

Pneumococcal Capsular Polysaccharide Is Immunogenic When Present on the Surface of Macrophages and Dendritic Cells: TLR4 Signaling Induced by a Conjugate Vaccine or by Lipopolysaccharide Is Conducive¹

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Previously, we reported that a peptide, p458, from the sequence of the mammalian 60-kDa heat shock protein (hsp60) molecule can serve as a carrier in conjugate vaccines with capsular polysaccharide (CPS) molecules of various bacteria. These conjugate vaccines were effective injected in PBS without added adjuvants. We now report that p458 conjugated to pneumococcal CPS type 4 (PS4) manifests innate adjuvant effects: it stimulated mouse macrophages to secrete IL-12 and induced the late appearance of PS4 on the macrophage surface in a TLR4-dependent manner; PS4 alone or conjugated to other carriers did not stimulate macrophages *in vitro*. The injection of macrophages manifesting PS4 on the surface into mice induced long-term resistance to lethal *Streptococcus pneumoniae* challenge. The TLR4 ligand LPS could also induce the late appearance on the surface of unconjugated PS4 and resistance to challenge in injected mice. Resistance was not induced by macrophages containing only internalized PS4 or by pulsed macrophages that had been lysed. Glutaraldehyde-fixed macrophages pulsed with PS4 did induce resistance to lethal challenge. Moreover, bone marrow-derived dendritic cells activated by LPS and pulsed with unconjugated CPS were also effective in inducing resistance to lethal challenge. Resistance induced by the PS4-pulsed bone marrow-derived dendritic cell was specific for pneumococcal CPS serotypes (type 3 or type 4) and was associated with the induction of CPS-specific IgG and IgM Abs. *The Journal of Immunology*, 2008, 180: 2409–2418.

Effective vaccines against bacterial capsular polysaccharide (CPS)³ Ags appear to require three components: 1) a CPS B cell epitope to induce the production of Ab that mediates opsonin-dependent phagocytosis and bacterial clearance; 2) a T cell epitope to induce T cell help for an IgG isotype switch and immune memory; and 3) an adjuvant to provide an Ag depot or a suitable cytokine environment (1). Classical adjuvants often contain bacterial or other ligands that stimulate TLR signaling of innate immune cells (2). Stimulation of TLR signaling enhances the production of proinflammatory cytokines and the up-regulation of MHC and costimulatory molecules (3, 4). Thus, TLR stimulation plays a key role in the interface between innate and adaptive immunity (5). Indeed, TLR molecules are considered to be “adjuvant receptors,” and the ligands that activate them are “adjuvants” (2,

6). Vaccines that incorporate ligands for TLR stimulation were shown to boost vaccine responses (7, 8).

Vaccines against encapsulated bacteria such as *Streptococcus pneumoniae* have included the CPS coat because the CPS is a major *S. pneumoniae* virulence factor (9). However, the *S. pneumoniae* CPS is, like other polysaccharides, poorly immunogenic, especially in young children, the elderly, and immunosuppressed adults (10, 11). The immune response to CPS Ags is T cell-independent, and is characterized primarily by IgM Ab, rare IgG isotype switching and no immunological memory. Conjugate vaccines have been designed using various protein carriers like tetanus toxoid as a source for T cell epitopes conjugated to the CPS with the aim of supplying the help needed for IgG switching and immune memory (12, 13). However, the conjugation of a CPS to a protein carrier alone may be insufficient to evoke a strong immune response and an added adjuvant is often needed.

Previously, we reported that a peptide derived from the human or mouse 60-kDa heat shock protein (hsp60), peptide p458, could serve as a carrier in conjugate CPS vaccines (14–17). We used the hsp60 peptide as a carrier because healthy humans mount a natural T cell response to hsp60 epitopes (18). Indeed, the p458 peptide conjugated to pneumococcal CPS type 4 (PS4)-p458 could induce protection in mice against a supra-lethal *S. pneumoniae* challenge (14, 15). Protection was associated with PS4-specific IgG Ab in most but not in all the mice, a T cell response to the p458 carrier and long-term memory. Vaccines composed of p458 conjugated to the CPS molecules of *S. pneumoniae*, *Salmonella* (16), or meningococcus B and C (17) were also immunogenic, even when injected in PBS without an added adjuvant. These findings suggested that CPS p458 conjugates might act as adjuvants, as well as

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³ Abbreviations used in this paper: CPS, capsular polysaccharide; PS4, pneumococcal CPS type 4; PS3, pneumococcal CPS type 3; hsp60, 60-kDa heat shock protein.

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specific immunogens. To test that idea, we examined the effect of the PS4-p458 conjugate on macrophages and bone marrow-derived dendritic cells (BMDC). In this study, we show that the PS4-p458 conjugate stimulates macrophages to produce IL-12 and induces the late appearance of PS4 on the cell surface for a prolonged time in a TLR4-dependent manner. Similarly, treatment of macrophages with the TLR4 ligand LPS could also induce the prolonged appearance of unconjugated PS4 on the cell surface. Injecting mice with PS4 present on the macrophage cell surface induced long-term resistance of mice to a lethal *S. pneumoniae* challenge that was not associated with readily detectable specific Abs. Finally, BMDC pulsed with PS4 or PS3 induced serotype-specific resistance to *S. pneumoniae* challenge; in contrast to macrophages, the resistance induced by injection of CPS-pulsed BMDC was associated with detectable CPS-specific IgG and IgM Abs.

Materials and Methods

Mice

Female BALB/c mice were purchased from Harlan Olac. Female C3HeB/Fej and C3H/HeJ were obtained from The Jackson Laboratory. All mice were used at the age of 8–10 wk. The mice were maintained in a specific pathogen-free facility and were used according to the guidelines and under the supervision of the Animal Welfare Committee.

Isolation of peritoneal macrophages

Mice were injected i.p. with 1 ml of thioglycolate 3% (Difco). After 4 days, the peritoneum was washed with 10 ml of ice-cold PBS. The cells were incubated on 24-well plates with full medium: DMEM (Invitrogen Life Technologies) containing 10% FCS (HyClone Laboratories), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and nonessential amino acids for 3 h at 37°C in 5% CO₂. The non-adherent cells were removed by washing.

Preparation of dendritic cells (DC) in culture

BMDC were prepared as described earlier (19) with minor modifications. Briefly, mice were sacrificed and bone marrow was extracted from femurs and tibias by flushing the shaft with PBS. RBC were lysed using red blood lysing buffer (Sigma-Aldrich), and the remaining cells were seeded into non-tissue culture plates at a density of 4×10^6 cells per plate in medium (RPMI 1640, 10% FCS, 5×10^{-5} M 2-ME, penicillin/streptomycin) containing 10% of supernatant derived from B16 cell cultures secreting GM-CSF. The medium was replenished every 3 days, and the loosely adherent DC were collected after 8–10 days and used for further studies. To induce DC maturation, day-8 cultures were treated with LPS (1 µg/ml) for 24 h. The BMDC were characterized for their surface marker expression profile by FACS. More than 90% of the cells had high expression of CD11c. The LPS-stimulated cells had up-regulation of CD86, CD40, and MHC class II (data not shown).

Cell lines

RAW 264.7 is a tumor macrophage cell line derived from BALB/c mice (20) that retains its phagocytic and Ag-presenting capabilities (21). 4T1 is a breast cancer carcinoma cell line derived from BALB/c mice. Both cell lines were obtained from American Type Culture Collection (ATCC) and were raised at 37°C, 5% CO₂ in full medium.

Reagents

Reagents were PS4 and PS3 (~100 kDa; ATCC), LPS *Salmonella Minnesota*, polymyxin B, glutaraldehyde, paraformaldehyde (Merck), cytochalasin D, dextran (~100 kDa), FITC (Sigma-Aldrich).

Peptides

Peptides were synthesized using a peptide synthesizer (model AMS 422; Abimed Analyses-Technik), according to the company's instructions for *N*-α-fluorenylmethoxycarbonyl synthesis. Peptide purity was determined by reversed-phase HPLC and by amino acid analysis. Peptide p458 was derived from murine hsp60, positions 458–474 (NEDQKIGIEIKRALKI). Peptide p30 was derived from tetanus toxoid protein, positions 947–967 (FNNFTVSWFLRVPKVSASHLE).

PS4 conjugation

The conjugates were made as previously described (14). Briefly, PS4 was dissolved in double-distilled water (5 mg/ml) and was activated with 0.1 ml of cyanogen bromide (20 mg/ml in acetone) in the presence of 30 mM triethylamine (Sigma-Aldrich) in acetone (pH 7). After 2 min, 6-aminohexanoic acid (10 mg/ml in double-distilled water) was added for 2 h of incubation at 4°C. We then added 12 mg of EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride; Sigma-Aldrich) and 7 mg of the peptide to the solution. The pH was adjusted (to pH 6) at room temperature. Four hours later, 12 mg of EDC was again added, the mixture was incubated overnight and then dialyzed at 4°C against double-distilled water. The following polysaccharide to peptide ratios were: PS4-p458, 1:1.3 w/w and 1:45 mol/mol; PS4-p30, 1:1.6 w/w and 1:35 mol/mol. In other words, 35–45 peptides are bound to each PS4 conjugate.

IL-12 cytokine determination

The presence of IL-12 in culture supernatants was determined by ELISA for IL-12 (OptiEIA kits; BD Pharmingen) following the manufacturer's instructions using Maxisorb 96-well plates. Standard curves were established using mouse recombinant IL-12.

Preparation of column-purified mouse anti-PS4 polyclonal Abs

Female BALB/c mice were injected s.c. three times with PS4-p458 conjugate (2 µg/mouse) emulsified in IFA at 2-wk intervals. Serum was collected after 3 wk from the last injection. Anti-PS4 Abs were purified by affinity chromatography: PS4 molecules, activated by cyanogen bromide, were coupled to 1.6 diaminoheptan-Sepharose provided by Prof. M. Wilchek (The Weizmann Institute of Science, Rehovot, Israel). The serum was loaded on the column and the effluent was collected. The column was washed with 10 volumes of PBS, and the anti-PS4 Abs were eluted with 0.1 M acetic acid (pH 3), and neutralized immediately with 1 M Tris (pH 10). The elution was tested for specificity in ELISA against the PS4. The purified anti-PS4 Ab was used in the flow cytometry and immunocytochemistry assays.

Flow cytometric analysis

The presence of PS4 on the macrophage surface was followed by flow cytometry. RAW cells or peritoneal macrophages (1×10^6 per tube) were pulsed with the conjugates or with PS4 alone as indicated in the experiment. The cells were washed with PBS/0.5% BSA and fixed with 1% paraformaldehyde. The cells were then incubated for 30 min with the anti-FcγRIII/RII 2.4G2 Ab (BD Pharmingen) to block nonspecific binding. After washing, the cells were incubated with the column-purified mouse anti-PS4 Abs for 45 min on ice. After washing, FITC-conjugated goat anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories) was added for an additional 45 min on ice. Flow cytometry analysis was done by FACSsort and CellQuest software (BD Biosciences).

PS4 labeling with FITC

PS4 was dissolved in double-distilled water (10 mg/ml), and 15 mg (0.1 mmol) of 1,3-diaminopropane dihydrochloride dissolved in double-distilled water was added. EDC · HCl (*N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride, 19 mg; 0.1 mmol; Sigma-Aldrich) was then added, and the reaction mixture was kept for 4 h at room temperature. Then another portion of EDC · HCl (9.5 mg; 0.05 mmol) was added. After 10 h at room temperature, the clear homogenous reaction mixture was extensively dialyzed against 0.1 N acetic acid at 4°C to remove low m.w. molecules. The pH was adjusted (to pH 7.5) with 0.1 N KHCO₃ solution followed by the addition of FITC (19 mg; ~0.05 mmol). After standing overnight at room temperature, the yellowish solution was dialyzed extensively against 0.1 N acetic acid at 4°C. The solution was divided into aliquots and frozen at –20°C.

Immunocytochemistry

RAW cells were cultured in full medium on 10-mm glass coverslips (Deckglaser) in 24-well plates (2×10^5 /well) overnight at 37°C. The cells were pulsed with conjugates or with PS4 alone, as indicated in the text. Then, the cells were washed and fixed with 1% paraformaldehyde for 20 min at room temperature. For detection of CPS on the cell surface, the cells were incubated with the column-purified mouse anti-PS4 Ab for 45 min at room temperature, washed with PBS twice and incubated with FITC-conjugated goat anti-mouse IgG secondary Ab (Jackson ImmunoResearch Laboratories) for an additional 45 min at room temperature. For detection of intracellular CPS, the cell membranes were permeabilized using 0.5% Triton X-100 (Sigma-Aldrich). In some experiments, we used FITC-labeled PS4

and the membranes were stained using anti-H-2D^d Ab (clone 3458), then, biotinylated affinity purified anti-mouse IgG secondary Ab (BioMakor) and finally, Cy5-streptavidin (Jackson ImmunoResearch Laboratories).

Injection with pulsed-macrophages or BMDC

RAW cells were cultured in 10-cm tissue-culture plates (5×10^6 /plate) in full medium. The next day, the cells were pulsed with conjugates or with PS4 alone for different time periods, as indicated in the text. Then the cells were harvested and extensively washed with ice-cold PBS to remove unbound PS4. The cells were then irradiated with 5000 rad and injected i.p. (2×10^6 cells) to naive female BALB/c mice. BMDC were prepared as described, and the cells were stimulated or not with LPS to induce maturation. The cells were then pulsed with the PS4 or PS3 for 1 h, washed and injected i.p. (5×10^6 cells) without irradiation to syngeneic mice.

S. pneumoniae bacteria

Lyophilized *S. pneumoniae* type 3 or type 4 bacteria and CPS Ag were obtained from the ATCC. *S. pneumoniae* bacteria were reconstituted and subcultured on sheep blood agar (Hy Laboratories), and the colonies were resuspended in brain-heart infusion broth (Hy Laboratories). After 6 h, the bacterial cultures were aliquoted and stored at -80°C in medium with 25% glycerol. For pneumococcal challenge, a frozen aliquot of *S. pneumoniae* bacteria was thawed in brain-heart infusion broth and was grown for 6 h at 37°C , and then transferred to ice until injection. Bacterial growth was estimated by the level of turbidity using an OD reader at 545 nm. The actual dose of viable bacteria injected in each challenge was determined by plating dilutions of the bacteria on sheep blood agar for 24 h at 37°C and counting the number of CFU. Pneumococcal virulence was obtained by periodic passage in mice: mice were injected with 100 *S. pneumoniae* CFU, and 20 h later the spleens were harvested, passed through a wire mesh, and seeded on sheep blood agar. The bacteria were then prepared as indicated above. To determine the minimal lethal dose, naive BALB/c mice (three mice per group) were injected i.p. with 200 μl of serially diluted bacteria, and survival was determined daily for 2 wk. All naive mice challenged with two or more CFU died within 2 days of challenge. The minimal lethal dose that killed half of the challenged mice (LD_{50}) was determined to be one bacterium per mouse.

Statistics

The InStat 2.01 program (GraphPad) was used for statistical analysis. Statistical analysis was performed using the two-sided Welch *t* test and Fisher's exact χ^2 test. Differences were considered statistically significant at $p < 0.05$.

Results

The PS4-p458 conjugate is effective in PBS

We previously reported that various conjugate vaccines of PS4, such as PS4-p458 and PS4 conjugated to a tetanus toxoid peptide p30 (PS4-p30), were effective in inducing protection against lethal *S. pneumoniae* challenge when the vaccine was administered three times in IFA (14) (Fig. 1A). The use of adjuvants, however, can evoke undesirable side effects, and it would be desirable to develop effective vaccines that need not be injected with adjuvants. Fig. 1B shows that the PS4-p458 conjugate was effective in protecting mice against *S. pneumoniae* challenge even when injected once in PBS; the PS4-p30 conjugate, in contrast, was markedly less effective in PBS. This suggests that the PS4-p458 conjugate might have an intrinsic adjuvant-like effect.

The PS4-p458 conjugate stimulates a macrophage cell line to secrete IL-12

Adjuvant components are known to stimulate innate immune cells via TLR molecules to secrete proinflammatory cytokines such as TNF- α and IL-12 (2, 8). To investigate whether the PS4-p458 conjugate has an adjuvant-like effect in vitro, we examined its ability to stimulate macrophages to secrete IL-12. A macrophage cell line (RAW 264.7) was pulsed for 24 h with the PS4-p458 conjugate, the PS4-p30 conjugate, unconjugated p458 peptide, unconjugated PS4, or LPS. Fig. 2 shows that only the PS4-p458 conjugate and LPS induced the macrophage line to secrete IL-12;

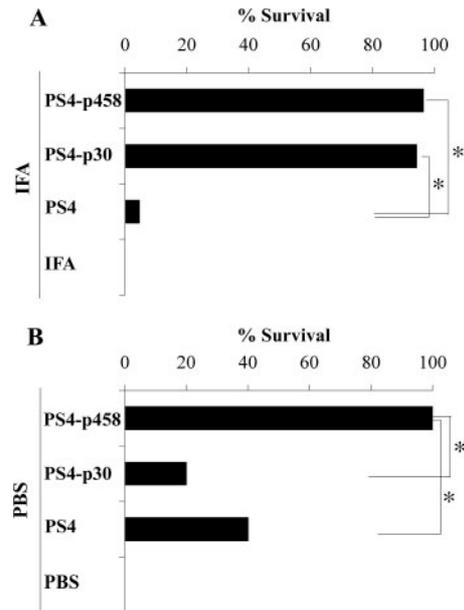


FIGURE 1. PS4-p458 induces protection when administered in PBS. Ten BALB/c mice per group were injected s.c. with 2 μg per mouse of PS4-p458, PS4-p30, or with PS4 alone in IFA three times at 2-wk intervals (A) or once in PBS (B). One month later, the mice were challenged with 10,000 *S. pneumoniae* CFU (A) or with 100 *S. pneumoniae* CFU (B). Data show the percentage of survival with indicated conjugate vaccine against challenge. 1 CFU = $\text{LD}_{50} \times 1$. *, $p < 0.02$.

unconjugated PS4 or p458 or the PS4-p30 conjugate failed to do so. The stimulatory effect of the PS4-p458 conjugate was not due to LPS contamination because an LPS inhibitor, polymyxin B (22), annulled the effect of LPS but not the effect of the PS4-p458 conjugate (Fig. 2). These findings suggest that the multivalent structure of the PS4-p458 conjugate has a stimulatory effect on macrophages that could not be elicited by the linear p458 peptide, PS4, or the PS4-p30 conjugate. The stimulatory effect of the PS4-p458 conjugate on macrophages could account for its effectiveness as a vaccine in PBS.

Macrophages activated by PS4-p458 manifest a late and prolonged appearance of PS4 on the surface

We examined whether the stimulatory effect of the PS4-p458 conjugate might influence the appearance of the PS4 moiety on

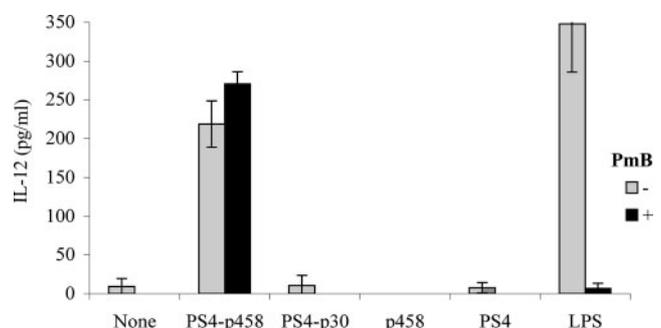


FIGURE 2. PS4-p458 induces the RAW macrophage cell line to secrete IL-12. RAW cells were incubated for 24 h with 20 $\mu\text{g}/\text{ml}$ PS4-p458, PS4-p30, p458, p30, PS4, LPS (100 ng/ml), or with serum-free medium alone (None). In parallel, RAW cells were also pretreated with polymyxin B (PmB) (10 $\mu\text{g}/\text{ml}$) for 30 min, and then incubated with the indicated treatments. Supernatants were collected and tested by ELISA for the presence of IL-12. Only conjugates PS4-p458 or LPS induced IL-12; polymyxin B inhibited the effect of LPS, but not that of PS4-p458.

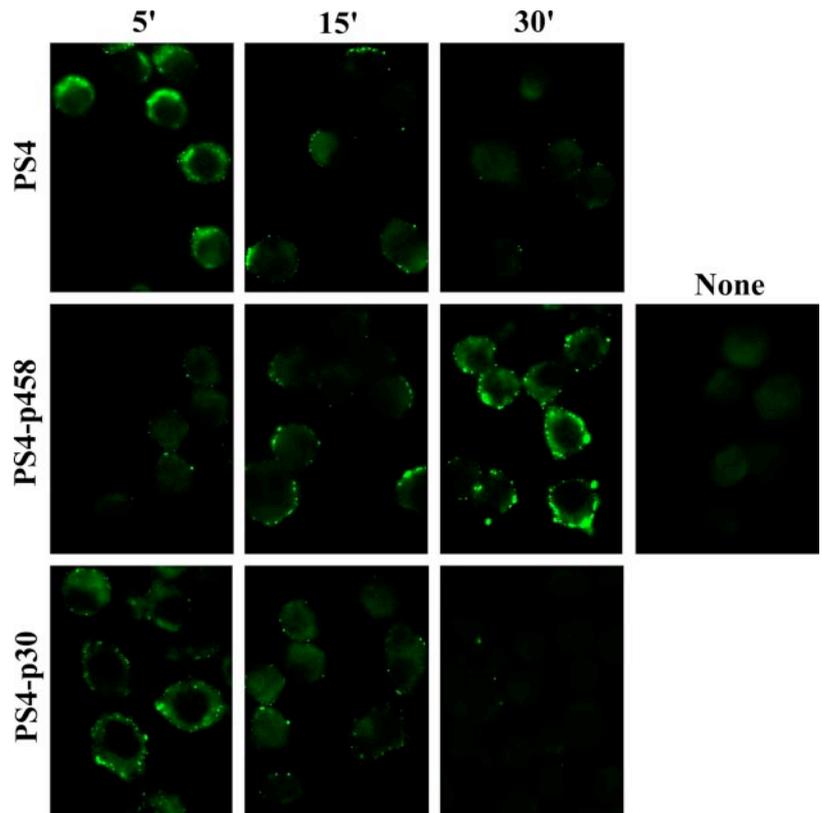


FIGURE 3. RAW cells pulsed with PS4-p458, but not with PS4-p30 or PS4, show late appearance of PS4 on the surface. RAW cells were pulsed with 100 $\mu\text{g}/\text{ml}$ PS4-p458, PS4-p30, or PS4 alone for 5, 15, or 30 min. Then the cells were fixed, and the presence of PS4 at the cell surface was detected by anti-PS4 Abs or FITC-conjugated anti-antibodies, followed by fluorescence microscope, or by no Ab (None). Only the cells that had been pulsed with PS4-p458 manifested PS4 at the surface at 30 min.

the macrophage surface. RAW cells were incubated for 5, 15, or 30 min with the PS4-p458 conjugate, the PS4-p30 conjugate, or with PS4 alone, then fixed and stained with FITC-conjugated anti-PS4 Abs to detect the presence of PS4 on the surface. To distinguish between surface and intracellular CPS, we permeabilized the cell membrane using Triton X-100 before incubating the cells with the Abs; the cells that had undergone permeabilization showed intracellular PS4, whereas the unpermeabilized cells showed only surface staining (data not shown). Fig. 3 shows that the PS4 was present on the surface following pulsing with PS4-p30 or unconjugated PS4 at 5 min and decreased thereafter; in contrast, pulsing with the PS4-p458

conjugate showed a late and prolonged appearance of PS4 on the surface. RAW cell line macrophages (A), peritoneal macrophages obtained from C3HeB/Fej mice with wild-type TLR4 (B), or from TLR4 mutant C3H/Hej mice (C) were incubated for various times with PS4-p458 or PS4 (20 $\mu\text{g}/\text{ml}$). The cells were then fixed and stained for surface PS4 by anti-PS4 Abs and FITC-conjugated anti-antibodies and followed by fluorescence microscopy. Fluorescence intensity was determined by FACS and expressed as the fluorescence index (FI): fluorescence intensity divided by the background of unpulsed macrophages (Background). Due to sampling differences between the different cultures, the absolute fluorescence index values between the cultures are not to be compared; attention is directed to the dynamics of the fluorescence index over time. Peritoneal macrophages from the TLR4 mutant C3H/Hej mice did not manifest the late and prolonged appearance of surface PS4.

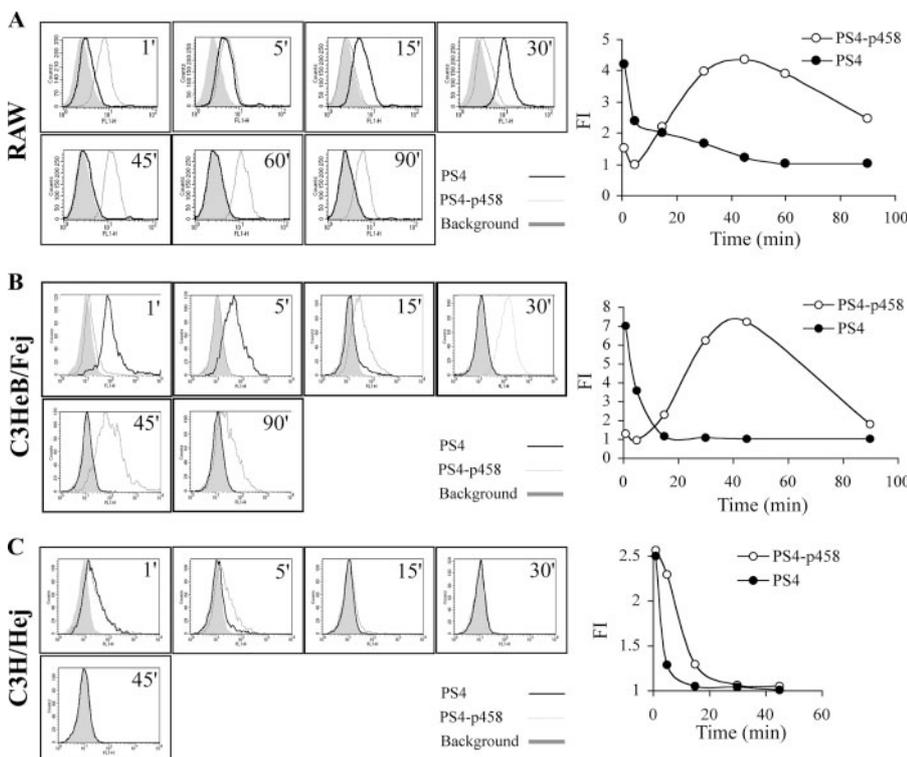


FIGURE 4. PS4-p458 induces the prolonged and late appearance of PS4 on the surface of macrophages with intact TLR4. RAW cell line macrophages (A), peritoneal macrophages obtained from C3HeB/Fej mice with wild-type TLR4 (B), or from TLR4 mutant C3H/Hej mice (C) were incubated for various times with PS4-p458 or PS4 (20 $\mu\text{g}/\text{ml}$). The cells were then fixed and stained for surface PS4 by anti-PS4 Abs and FITC-conjugated anti-antibodies and followed by fluorescence microscopy. Fluorescence intensity was determined by FACS and expressed as the fluorescence index (FI): fluorescence intensity divided by the background of unpulsed macrophages (Background). Due to sampling differences between the different cultures, the absolute fluorescence index values between the cultures are not to be compared; attention is directed to the dynamics of the fluorescence index over time. Peritoneal macrophages from the TLR4 mutant C3H/Hej mice did not manifest the late and prolonged appearance of surface PS4.

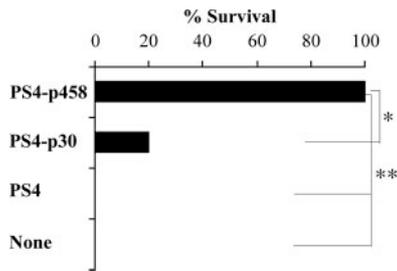


FIGURE 5. RAW cells pulsed with PS4-p458 induce resistance to a lethal *S. pneumoniae* challenge. RAW cells were pulsed with 20 $\mu\text{g/ml}$ PS4-p458, PS4-p30, PS4 alone, or with medium alone (None) for 30 min. The cells were then washed with PBS, irradiated and injected i.p. to five BALB/c mice per group. The mice were challenged 3 wk later with 1000 *S. pneumoniae* CFU. Data show the percentage of survival with indicated conjugate vaccine against challenge. *, $p < 0.05$; **, $p < 0.01$.

conjugate led to the appearance of PS4 on the cell surface at 30 min.

To examine the kinetics of PS4 presence on the surface in more quantitative terms, we used flow cytometry. RAW cells were incubated with PS4-p458 or with unconjugated PS4 for various time periods, then fixed and stained with FITC-conjugated anti-PS4 Ab. Fig. 4A shows that the presence of PS4 on the surface of PS4-pulsed macrophages decreased sharply within minutes and then gradually declined over the next hour. The appearance of PS4 on the surface of the PS4-p458-pulsed macrophages differed; PS4 continued to increase over 45 min, remained high for 1 h, and then gradually decreased, but was still relatively high at 90 min. The PS4-p30 conjugate did not show this prolonged appearance of surface PS4 and behaved as did unconjugated PS4 (data not shown). Thus the stimulatory effect of the PS4-p458 conjugate not only induced the macrophage line to secrete IL-12, it also led to the late and prolonged appearance of PS4 on the cell surface.

Effects of the PS4-p458 conjugate depend on TLR4 signaling

Because the p458 carrier originated from the hsp60 molecule, which has been reported to activate immune cells via TLR4 (23–25), we tested whether the prolonged appearance of PS4 on the cell surface might also require functional TLR4. We isolated peritoneal macrophages from wild-type C3HeB/Fej mice or from TLR4-mutant C3H/HeJ mice (26), and repeated the assay for the detection of surface PS4 using anti-PS4 Ab, as we had done with the RAW cell line. Fig. 4, B and C, show that the macrophages from the wild-type C3HeB/Fej mice also manifested a prolonged appearance of PS4 on the cell surface in response to the PS4-p458 conjugate. However, the TLR4 mutant macrophages failed to do so: there was no difference in the appearance of PS4 in the TLR4 mutant macrophages irrespective of whether they had been incubated with the PS4-p458 conjugate or with PS4 alone. These results suggest that the late, prolonged appearance of PS4 induced by the PS4-p458 conjugate is a process that is dependent on stimulation via intact TLR4. Moreover, the phenomenon is not limited to the RAW macrophage line, but can be induced by the PS4-p458 conjugate in primary peritoneal mouse macrophages.

Macrophages pulsed with PS4-p458 induce protection against lethal challenge

We studied whether late-appearing, surface PS4 can induce resistance to *S. pneumoniae* challenge. RAW cells were pulsed with PS4-p458, PS4-p30, or with unconjugated PS4 for 30 min;

a time when PS4 can be detected on the surface of the cells following incubation with the PS4-p458 conjugate but not after incubation with PS4-p30 or with unconjugated PS4. The cells were irradiated to block their proliferation, and were injected i.p. into naive BALB/c mice. Three weeks later the mice were challenged with a lethal dose of *S. pneumoniae* ($\text{LD}_{50} \times 1000$).

Fig. 5 shows that the mice that had been injected with the PS4-p458-pulsed macrophages were completely protected against the lethal *S. pneumoniae* challenge. In contrast, the mice that had been injected with macrophages pulsed with the PS4-p30 conjugate or with unconjugated PS4 were significantly less protected. Thus it appears that the PS4 present on the macrophage surface can induce protection against *S. pneumoniae* challenge.

Prestimulation with LPS induces late appearance of unconjugated PS4 on the surface of macrophages

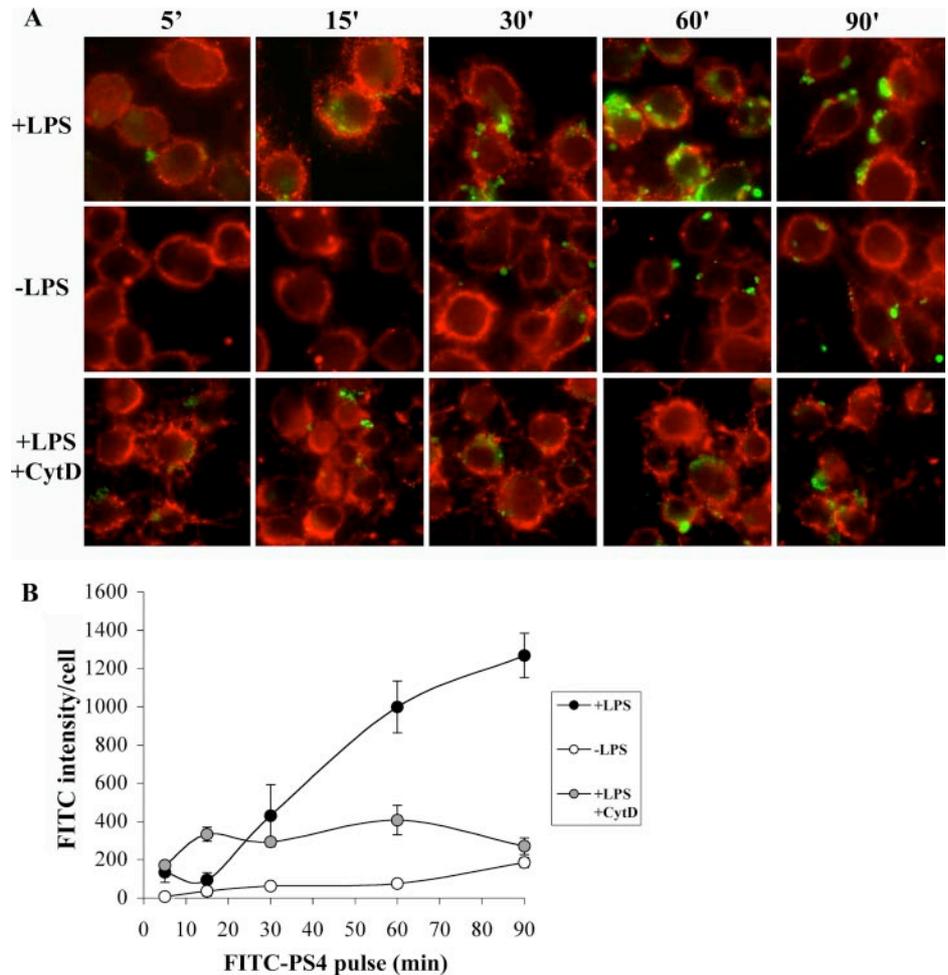
As described (Fig. 4), the ability of the PS4-p458 conjugate to induce the prolonged appearance of PS4 on the macrophage surface depends on a functional TLR4 molecule. If TLR4 activation is sufficient as well as necessary for such prolonged appearance of PS4, then it should be possible to induce the effect using unconjugated PS4 along with a known TLR4 activator. To test this idea, RAW cells were prestimulated or not with bacterial LPS, a classic TLR4 ligand (27), before incubating the cells with unconjugated PS4. We used PS4 internally labeled with FITC to follow PS4 localization. Fig. 6A shows that RAW cells that had been prestimulated with LPS internalized the labeled PS4 within 15 min; the PS4 appeared to be inside the cells. From 30 to 90 min, however, the labeled PS4 molecules appeared on the cell surface, as clustered nodules. In contrast, the RAW cells that had not been pretreated with LPS (Fig. 6A) manifested less PS4 at the cell surface. A quantitative analysis of FITC intensity on the cell surface is presented in Fig. 6B; the FITC intensity of the PS4 on the surface of LPS-treated macrophages decreased slightly after 15 min of incubation and then increased at 60 and 90 min. These results demonstrate that prestimulation with LPS can induce the prolonged appearance of PS4 on the cell surface following pulsing with unconjugated PS4, similar to that observed following pulsing with the PS4-p458 conjugate (Fig. 4).

Endocytosis and exocytosis are processes that require cytoskeletal rearrangement and polymerization. We examined whether cytochalasin D, an inhibitor of cytoskeleton rearrangement (28), might affect the uptake and appearance of PS4 on the macrophage surface. Prior treatment with cytochalasin D indeed inhibited the appearance of PS4 on the surface of LPS-stimulated RAW cells (Fig. 6). Thus, the uptake and expression of surface PS4 following LPS stimulation would seem to require rearrangement of the cytoskeleton.

The appearance of FITC-labeled PS4 on the surface is specifically blocked by unlabeled PS4

To investigate whether the late, prolonged appearance of PS4 at the macrophage surface involves a specific interaction of PS4 with the cell, we conducted competition experiments using unlabeled PS4 or dextran, an oligosaccharide of glucose with a similar average m.w. as PS4. RAW cells were preactivated with LPS and incubated with various amounts of unlabeled competitor for 1 h, and then FITC-labeled PS4 was added for additional hour, at the time the unlabeled PS4 should be presented on the cell surface. The PS4 binding appeared to be specific (Fig. 7); the FITC-labeled PS4 at the cell surface was greatly inhibited by a 100-fold excess of unlabeled PS4, whereas preincubation with the same amount of dextran had no inhibitory effect. These results suggest that the PS4 appearing on the cell

FIGURE 6. LPS stimulation induces late appearance of PS4 on the macrophage surface, inhibitable by cytochalasin D. RAW cells were stimulated with 5 $\mu\text{g/ml}$ LPS (+LPS) for 2 h or not (-LPS). A third group of RAW cells were stimulated with LPS and then washed and incubated with 20 μM cytochalasin D for 1 h (+LPS +CytD). Then the cells were pulsed with 2.5 $\mu\text{g/ml}$ FITC-labeled PS4 (mixed with 100 $\mu\text{g/ml}$ unlabeled PS4) for 5, 30, 60, or 90 min. The cells were then fixed and stained with Cy5 for H-2D^d (MHC-I). A, Representative images are PS4-FITC (green), MHC-I-Cy5 (red). B, A quantitative analysis of PS4-FITC intensity on the cell surface. The analysis was done using SimplePCI software. Data represent mean FITC intensity of five random pictures at each time point.



surface results from an interaction with a specific molecule or molecules, and is not the result of unspecific glycan binding.

Macrophages stimulated with LPS and pulsed with PS4 induce protection to S. pneumoniae challenge

As shown in Fig. 5, macrophages pulsed with the PS4-p458 conjugate induced protection to lethal *S. pneumoniae* challenge. Accordingly, we examined whether the late appearance of PS4 following LPS stimulation is also able to induce functional resistance to lethal *S. pneumoniae* challenge. Mice were injected with LPS-stimulated

RAW cells that had been pulsed with PS4 for 5, 30, or 60 min; see the kinetics of the surface appearance of PS4 in Fig. 6. Three weeks later, the mice were challenged i.p. with a lethal dose of *S. pneumoniae* bacteria ($\text{LD}_{50} \times 100$). The RAW cells stimulated with LPS and pulsed with PS4 for 60 min induced significantly more protection than those pulsed with PS4 for only 5 or 30 min (Fig. 8A). PS4-pulsed macrophages that had not been treated with LPS were significantly less protective. These results indicate that the cell-induced protection to *S. pneumoniae* challenge is associated with the presence of PS4 on the cell surface induced by LPS stimulation.

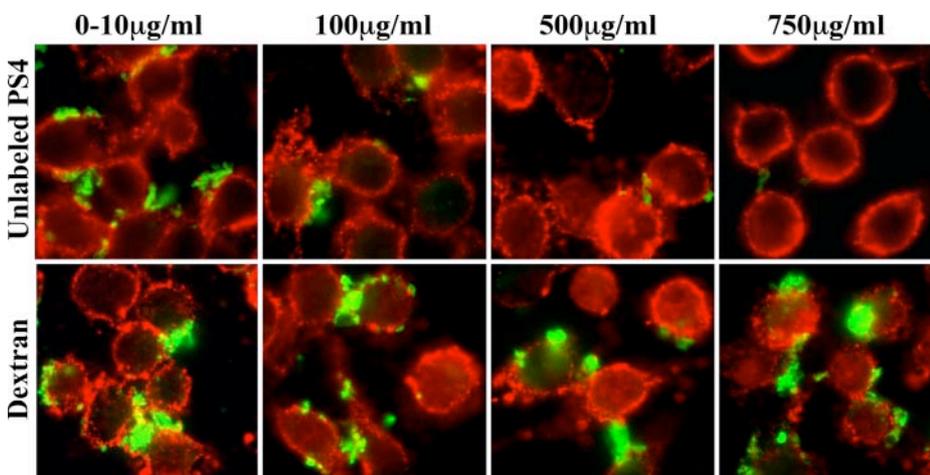


FIGURE 7. Presence of labeled PS4 on the RAW cell surface is specifically inhibited by incubation with cold PS4. RAW cells were stimulated with LPS (5 $\mu\text{g/ml}$) for 2 h, and then incubated with various concentrations (0–750 $\mu\text{g/ml}$) of unlabeled PS4 or dextran for 1 h. FITC-labeled PS4 (2.5 $\mu\text{g/ml}$) was then added for an additional hour. The cells were fixed and stained for H-2D^d (MHC-I), PS4-FITC (green) and MHC-I-Cy5 (red) are shown. Unlabeled PS4 specifically inhibited the surface appearance of labeled PS4.

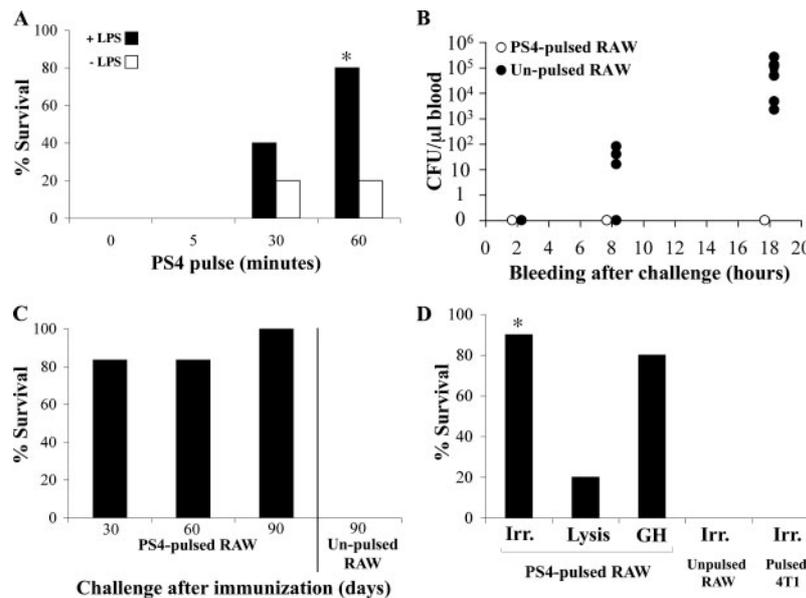


FIGURE 8. Mice injected with macrophages pulsed with PS4 are long-term resistant to lethal *S. pneumoniae* challenge, and have no *S. pneumoniae* bacteria in the bloodstream after the challenge. Intact but not lysed PS4-pulsed RAW cells induce resistance. **A**, RAW cells were either prestimulated with LPS (5 $\mu\text{g}/\text{ml}$) for 2 h or not prestimulated, and then pulsed with PS4 for 0, 5, 30, or 60 min. The cells were then irradiated and injected i.p. (2×10^6) into 5–10 BALB/c mice per group. After 3 wk, the mice were challenged i.p. with 100 *S. pneumoniae* CFU per mouse, and followed for survival. The graph summarizes three different experiments. *, $p < 0.01$ compared with no LPS, 60 min, and to plus LPS, 0 and 5 min. **B**, After the challenge, blood samples were taken from each mouse 2, 8, and 18 h and plated for CFU number determination. Data are presented in a semilogarithmic scale and includes six different blood samples from each group. **C**, Mice were challenged 30, 60, or 90 days after the injection. $p < 0.02$ PS4-pulsed RAW groups compared with unpulsed RAW group. **D**, RAW cells or a breast cancer cell line (4T1) were activated with LPS and pulsed with PS4, then irradiated (Irr.), glutaraldehyde-fixed (GH), or hypotonically lysed (Lysis), as indicated. The cells were then injected i.p. to 5–10 BALB/c mice per group. After 3 wk, the mice were challenged with 100 *S. pneumoniae* (Pn) CFU and followed for survival. Hypotonic cell lysis was done by incubating the cells in ice-cold double-distilled water for 15 min and then passing the cells through a 27-gauge syringe needle several times. Glutaraldehyde-fixation was done by incubating the cells with 1% glutaraldehyde for 20 min at room temperature. *, $p < 0.02$ compared with unpulsed RAW cells, lysed PS4-pulsed RAW cells, and irradiated PS4-pulsed 4T1 cells.

Cell-vaccinated mice clear *S. pneumoniae* bacteria efficiently

We examined the effect of cell vaccination by monitoring the bacterial counts in the blood, and not only by survival. Mice were injected with RAW cells that had been stimulated with LPS and pulsed with PS4 or with unpulsed RAW cells, and 3 wk later, the mice were challenged i.p. with a lethal dose of *S. pneumoniae* bacteria ($\text{LD}_{50} \times 100$). The number of CFU in the blood of the challenged mice was assayed by plating. Fig. 8B shows that the mice that had been injected with the PS4-pulsed RAW cells had an undetectable number of bacteria in the blood 2, 8, and 18 h after the challenge. In contrast, the mice that had been injected with unpulsed RAW cells manifested time-dependent elevation of the CFU counts, leading to death within 24 h. These results demonstrate that the mice protected by LPS-activated PS4-pulsed RAW cells rapidly eradicate the *S. pneumoniae* challenge, and prevent the bacteria from reaching the bloodstream.

Induced resistance persists

To test whether the resistance induced by LPS-activated, PS4-pulsed RAW cells persists, mice were challenged with *S. pneumoniae* bacteria 1, 2, or 3 mo after injection with the treated cells. A control group of mice was injected with unpulsed, but LPS-activated cells. Fig. 8C shows that the induced protection lasted at least 3 mo after injection; the unpulsed RAW cells did not induce protection.

Lysed PS4-pulsed RAW cells cannot induce resistance to *S. pneumoniae* challenge

These experiments demonstrated the ability of PS4-pulsed RAW cells to induce resistance to *S. pneumoniae* challenge; the cells were most effective after 60 min of incubation when the

CPS was on the cell surface. Colino et al. (29) reported, however, that BMDC pulsed ex vivo with whole, heat-killed *S. pneumoniae* were immunogenic and did not require that the CPS be present on the cell surface. Therefore, we examined in greater detail whether intact RAW cells manifesting PS4 on the surface are required to induce resistance to *S. pneumoniae* challenge. We injected mice with LPS-stimulated, PS4-pulsed RAW cells treated in three ways: cell irradiation, lysis of the cells or glutaraldehyde fixation of the cells, which leads to the death of intact cells. We also injected mice with LPS-stimulated RAW cells unpulsed with PS4, or with PS4-pulsed 4T1 cells, a breast cancer tumor cell line, which is a nonphagocytic cell line. Fig. 8D shows that LPS-activated macrophages effectively induced resistance to challenge when the cells were intact, either irradiated or glutaraldehyde-fixed. Lysed RAW cells, in contrast, were significantly less protective. Unpulsed, but LPS-activated RAW cells, or PS4-pulsed 4T1 cells were not at all effective. These results indicate that the induction of resistance to *S. pneumoniae* challenge is a property of RAW cells that have been activated via TLR4 and have PS4 on their intact surface; intact cells are effective even when killed by fixation. The mere injection of PS4 with other cells or with lysed RAW cells is not sufficient.

Immunization with CPS-pulsed BMDC induces specific anti-CPS IgM and IgG Abs and resistance to challenge

DC are important in the induction of specific immunity. To learn whether such cells too could induce CPS type-specific Ab resistance, we used the model of BMDC. Because immature and mature BMDC behave differently in Ag processing and other functions, we studied unstimulated, immature BMDC and LPS-stimulated, mature BMDC.

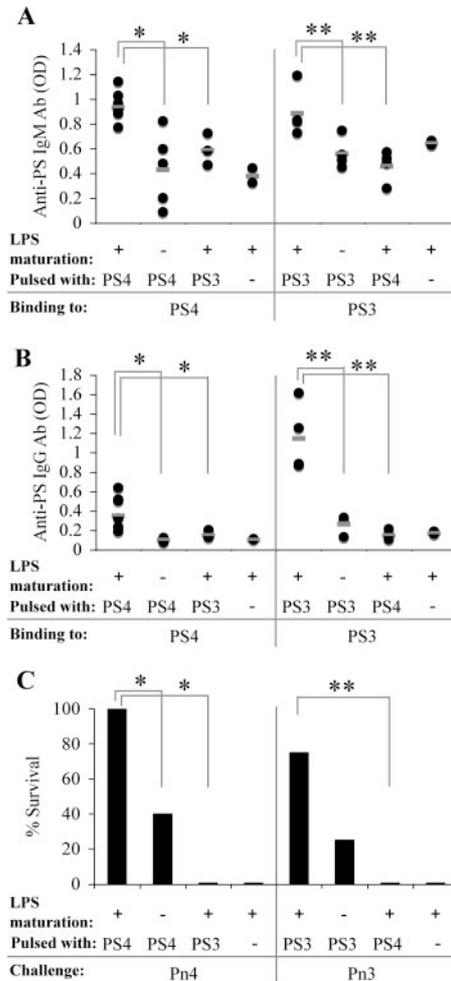


FIGURE 9. Pulsed BMDC induce specific IgM and IgG Abs and specific resistance to challenge. BMDC were either stimulated or not with LPS (1 $\mu\text{g}/\text{ml}$) for 24 h, and then pulsed with PS4 or PS3 for 1 h (100 $\mu\text{g}/\text{ml}$). The cells were then injected i.p. (5×10^6) into 5–10 mice per group. After 3 wk, the mice were bled, and their sera were tested for anti-CPS (type 3 or type 4) IgM (A) and IgG (B) Abs by ELISA. The amount of Ab is shown by OD at 405 nm. C, The mice were then challenged i.p. with $\text{LD}_{50} \times 10,000$ *S. pneumoniae* type 3 (Pn3) or type 4 (Pn4) per mouse, and scored for survival. *, $p < 0.01$; **, $p < 0.05$.

BMDC were prepared and a fraction of them was stimulated with LPS. Both the LPS-stimulated and the unstimulated cells were pulsed with CPS type 3 or type 4 for 1 h and then injected i.p. into BALB/c mice. Three weeks later, we examined the levels of anti-PS4 and anti-PS3 IgM and IgG Abs in the sera of the injected mice. Fig. 9, A and B, show that BMDC pulsed with either PS3 or PS4 induced specific IgM and IgG Ab responses to the respective CPS. PS3-pulsed BMDC induced a greater amount of specific IgG Ab than did PS4-pulsed BMDC. IgG Ab production was also significantly higher in mice injected with LPS-stimulated CPS-pulsed BMDC than in mice injected with unstimulated BMDC.

To test for the induction of specific resistance, 3 wk after immunization with BMDC pulsed with PS3 or PS4, the mice were challenged i.p. with $\text{LD}_{50} \times 10,000$ *S. pneumoniae* bacteria of type 3 or type 4. Fig. 9C shows that LPS-stimulated BMDC were significantly more effective in inducing protection than were the unstimulated BMDC. Moreover, the protection was serotype-specific; only the mice immunized with PS3 were protected against *S. pneumoniae* type 3 and not against *S. pneumoniae* type 4 and vice versa.

Discussion

In this study, we demonstrate that the p458 peptide conjugated to the pneumococcal CPS PS4 was effective as a vaccine administered in PBS in inducing resistance to lethal *S. pneumoniae* challenge (Fig. 1B). The effectiveness of PS4-p458 conjugates administered in PBS drew our attention to the possibility that the PS4-p458 conjugate might work as adjuvants, and not only as a combination of T cell and B cell epitopes. It was recently reported that an outer membrane protein complex from the meningococcus (outer membrane protein complex) conjugated to the CPS of *Haemophilus influenzae* acted as an adjuvant-like molecule by inducing cytokine secretion in vitro in mice with intact TLR2; the induction of resistance to challenge was not examined in that study (30). The adjuvant-like property of PS4-p458 was demonstrated in this study by its ability to induce the macrophage cell line RAW to secrete IL-12 in vitro (Fig. 2). This innate adjuvant-like effect could not be induced by either the unconjugated p458 peptide or the PS4 alone, or by the PS4 conjugated to the T cell epitope peptide p30, PS4-p30. The parent hsp60 molecule of p458 has also been reported to activate innate effects on macrophages and DC via TLR4 (23); however, some reports claim that the innate TLR4 stimulatory effect of hsp60 might actually be caused by LPS bound to the hsp60 molecule (31). Nevertheless, the innate effects of the PS4-p458 conjugate could not be attributed in this study to LPS because an inhibitor of LPS did not inhibit the PS4-p458 conjugate effect (Fig. 2); it is not likely that the PS4-p458 conjugate contained any LPS, and it appears that neither the PS4 nor the p458 peptide are contaminated with any other TLR ligands.

In the course of these investigations, we discovered that the conjugate activated the appearance of PS4 molecule for a late and prolonged period on the cell surface of both RAW line cells and peritoneal macrophages (Fig. 4); pulsing the cells with PS4 alone or with the PS4-p30 conjugate did not induce this effect. The ability of the PS4-p458 conjugate to influence the presence of the PS4 on the macrophage cell surface was found to be dependent on a functional TLR4 molecule (Fig. 4, B and C). Thus, the adjuvant-like effect of PS4-p458 conjugates could be explained by TLR4 innate signaling. This signaling would appear to require cross-linking because neither the unconjugated p458 peptide nor the PS4 CPS alone could stimulate macrophages. Indeed, the PS4-p458 conjugate features 45 p458 peptides on each PS4 backbone (see Materials and Methods). The TLR4 signaling would appear to be induced by the p458 moiety because the PS4-p30 conjugate, which is also multivalent, was not effective.

Importantly, we found that the presence of the PS4 at the macrophage or RAW cell surface endowed the cells with the ability to induce resistance to lethal *S. pneumoniae* challenge when the cells were injected i.p. in PBS into syngeneic mice; cells that had internalized unconjugated PS4, or PS4 conjugated to p30 were much less protective (Fig. 5).

Because the induction of protection in vivo by PS4 present on the surface of cells had not been reported previously, we explored various aspects of this phenomenon. We followed the prolonged PS4 appearance on the macrophage surface using mouse anti-PS4 Abs as probes (Figs. 3 and 4). We confirmed this observation using PS4 internally labeled with FITC (Fig. 6). Subsequently, we reasoned that if the prolonged surface presence of the PS4 was due to innate TLR4 signaling, then one might be able to induce the phenomenon with unconjugated PS4 provided that the TLR4 pathway was activated. To test this

hypothesis, we used the classic TLR4 activator LPS. In this study too, we could confirm that the prolonged surface presence of PS4 could be induced with unconjugated PS4 following LPS stimulation of the RAW macrophage line (Fig. 6). The presence of surface PS4 was blocked by cytochalasin D treatment (Fig. 6). Pretreatment with the cytochalasin D inhibitor could block the active internalization of the CPS into the cells and the late appearance of the CPS on the surface after 60 and 90 min. These results suggest that active rearrangement of the cytoskeleton is essential for the uptake and late surface appearance of the CPS; the surface CPS is not likely to be only passively absorbed from the culture medium. Moreover, the specificity of the surface expression of the PS4 was demonstrated by inhibition of the effect by pretreatment of the cells with unlabeled PS4; dextran, a ligand for the multilectin receptors (32, 33) did not block the prolonged surface appearance of the PS4 (Fig. 7). Most importantly, the surface appearance of the PS4 was demonstrated to be capable of inducing resistance to lethal challenge (Fig. 8A). The resistance appeared to require some intact structural arrangement because lysing the PS4-pulsed macrophages abolished the induction of protection (Fig. 8D). The injection of the PS4 Ag associated with disrupted cellular debris also did not induce resistance. Glutaraldehyde fixation of the macrophages with surface PS4, in contrast, did induce resistance. Finally, the resistance induced by surface PS4 lasted for at least 3 mo (Fig. 8C), indicating that the effect was relatively stable. T cell-independent Ag vaccines are known to induce short-time protection that wanes in about a month (34). Indeed, unconjugated PS4 injected alone fails to induce long-term resistance (15).

We also confirmed that BMDC pulsed with CPS could induce type-specific resistance to *S. pneumoniae* challenge. Immature BMDC express a variety of sensors of inflammation including TLRs and are very active in Ag uptake, but maturation of the BMDC by LPS activation significantly enhanced their effectiveness in inducing resistance and specific IgG anti-CPS Abs. Colino et al. (29) too have shown that Ab responses to bacterial proteins and CPS Ags can be induced by BMDC pulsed ex vivo with whole, heat-killed *S. pneumoniae*, but these authors did not test the recipients of the cells for resistance to *S. pneumoniae* challenge. The induction of resistance to challenge by CPS-pulsed BMDC was associated with the appearance of specific Abs (Fig. 9). However, Ab production is not always correlated with induced protection; it can be seen in Fig. 9 that some of the mice vaccinated with BMDC produced very little or no detectible IgG or IgM Abs, but were, nevertheless, protected against the challenge. We have previously reported that some mice vaccinated with the PS4-p458 conjugate made very little detectible Ab, yet were still resistant to supralethal *S. pneumoniae* challenge (14). Similarly, RAW cells pulsed with PS4 induced protection to $100 \times \text{LD}_{50}$ bacteria for 3 mo, but no IgG or IgM Ab could be detected in the sera of the injected mice by a variety of assay systems (data not shown). These results suggest that the resistance induced by vaccination with CPS-p458 conjugates or CPS-pulsed APC may involve mechanisms not dependent on readily detectible serum Ab. The serotype-specificity of induced resistance and its persistence for at least 3 mo suggest that adaptive immunity is involved.

Recent reports indicate that carbohydrates may be presented to T cells: a zwitterionic CPS can be presented to T cells via the canonical MHC class II pathway (28). Additionally, it was shown by Meltzer and Goldblatt (35) that Pneumococcal CPS is taken-up by immature human DC and transported to HLA-DR and late endosomal compartments; however, no functional as-

say was done to show priming of T cells by the presented CPS. We are presently investigating the role of T cells in this resistance.

The late and prolonged surface presence of the PS4 CPS induced by TLR4 signaling is another observation that needs further study: How does TLR4 activation bring a carbohydrate moiety to the macrophage surface? What molecular arrangement holds the CPS at the surface and presents it to induce immune resistance to challenge?

Disclosures

The authors have no financial conflict of interest.

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