccine, Prevnar, to enhance both primary and secondary IgG anti-PPS responses was somewhat surprising, because Prevnar is injected with aluminum phosphate adjuvant and, unlike isolated PPS Ags, promotes recruitment of classical cognate $CD4^+$ T cell for anti-PPS responses. The preferential enhancement of IgG3 and IgG2a by the associated TLR2 ligand is consistent with our recent data demonstrating a role for TLR2 in promoting type 1 IgG isotype production (i.e., IgG3, IgG2b, and IgG2a, but not IgG1) in response to intact Pn14 (13). TLR stimulation typically leads to production of IFN-γ (8), which we previously demonstrated promotes switching to IgG3 and IgG2a (34, 35). The relatively greater impact of TLR2-mediated stimulation using the higher dose of Prevnar (4.0 μg) probably reflects the proportionately greater amount of TLR2 ligand injected. Consistent with our data, a recent study by Latz et al. (36) demonstrated that a soluble conjugate of meningococcal outer membrane protein complex and capsular PS of *Haemophilus influenzae* type b was less immunogenic in $TLR2^{-/-}$ mice, producing lower anti-Hib PS IgG and IgM titers compared with wild-type mice. The outer membrane protein complex, the carrier protein was shown to be a TLR2 ligand, acting as an adjuvant for augmenting the anti-PS Ab response induced by the glycoconjugate vaccine.

Finally, these data may call into question the physiologic relevance of classifying PS Ags as either TI-1 or TI-2. Thus, classical TI-1 Ags, such as LPS, consist of a PS Ag linked to a polyclonal B cell activator (PBA; i.e., TLR ligand such as lipid A), whereas TI-2 Ags lack PBA activity (23). However, our current study showing a critical role for contaminating TLR ligands in TI-2 Ag-mediated IgG induction suggests that the distinction between TI-1 and TI-2 Ags may reflect the level of associated TLR activity in isolated PS Ag preparations, not its presence or absence. More importantly, this distinction may become less important when considering an intact bacterial pathogen, where capsular PS (TI-2) Ags are typically associated with the bacterial cell wall, the latter containing several TLR ligands (i.e., PBAs).

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Disclosures

The authors have no financial conflict of interest.

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