



The contribution of naturally occurring IgM antibodies, IgM cross-reactivity and complement dependency in murine humoral responses to pneumococcal capsular polysaccharides

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ABSTRACT

Immunogenicity of 12 capsular polysaccharides (CPS) from *Streptococcus pneumoniae* did not correlate with pre-existing levels of natural IgM anti-CPS antibodies in mice. Immunization of mice with individual CPS, with the exception of type 14 (the only neutral CPS tested), increased serum IgM that also bound other CPS serotypes independent of structural similarity or commonly known contaminants. Surprisingly only IgM response to type 4 (which has a small immunodominant epitope) was dependent on either complement C3 or complement receptors CD35/CD21. IgG anti-CPS responses were infrequently induced, but critically dependent on complement. Our results have clarified the role of complement in the induction of IgM and IgG anti-CPS antibody responses in mice and have implications for CPS vaccine development.

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1. Introduction

Human infections due to extracellular encapsulated bacteria are still responsible for significant morbidity and mortality worldwide despite the availability of antimicrobial agents. Protective immunity correlates with serum levels of antibodies specific for serotype-defining capsular polysaccharides (CPS) [1]. CPS vaccines are available for both Gram-negative and Gram-positive bacteria including *Haemophilus influenzae* (Hi), *Neisseria meningitidis* (Nm) and *Streptococcus pneumoniae* (Sp). These vaccines are generally effective in older children and adults, but ineffective in children less

than 2 years old. The deficiency in antibody responses toward CPS in young children can be overcome by vaccination with CPS covalently attached to a protein carrier such as tetanus toxoid or diphtheria CRM197 protein. Indeed CPS–protein conjugate vaccines for Hi and Nm have been shown to be extremely successful in reducing disease incidence [2,3]. Unfortunately conjugate vaccines are not affordable to those in developing countries where disease burden is highest. In addition, conjugate vaccines are much more difficult to produce against bacteria such as Sp where 23 serotypes are required [4]. Studies of a 7-valent conjugate vaccine for Sp which covers between 65 and 80% of serotypes that cause invasive infections [5] have revealed two complications: there was an increase in disease caused by nonvaccine serotypes [6] and there were cases of serotype-specific unresponsiveness in immunized infants [7]. Therefore, understanding of CPS immunogenicity in greater detail is still required.

Extensive studies have been performed to define humoral responses to CPS and the underlying basis for hypo- or non-responsiveness to CPS in newborns and infants, but no consensus mechanisms have emerged. One of the difficulties in understanding CPS immunogenicity is due to CPS heterogeneity. CPS are large ((0.5–2) × 10⁶ Da) polymers, composed of hundreds of repeating units of up to eight sugar residues. They are structurally diverse as exemplified by those from Sp in which 90 different serotypes have been identified. Immunogenicity of CPS in humans and mice is known to be variable depending on serotypes/serogroups [8] and references therein). CPS structures antigenically similar to host glycans (e.g. CPS from serogroup B meningococci [9]) are gener-

Abbreviations: BCR, B cell antigen receptor; CPS, capsular polysaccharide(s); CWPS, cell-wall polysaccharide; DNP, dinitrophenol; LPS, lipopolysaccharide; MZ, marginal zone; PC, phosphocholine; PtC, phosphatidylcholine; Sp, *Streptococcus pneumoniae*; TI-1, T cell independent type 1; TI-2, T-independent type 2.

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ally poor immunogens presumably due to central and peripheral tolerance mechanisms that regulate autoimmune B cell responses, but contribution of factors such as chemical composition, conformation and specific antigenic determinants to immunogenicity for other CPS is less clear.

Despite their heterogeneity, CPS are classified together as type 2 T cell-independent (TI-2) antigens. CPS and other TI-2 antigens, unlike protein antigens, are not processed and presented by MHC molecules and do not activate follicular B (B2) cells which require T cell help to proliferate and differentiate. TI-2 antigens with their repetitive structures can directly activate mature B cells (B1 or marginal zone B cell subsets) by cross-linking specific B cell antigen receptors (BCR), but in the absence of T cell help, anti-TI-2 antibody responses in general do not undergo affinity maturation and Ig class switching. Consequently TI-2 antigens elicit predominantly low affinity IgM antibodies and fail to develop immunological memory. Much of the current knowledge on mechanisms of CPS immunogenicity is inferred from many studies utilizing model TI-2 antigens such as dextran and DNP–Ficoll (a haptenated synthetic polysaccharide). It is debatable whether antibody responses to these model TI-2 antigens accurately reflect those to CPS as there are important differences between them. Unlike dextran and DNP–Ficoll, CPS are almost always anionic due to the presence of acidic components such as uronic acids, phosphate or O-acetyl groups. CPS are also different from model TI-2 antigens in terms of structure/conformation and size of B cell epitopes. These differences could affect critical interactions of CPS with natural IgM antibodies, complement, cell surface lectins on innate immune cells [10] and specific B cell subsets [11–13].

In addition to problems of inherent CPS heterogeneity and using model TI-2 antigens to generalize anti-CPS antibody responses, the contribution of contaminants in CPS preparations to CPS immunogenicity has not been properly addressed until recently [14,15]. This is due to the fact that majority of experiments on antibody responses toward TI-2 antigens and CPS were performed prior to the discovery of toll-like receptors (TLR) and our appreciation of their potent effects on innate and adaptive immune responses [16]. Natural or environmental contamination of CPS or TI-2 antigens with varying amounts of TLR agonists including unmethylated CpG nucleotides, peptidoglycan, lipoteichoic acid and lipopolysaccharide (LPS) would most likely have contributed to some of the discrepancy in anti-CPS or anti-TI-2 antibody responses reported in the literature [14,15]. However, this is difficult to confirm because purity of CPS preparations was generally not documented fully in previous reports. In view of these difficulties, factors such as complement that have been implicated as important in the induction or modulation of anti-TI-2 or anti-CPS antibody responses need to be re-assessed.

Complement plays a major effector role in innate host defence against bacterial infections and genetic deficiencies in complement components in humans are associated with an increased risk of infection with encapsulated bacteria [17,18]. Importance of complement in anti-CPS antibody responses has been suggested by studies in which C3 depletion and blockade of complement receptors CD35/CD21 by neutralizing antibodies resulted in a reduction in TI-2 responses in mice [19–21]. However, the role of complement in anti-CPS antibody responses is not entirely consistent. C3 deficiency in human subjects is associated with a large decrease in anti-*Sp* CPS antibodies, but not in antibodies against the CPS of either *Nm* or *Hi* [22]. Although more susceptible to infection, antibody responses against a tetravalent meningococcal CPS vaccine are also normal in C3 deficient individuals [23,24]. Contradictory results on the role of complement in antibody responses to the same CPS have also been reported. Impaired antibody response to the CPS and increased susceptibility to group B type III streptococcal infection have been shown in C3^{−/−} and CD35/CD21 deficient (Cr2^{−/−})

mice [25,26] although an earlier study suggested that responses to this CPS were complement independent [19].

Here we used a panel of 12 *Sp* CPS, all shown to lack contaminating TLR activities, to investigate factors contributing to CPS immunogenicity. After ensuring that only CPS-specific antibodies were measured, we determined whether or not CPS structures, natural IgM anti-*Sp* CPS levels or complement influence the production of adaptive IgM and IgG anti-CPS antibodies by analyzing serum level and specificity of these antibodies. We also investigated differences in antibody responses to CPS with those to a model TI-2 antigen, DNP–Ficoll. Our results have implications for future CPS vaccine development as they suggest involvement of different B cell subsets in IgM and IgG anti-CPS antibody responses and that certain biochemical features of CPS contribute to cross-reactivity and complement dependency of murine humoral responses to pneumococcal CPS.

2. Materials and methods

2.1. Polysaccharides and reagents

Sp CPS, serotypes 1, 2, 3, 4, 6B, 9N, 9V, 14, 18C, 19A, 19F and 23F (Danish nomenclature) were provided either by Dr. Chris Jones (National Institute for Biological Standards and Controls, Hertfordshire, UK) or purchased from the American Type Culture Collection (LGC, UK distributor). The structures of the *Sp* CPS used in this study have been illustrated previously [27]. The *Sp* cell-wall polysaccharide (CWPS), rabbit anti-CPS-specific antisera, rabbit anti-CWPS antiserum and anti-phosphocholine (PC) specific monoclonal antibody (HAS) were purchased from the Statens Serum Institute, Copenhagen, Denmark. Horseradish peroxidase (HRP) conjugates of anti-rabbit IgG Fc-specific and anti-rabbit IgM μ -chain specific antisera were obtained from Cambridge Bioscience Ltd., Cambridge, UK and HRP conjugates of anti-mouse IgG Fc and IgM μ -chain specific antisera from Stratech Scientific Ltd., Luton, Bedfordshire, UK. Phosphocholine chloride calcium salt tetrahydrate and 1-palmitoyl-glycero-3-phosphocholine was obtained from Fluka. DNP–Ficoll was purchased from Biosearch Technologies, Novata, CA, USA.

2.2. Analysis of CPS samples

Commercial preparations of *Sp* CPS all contain varying amounts of contaminating teichoic acid or CWPS. To assess the size, purity and level of contamination of the CPS used in this study, aliquots of each were analyzed by HPLC gel filtration chromatography. Aliquots of CPS (100 μ g) were separated on a TSK G5000 PW xl column (300 mm $\times$ 7.5 mm) in PBS at 40 °C and at a flow rate of 0.25 ml/min. The eluant was monitored at 280 and 205 nm to detect protein and at 205 nm for refractive index to detect carbohydrate. LPS contamination was not expected because CPS were purified from a Gram-positive bacterium. This was confirmed by a kinetic turbidometric limulus amoebocyte lysate (LAL) assay (sensitivity 0.05 EU/ml), using reagents from Charles River Endosafe and *E. coli* 055 LPS as standard according to the manufacturer's instructions. The presence or absence of CWPS in CPS preparations was confirmed by titration of CPS against rabbit anti-CWPS-specific antiserum and by reaction with anti-PC specific monoclonal antibody. Briefly, CPS were coated onto ELISA plates at concentrations from 50 to 0.01 μ g/ml and relative levels of CWPS for each CPS sample were determined.

2.3. Removal of CWPS and modification of CPS by periodate oxidation

Periodate oxidation cleaves the CWPS polysaccharide backbone at ribitol residues [28] thereby cleaving the polysaccharide into

smaller fragments which can be removed by dialysis. CPS samples were oxidized at 500 µg/ml with 10 mM sodium periodate in 0.1 M sodium acetate pH 5.6 at 4 °C in the dark for 8 h. The reaction was quenched by the addition of ethanediol to a concentration of 25 mM followed by dialysis against water (25 kDa molecular weight cut off). The oxidized samples were then reduced with sodium borohydride at 10 mg/ml in water for 4 h at room temperature. Excess borohydride was destroyed by the addition of acetic acid followed by dialysis against water (25 kDa molecular weight cut off). After treatment samples were tested with either anti-CWPS antiserum or anti-PC antibody to confirm the removal of CWPS. Periodate oxidation also modifies CPS depending on structure with types 3, 4 and 9N the least sensitive to this treatment.

2.4. Removal of immunodominant epitope of serotype 4 CPS

Mild acid hydrolysis was used to remove cyclic pyruvate from serotype 4 CPS [29]. Hydrolysis was carried out with 0.01 M HCl at 100 °C for 10 min. The reaction was cooled and the sample neutralized with sodium hydroxide followed by dialysis against water. Neutralization and minimal depolymerization of the sample was confirmed by standard QAE-Sephadex ion-exchange chromatography and gel filtration chromatography, respectively. Titration with anti-CWPS antiserum and anti-PC monoclonal antibody confirmed that the CWPS content of the sample had not been affected by hydrolysis.

2.5. Mice

Female or male C57Bl/6 (H-2^b) and rederived Cr2^{-/-} [complement receptors 1 and 2-deficient (H-2^b)] mice were obtained from Charles River, UK (Margate, Kent, UK). The line of Cr2^{-/-} mice used in this study was obtained from Dr. MC Carroll (Harvard Medical School) has been described previously [30]. C3^{-/-} [complement 3-deficient (H-2^b)] mice were kept at Oxford University. All animals were kept under specific pathogen free conditions and handled according to institutional guidelines. All mice were used between 8 and 12 weeks of age.

2.6. Immunizations

Optimal dose for each CPS was determined to be equal or greater than 5 µg. Groups of three mice were immunized i.p. with 5 µg of CPS in 500 µl of sterile PBS. Blood was taken from the tail vein of C57Bl/6 wild type and Cr2^{-/-} mice 1 day before immunization and again either 7 or 21 days after immunization. Blood was collected from C3^{-/-} mice 15 days after immunization. Samples were left at 4 °C overnight, centrifuged briefly, and the serum removed and stored either at -18 °C until required or at 4 °C with 0.01% (w/v) azide. Control mice were injected with PBS only.

2.7. Determination of serum antibody levels by enzyme-linked immunosorbent assay (ELISA)

Prior to measuring anti-CPS responses, antibodies to CWPS were absorbed with at least 200 µg/ml of CWPS at a 1/50 serum dilution for 1 h at room temperature. Further doubling dilutions were then carried out in buffer without added CWPS. In some experiments anti-phosphocholine (PC) specific antibodies were absorbed by the addition of PC (50 µg/ml) to the dilution buffer. Wells coated with CWPS were included in all assays as a control to confirm absence of CWPS binding. The coating of CWPS or 1-palmitoyl-glycero-3-PC was confirmed by ELISA using a CWPS-specific antiserum or PC-specific monoclonal antibody. All incubations and washings were carried out in PBS and 0.05% (w/v) Tween 20. Polysaccharides were coated onto the wells of ELISA plates (Nunc, Maxisorb) by incubation in 0.154 M NaCl at 5 µg/ml overnight at 37 °C (50 µl/well) in

a sealed damp box. Plates were coated with phosphatidyl choline (PtC) and phosphatidyl ethanolamine in 50% ethanol overnight at 4 °C. After coating, plates were washed 5 times and those for analysis of mouse IgG were blocked by incubation with PBS and 4% (w/v) skimmed milk powder.

Serum dilutions were incubated in the wells of coated plates (50 µl/well) in duplicate for 2 h at room temperature. Plates were then washed 5 times and bound antibodies detected with either anti-mouse IgG-specific or anti-mouse IgM µ-chain specific HRP conjugates. After washing, the reaction was developed with o-phenylenediamine dihydrochloride (50 µl/well). Reactions were stopped after 10 min with 15 µl/well of 3 M sulphuric acid and absorbance measured at 490 nm.

2.8. Statistics

According to institutional and UK Home Office guidelines, number of mice used in each experiment was kept to as minimum as statistical analysis principle allowed. Statistical analysis was performed according to instructions given by Dr. Sandro Leidi, Statistical Services Centre, University of Reading, Berkshire, UK. Data were analyzed using a general linear model of ANOVA comparing sample, mouse strain (naïve vs. immunized) and experiment. Statistical differences with *p* values <0.05 were considered significant.

3. Results

3.1. CPS used in this study do not induce signature activities of toll-like receptor agonists

We believe that much of the discrepancy in anti-CPS or anti-TI-2 antibody responses reported in the literature was due to varying amounts of TLR agonist contaminants in CPS or TI-2 antigen preparations. TLR activation has profound effects on innate and adaptive immune responses [16] and has been shown recently to alter antibody responses to Sp CPS in mice [15]. We took great care to analyze our CPS samples for TLR activities. Up-regulation of co-stimulatory molecules CD40, CD80 and CD86 by macrophages and dendritic cells is a signature response to the presence of TLR agonists. Our CPS samples tested at 10 µg/ml in culture had no effect on the expression of these molecules in mouse or human macrophages or dendritic cells (data not shown). Types 3, 9N, 9V, 14 and 23F, but not the other seven CPS or CWPS stimulated the release of TNF-α. Consistent with the lack of detection of LPS in CPS samples by the sensitive LAL assay, none of the CPS induced IL-12 production (Fig. S1, supplementary data).

We also assessed the purity of CPS by HPLC. All samples showed a single polysaccharide peak of approximately 500 kDa that co-eluted with a trace absorbance at 280 nm (Fig. S2, supplementary data). This trace contaminant is probably peptidoglycan that links CPS with CWPS [31]. CWPS is a well known contaminant of CPS preparations and we have found that CWPS level in our 12 CPS preparations varied, with type 4 having the highest and type 3 the lowest (data not shown). Although CWPS by itself was found to be a poor immunogen in C57Bl/6 mice, greater increases in anti-CWPS IgM were seen after immunization with CWPS-containing CPS (data not shown). We initially used the common practice of absorbing out anti-CWPS antibodies with concentration of CWPS up to 100 µg/ml prior to assessing anti-CPS antibody levels. However, we found that CWPS concentration of 200–500 µg/ml at 1/50 serum dilution was required to ensure adequate absorption of anti-CWPS in all cases. Complete absorption of anti-CWPS IgM antibodies in immune sera which typically resulted in a reduction of from 20 to 30% in binding to CPS enables us to compare CPS-specific antibody responses only. In addition to the purity of CPS, immunogenicity of CPS could also be influenced by the pre-immunization level of natural IgM in

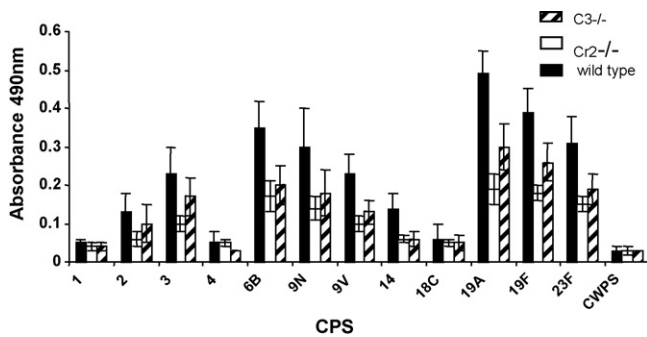


Fig. 1. Natural IgM antibodies against CPS in naïve mice. The sera from naïve C57Bl/6 mice (8–10 weeks of age) were absorbed with either CW-PS or PC and the levels of natural IgM antibodies recognizing CPS measured by ELISA at a 1/200 serum dilution as described in Section 2. The results are for sera taken from 12 individual mice of each type (wild type, $Cr2^{-/-}$ and $C3^{-/-}$) $\pm$ SD.

mice. Therefore, we next examined level and specificity of natural IgM antibodies to *Sp* CPS and CWPS.

3.2. Natural IgM antibodies with specificity for *Sp* CPS do not affect CPS immunogenicity

Natural IgM antibodies with specificity for TI-2 antigens have a critical role in the immediate defence against systemic bacterial infection in mice including *Sp* [12,32]. These IgM antibodies are spontaneously induced in naïve animals and could influence CPS immunogenicity by a number of mechanisms including reducing serum level of CPS and activating complement via the classical pathway. It was thus necessary to establish levels of natural IgM with specificity for *Sp* CPS in naïve sera from different mouse strains and determine whether or not levels correlate with CPS immunogenicity. Normal mouse sera from C57Bl/6 wild type, $C3^{-/-}$ and $Cr2^{-/-}$ (CD35/CD21-deficient) mice were screened against the panel of CPS by ELISA. Fig. 1 shows that all the sera contained natural IgM antibodies recognizing CPS, but not CWPS or PC (a component of CWPS and lipoteichoic acid). In agreement with this finding, absorption of normal sera with either CWPS or PC had no effect on IgM binding to any of the CPS. The observed binding was CPS-specific as carbohydrate destruction of CPS by periodate treatment decreased binding of natural IgM antibodies. The extent of binding to different CPS varied, but was consistent between individual mice, for example, binding to type 4 CPS was consistently low whereas levels of natural IgM against 19A and 19F CPS were consistently high (Fig. 1). However, *Sp* CPS immunogenicity did not depend or correlate with levels of natural IgM anti-*Sp* CPS antibody.

3.3. Analysis of IgM anti-CPS antibodies revealed unexpected cross-reactivity among CPS with no obvious structural relatedness

Results from our analysis of CPS samples have indicated that antibody responses induced by individual CPS alone in different mouse strains could be compared without the complications introduced by different levels of TLR activities in individual CPS preparations or of natural IgM antibodies with specificity for *Sp* CPS. CPS immunization resulted in an early IgM response that peaked at 5–7 days, as expected from TI-2 antigens. A high level of IgM antibodies was maintained for at least 6 months and was not boostable (data not shown). However, with the exception of type 4, IgM anti-CPS antibodies were not specific for the immunizing CPS antigen and cross-reactions were observed independent of obvious structural similarity between the polysaccharides. Cross-reactions induced by types 23F and 3 CPS are shown in Fig. 2. Cross-reactions were not due to IgM antibodies specific for PC as demonstrated by CPS reactivity despite extensive absorption of anti-serum with

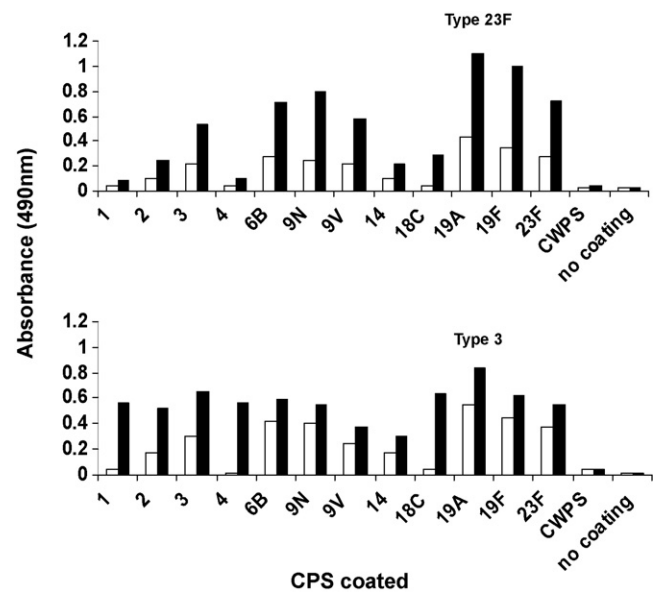


Fig. 2. Cross-reactions of CPS binding IgM antibodies. All CPS were coated onto the wells of ELISA plates at 5 μ g/ml and IgM cross-reactions were determined at a 1/200 serum dilution after absorption of PC binding antibodies as described in Section 2. Cross-reactions after immunization with types 23F and 3 are shown. Open bars, sera from pre-bleed C57Bl/6 mice; solid bars, immune sera. The results from one experiment are shown. These are representative of data obtained from 3 to 5 separate experiments.

either CWPS or PC. In contrast, there were reductions in binding by cross-reacting IgM antibodies to periodate-treated CPS. More importantly CPS with an oxidizable side-branch forming part of the immunodominant structure, such as present in types 2 and 14 lost almost all reactivity whereas those with only one oxidizable bond in a linear polysaccharide, for example, types 3 and 19A, were only moderately affected (Fig. 3). The decreases in binding to periodate-treated CPS were similar between mouse IgM antibodies and commercial rabbit anti-CPS-specific antiserum (data not shown). Together these results are consistent with binding of IgM to the CPS structure and indicate cross-reactions observed were not due to antibodies to a common contaminant.

Extensive cross-reactions of CPS without obvious structural relatedness were unexpected. As this result may have implications for CPS vaccine development, in particular where multiple serotypes are targeted, we analyzed qualitatively the property and types of cross-reaction induced and divided the CPS tested into two groups (Table 1). Each CPS in group 1 induced relatively good increases in IgM antibodies recognizing all CPS from this group. More importantly absorption of the immune sera with any one CPS (50 μ g/ml) from this group was sufficient to remove all binding to CPS. Type 19A was additionally able to induce IgM antibodies cross-reacting to a few other *Sp* CPS types. CPS in group 2 induced higher levels of IgM anti-CWPS antibodies than those in group 1, and CPS-specific cross-reactions for CPS in group 2 were more diverse (Table 1). Serotype 1 *Sp* CPS was not used as an immunogen because of its structural similarity with CWPS. Interestingly antibodies to the only neutral CPS in this panel, type 14, showed no cross-reactions with any of the other 11 CPS. This CPS also did not elicit an increase in IgM antibodies other than those specific for CWPS.

3.4. Differential dependency on complement in IgM responses toward CPS and DNP-Ficoll

We next wished to clarify the role of complement in anti-CPS antibody responses by comparing antibody levels induced by individual CPS or DNP-Ficoll in wild type C57Bl/6, $Cr2^{-/-}$ and $C3^{-/-}$ mice. All the sera from these experiments were absorbed with

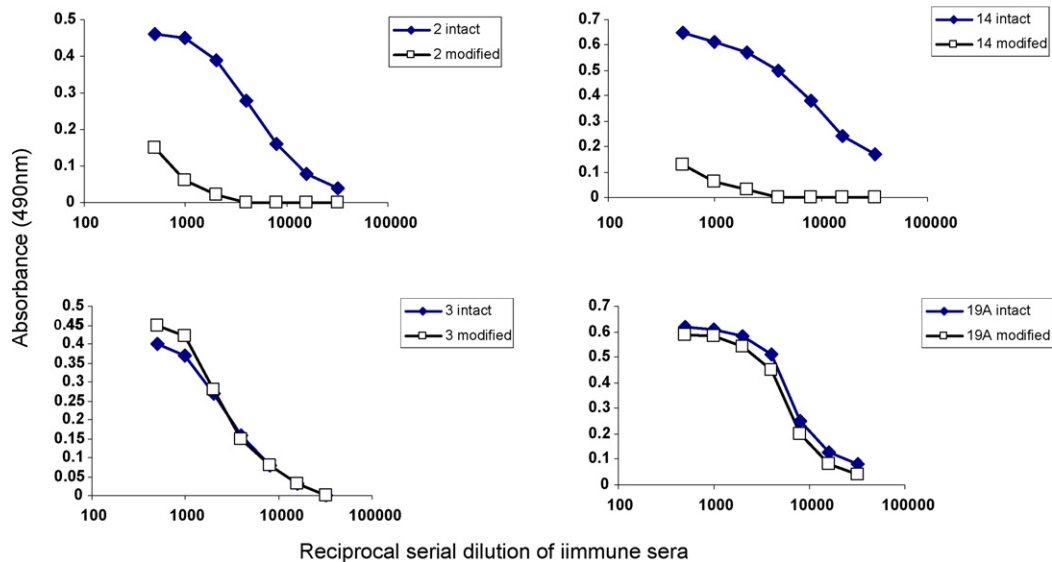


Fig. 3. Reductions in binding by cross-reacting IgM antibodies to periodate-treated CPS. Untreated or periodate-treated CPS was coated onto the wells of ELISA plates at 5 µg/ml and a serial dilution of serum from mice immunized with individual CPS was applied. IgM binding to untreated and periodate-treated CPS was determined as described in Section 2. Strong reductions in IgM binding were observed for types 2 and 14 CPS due to an oxidizable side-branch forming part of the immunodominant structure, and slight reductions were observed for types 3 and 19A CPS because they have only one oxidizable bond in a linear polysaccharide.

CWPS to remove anti-CWPS antibodies. The effect of CPS immunization was measured as a relative increase between naïve and immune sera because both naïve C3^{-/-} and Cr2^{-/-} mice contained lower level of natural IgM anti-CPS antibodies than in wild type mice (Fig. 1). After injection with CPS, increases in serum IgM were seen in C3^{-/-} and Cr2^{-/-} mice although the total level of anti-CPS IgM was always lower than in the wild type mice. The relative increase in IgM between pre-bled mice and after injection was not significantly different between wild type and complement deficient mice in all cases (as shown for types 2 and 3 CPS in Fig. 4) except for type 4 CPS which consistently gave a significantly reduced response in both Cr2^{-/-} and C3^{-/-} mice (Fig. 4). Although only 3 mice per group were used for each of the 12 CPS tested in each experiment, and the response was somewhat variable between mice and experiments, statistically significant reduction of anti-CPS antibody response in complement-deficient mice was only observed for type 4 in three repeat experiments.

IgM responses to DNP–Ficoll were consistently good and always specific for the hapten and not Ficoll (data not shown). In contrast to CPS, except type 4, IgM responses to DNP–Ficoll were significantly reduced in C3^{-/-} mice indicating a dependence on C3. But unlike type 4, IgM anti-DNP–Ficoll responses were not dependent on CD35/CD21 (Fig. 4). These results suggest that complement contributes to IgM anti-DNP and anti-type 4 CPS antibody responses, but has no significant influence on the induction of IgM anti-CPS antibody.

3.5. Induction of IgG anti-CPS and anti-DNP–Ficoll antibody responses differed markedly in frequency but critically dependent on complement

In contrast to IgM responses to CPS, IgG responses to the same set of Sp CPS in C57Bl/6 wild type mice were highly variable, infrequently observed and mostly against serotypes 3, 4, 14 and 19A

Table 1
Cross-reactions of anti-pneumococcal capsular polysaccharide antibodies.

	Cell-wall polysaccharide-independent IgM anti-CPS antibodies cross-reactions										
	6B	9N	9V	19A	19F	23F	2_	3_	18C	14_	4_
Group 1 CPS											
6B	**	**	**	**	**	**	neg	*	neg	neg	neg
9N	**	**	**	**	**	**	neg	*	neg	neg	neg
9V	**	**	**	**	**	**	neg	*	neg	neg	neg
19A	**	**	**	**	**	**	*	**	*	neg	neg
19F	**	**	**	**	**	**	neg	*	neg	neg	neg
23F	**	**	**	**	**	**	neg	*	neg	neg	neg
Group 2 CPS											
2_	*	**	**	**	**	**	*	neg	neg	neg	**
3_	neg	neg	neg	neg	neg	neg	**	**	**	neg	**
18C	*	*	neg	*	*	neg	neg	neg	*	neg	neg
Unclassified CPS											
14_	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
4_	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	**

IgM anti-CPS antibodies elicited by immunization with individual CPS were tested for cross-reactions with other serotypes after absorption of cell-wall polysaccharide. **Denotes an increase of >0.3, *an increase of between 0.2 and 0.3, neg: an increase of <0.2 absorbance units at 1/200 serum dilution. Group 1 CPS: higher level of natural IgM, lower level of induced anti-CWPS antibodies, cross-reactions mainly with group 1 CPS. Group 2 CPS: lower level of natural IgM, higher level of induced anti-CWPS antibodies, cross-reactions more diverse. Unclassified CPS: type 14 is the only neutral CPS in this panel. Elicited IgM antibodies were against CWPS and as such show no cross-reactions to any of other CPS types; type 4 is the only one in which IgM elicited is type-specific.

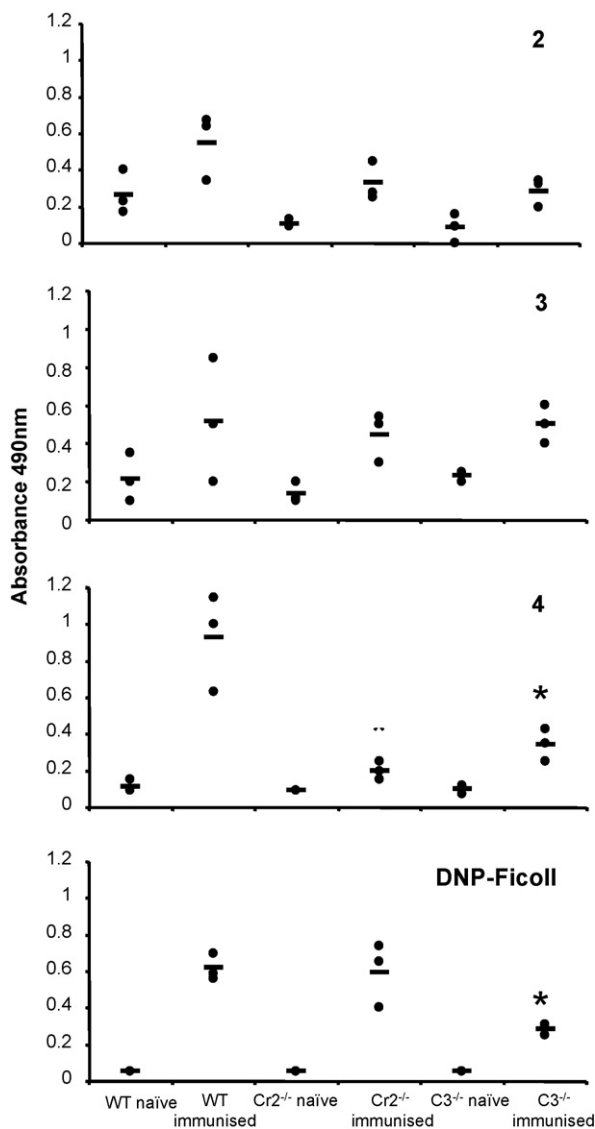


Fig. 4. Comparison of anti-CPS IgM responses in wild type, $Cr2^{-/-}$ and $C3^{-/-}$ mice. Groups of three C57Bl/6 mice were injected i.p. with 5 μ g of each CPS. Mice were pre-bled and blood taken again either 7 or 15 days after immunization. Experiments were repeated 2 or 3 times. Anti-CPS serum IgM was assayed for individual mice. The figure shows representative data from one experiment for 3 CPS, together with results for DNP–Ficoll. The sera were assayed at a 1/200 dilution. Statistical analysis was carried out using a general linear model of ANOVA comparing each experiment, antigen, and each mouse type (naïve or pre-bled vs. immunized). p -Values <0.05 were considered significant (*). The results obtained for serotypes 2 and 3 are representative of those obtained for other serotypes 1, 6B, 9N, 9V, 18C, 19A, 19F and 23F.

(responses to types 4 and 14 are shown in Fig. 5). Elicited IgG responses, also in contrast to IgM responses, were serotype-specific because no IgG anti-CPS antibodies were detected in normal sera, and CPS immunization did not generate IgG anti-CWPS antibodies. IgG cross-reaction was only observed between the structurally

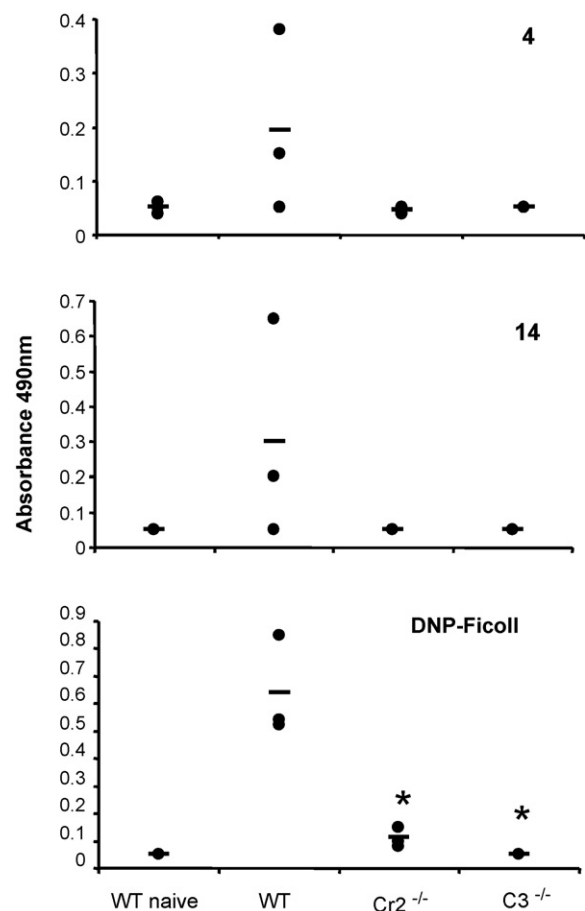


Fig. 5. Comparison of anti-CPS IgG responses in wild type, $Cr2^{-/-}$ and $C3^{-/-}$ mice. Groups of three C57Bl/6 mice were injected i.p. with 5 μ g of each CPS and sera analyzed as described for Fig. 4. Anti-CPS serum IgG was assayed as described in Section 2. IgG responses were variably observed only against serotypes 3, 4, 14 and 19A, during this study. In contrast, IgG responses against DNP–Ficoll were constant. The data show absorbance readings obtained for individual mice at a 1/50 serum dilution against serotypes 4, 14 and at a 1/100 dilution against DNP–Ficoll. Statistical analysis was carried out using a general linear model of ANOVA comparing each experiment, antigen, and each mouse type (naïve or pre-bled vs. immunized). p -Values <0.05 were considered significant (*).

related 19A and 19F. Furthermore, IgG recognition of CPS showed sensitivity to periodate oxidation (data not shown) except for type 4 whose structure is resistant (Table 2). These results together strongly suggest that the IgG induced was against the CPS and not against a contaminant, in particular a protein component, in the CPS preparation. In marked contrast, injection with DNP–Ficoll consistently generated a good IgG response. As it was the case for IgM anti-DNP–Ficoll antibodies, the targeted epitope was the DNP hapten and not Ficoll. Despite differences in frequency and levels, IgG responses against CPS and DNP–Ficoll were critically dependent on complement. In no instance was an IgG response against any CPS detected in either $Cr2^{-/-}$ or $C3^{-/-}$ mice. There was also a total

Table 2

Contribution of cell-wall polysaccharide and cyclic pyruvate of type 4 CPS in IgM and IgG antitype 4 CPS antibody response.

Coating antigen	Anti-type 4 CPS serum			
	IgM (–)	IgM (+)	IgG (–)	IgG (+)
Native type 4 CPS	1.7	0.56	0.65	0.61
Periodate oxidized (CW-PS destroyed)	0.65	0.54	0.59	0.56
De-pyruvylated (cyclic pyruvate destroyed)	0.85	0.03	0.1	0.05
CW-PS	1.6	0.04	0.1	0.05

(+) absorbed with cell wall polysaccharide (CW-PS); (–) not absorbed. Numbers represent absorbance units in standard ELISA for CPS reactivity.

absence of an IgG response toward DNP–Ficoll in C3^{-/-} mice and a significant reduction in Cr2^{-/-} mice (Fig. 5).

3.6. Immunodominant epitope of type 4 contributes significantly to its uniqueness to other CPS

Type 4 CPS contrasted with other serotypes in showing poor binding to natural IgM antibody, complement dependence of the IgM response, and in specificity of IgM binding to type 4 after removal of PC- (thus CWPS-) binding IgM. Therefore, we wished to determine how type 4 CPS might be different from the other 11 CPS tested. This CPS differs from other *Sp* CPS in containing a cyclic pyruvate modification in its structure [27], which has previously been reported to be an immunodominant epitope [29,33]. The binding of IgM and IgG anti-type 4 CPS antibodies to native, oxidized (CWPS removed) and de-pyruvylated (cyclic pyruvate removed) samples were thus investigated. As shown in Table 2, de-pyruvylation resulted in a loss of binding of IgM and IgG anti-type 4 antibodies with absorbance decreased from 0.56 to 0.03 and 0.61 to 0.05, respectively. This confirms the immunodominance of the epitope. Unlike IgM, none of the IgG was generated against PC as shown by the similar binding (with absorbance of 0.56 and 0.54, respectively) to non-modified and oxidized type 4 CPS samples. Hence all serotype 4-specific IgM and IgG binding was lost after CPS de-pyruvylation while CWPS-specific IgM binding was unaffected.

Discussion

Understanding of humoral responses to *Sp* CPS would provide ways to improve or expand options to treat and prevent infections by *Sp* and other encapsulated bacteria, but precise mechanisms responsible for the generation and regulation of antibody responses to CPS *in vivo* are poorly defined. In this study, we investigated the contribution of natural IgM and complement in antibody responses to *Sp* CPS because these two serum factors have been implicated to play an important role in modulating CPS immunogenicity. Although natural IgM and complement have been assessed previously using either model TI-2 antigens or a small number of CPS in a single study, their role in the induction of anti-*Sp* CPS antibodies remains unclear or contradictory. By overcoming problems associated with previous studies such as the presence of contaminants in CPS immunogens and comparing antibody responses to 12 *Sp* CPS structures, we aimed to clarify the role of natural IgM and complement in anti-CPS antibody responses.

A major problem in studying CPS immunogenicity is the presence of variable amounts of contaminants in CPS preparations that are often extremely difficult to remove either due to covalent attachment (e.g. CWPS) or strong non-covalent associations (e.g. LPS). These potential contaminants could affect the determination of CPS-specific antibody levels and the interpretation of the mechanisms involved. Our analysis suggests that all 12 CPS immunogens used in this study consist of both *Sp* CPS and CWPS, likely covalently linked through a fragment of peptidoglycan [31]. This however does not present a problem because the immunogenicity of CPS in mice is not enhanced by these contaminants ([31] and this study). In contrast we found that CWPS associated with CPS was more immunogenic than CWPS alone and levels of CWPS-specific antibody induced by CPS varied depending on serotype. We thus caution against the assumption that certain concentration of CWPS (i.e. 100 µg/ml) will absorb out all CWPS-specific antibodies in every CPS immune serum. Periodate oxidation to destroy epitopes in CPS was used to ensure that antibody level comparison was specific for CPS and not for protein contaminants. Unlike CWPS which does not affect CPS-specific antibody responses, a recent study clearly demonstrated that TLR-activating contaminants (TLR2 and TLR4 agonists) in *Sp* CPS and conjugate vaccine samples (i.e.

type 3, 14, 23-valent vaccine Pneumovax-23 and 7-valent conjugate vaccine Prevnar) were critical in the induction of IgG anti-CPS antibodies *in vivo*. IgM anti-CPS antibody production was also reduced, albeit variably depending on serotype, in TLR-deficient mice [15]. These findings imply that previous studies using CPS immunogens containing TLR-activating contaminants could possibly be describing polyclonal B cell responses activated by TI-1 antigens such as LPS instead of those induced by TI-2 antigens [15]. This is not an issue in our study because none of the 12 CPS samples up-regulated the expression of co-stimulatory molecules on macrophages or dendritic cells, a signature response of TLR activation. Although a few CPS induced TNF-α secretion by macrophages and dendritic cells *in vitro* this was not consistent for all CPS and does not appear to be sufficient to serve as an adjuvant based on *in vivo* studies. Lack of TLR agonist activities in our CPS samples enabled us to analyze adaptive CPS-specific antibody responses that are activated by CPS alone and to clarify the influence of natural IgM and complement to these responses.

Natural IgM antibodies [13] including those specific for *Sp* CPS [12] are produced mainly by B1 cells in the pleural and peritoneal cavities in mice. Several studies have indicated an important role for B1 cells in anti-TI-2 or anti-carbohydrate responses [12,34–38] in mice. Furthermore natural IgM antibodies contribute to the induction of adaptive immune responses to vaccine antigens [39–41]. We found the presence of varying levels of natural IgM antibodies against *Sp* CPS in naïve mice, but surprisingly their presence prior to immunization was not necessary to generate a good anti-*Sp* CPS antibody response. Thus, natural *Sp* CPS-specific IgM antibody level, despite their importance in innate defence against *Sp* infection, does not appear to influence the induction of adaptive anti-*Sp* CPS antibodies. Our results also indicated that PC, as presented on CWPS, is not a major epitope for natural IgM antibodies in C57Bl/6 mice. This was initially surprising because B1 cells expressing IgM specificity for phosphatidylcholine (PtC) represent 5–15% of murine B1 cells [42,43]. An explanation for this is that glycerol backbone and fatty acid side chains but not phosphocholine of this glycolipid form the epitope recognized by natural IgM PtC-specific antibodies. A study showing that PtC- but not PC-specific antibodies protect against systemic microbial infection in the cecal ligation and puncture model also suggests different epitopes are being recognized [32]. Although not part of the natural IgM repertoire in C57Bl/6 mice as shown in this study and suggested previously [44], PC-specific antibodies induced by immunization with whole *Sp* have been shown to contribute to protection during *Sp* infection [45].

Adaptive antibodies specific for *Sp* CPS are also important in protective immune responses against *Sp* [46] in particular in mice deficient in natural IgM antibodies [12]. It has been shown that different B cell subsets are involved in the induction of natural and adaptive IgM antibody responses to different TI-2 antigens including *Sp* CPS. B1a (CD5⁺) cells provide natural IgM against type 3 *Sp* CPS, whereas B1b (CD5⁻) but not splenic marginal zone (MZ) B cells are the key population producing anti-*Sp* CPS antibodies after type 3 CPS immunization in mice [12]. Two other reports support the role of B1b in adaptive IgM antibody responses to CPS and other clinically relevant antigens [35,47]. In contrast, one report indicates the importance of B1a subset in anti-Pneumovax-23 antibody responses [37]. Our study implicates the involvement of B1b cells in adaptive anti-*Sp* CPS antibody responses as immunogenicity of CPS did not depend or correlate with pre-immunization level of *Sp* CPS-specific IgM that are produced by B1a cells. Considering that B1b cells have been shown to confer T cell-independent long-lasting immunity against a bacterial pathogen [47], it was intriguing to find in our study that high levels of *Sp* CPS-specific IgM antibodies were maintained for at least 6 months. Further investigations are required to confirm that B1b cells are the main source of adaptive IgM antibodies induced by our panel of *Sp* CPS.

Another interesting observation on CPS immunogenicity from our study was that IgM antibodies elicited by individual CPS cross-reacted to varying degrees with other CPS. Cross-reactions exhibited by CPS in group 1 (Table 1) suggest that IgM antibodies induced may be the product of one B cell population that could be activated by any one of the CPS antigens. Poly-reactivity is a well-known phenomenon commonly associated with natural IgM, but the basis for this property is unclear. It has been proposed that poly-reactivity could be due to the structural nature of the antigen-binding site rather than antigenic similarity [48]. We hypothesize that antibodies with specificity for CPS in group 1 contain a positively charged antigen-binding groove which could only bind strongly to negatively charged CPS structures in group 1 because type 14, the only neutral CPS included in the study, did not induce antibodies that are cross-reactive to any other CPS. Group 2 CPS structures, although negatively charged, do not fit as well into the groove as CPS structures in group 1. In contrast we speculate that BCR specific for type 4 CPS contains an antigen-binding cavity that bind strongly to the small immunodominant epitope, cyclic pyruvate, of this CPS. Groove and cavity types of antigen-binding sites for a polysaccharide have been described previously [49]. An important implication of cross-reactions is that multiple *Sp* serotypes in group 1 could be targeted by any group 1 CPS vaccine. A similar cross-reactions study that should also include the remaining 11 serotypes of the current 23-valent *Sp* CPS vaccine is thus warranted. Cross-reactive IgM anti-*Sp* CPS antibodies were also found in humans following immunization with either 23-valent *Sp* CPS or 7-valent *Sp* CPS conjugate vaccines [50]. These antibodies share characteristic features with natural IgM antibodies (unmutated heavy chains and long CDR3), but their origin (B1a or B1b) was not determined as B1 cells are less defined in humans. In contrast to our study, these antibodies were more polyreactive as some of them not only bound CPS, PC but also endogenous carbohydrate and protein antigens. These polyreactive antibodies bound type 14 CPS the strongest which suggests that their antigen-binding site may interact better with uncharged than negatively charged CPS. Importantly two heterohybridoma clones produced antibodies that were protective against invasive *Sp* disease in mice [50].

Complement has also been implicated to augment CPS immunogenicity by a number of studies. CPS including those from *Sp* can activate complement by the alternative pathway [51] and the degree of activation depends on species, polysaccharide size and structure [52–54] and whether the polysaccharide is surface bound or in soluble form [51]. Moreover, complement fragment C3d has been shown to enhance humoral responses to T cell-dependent and to some TI-2 antigens [55]. One proposed mechanism is that CPS activate complement factor C3d via the alternative pathway and C3d–CPS complex then interacts with CD35/CD21 on splenic MZ B cells to promote the production of CPS-specific antibodies. This scenario is suggested by many studies demonstrating the importance of the spleen and MZ B cells in resistance to encapsulated bacteria, in response to TI-2 antigens and in the generation of anti-CPS or anti-PC responses to whole *Sp* ([11,56–58] and references therein). The acquisition of anti-CPS antibody responses in young children also appears to coincide with MZ B cell maturation and an increase in cell surface expression of CD35/CD21 [59,60]. However, the role of complement in anti-CPS antibody responses is not unequivocal and to our knowledge has not been studied with a large panel of *Sp* CPS structures as presented here. We found that natural IgM anti-*Sp* CPS antibody levels were reduced in C3- and CD35/CD21-deficient mice. Decreased serum concentration of natural IgM including those for *Sp* CPS is probably due to a reduction in B1-a (CD5⁺) in C3- and CD35/CD21-deficient mice [30]. The reduction in binding to CPS may also be in part due to changes in the B1 cell repertoire, as the presence of CD35/CD21 has also been shown to be important in the maintenance of some, but not all, B1 cell antigen specificities

[61,62]. This does not pose a problem because natural IgM anti-*Sp* CPS antibody levels do not affect CPS immunogenicity [this study].

Our results indicate surprisingly that the IgM responses to 12 *Sp* CPS, with the exception of serotype 4, were not significantly affected either by the absence of C3 or CD35/CD21. In contrast, IgM responses to DNP–Ficoll were dependent on C3, but not CD35/CD21. A CD35/CD21-independent mechanism for C3d to augment humoral responses to a protein antigen has previously been demonstrated [63]. In agreement with a recent report [15], induction of IgG responses by purified CPS was infrequently observed in mice which made statistical analysis extremely difficult. Nevertheless when observed CPS-specific IgG responses, similar to DNP–Ficoll, were C3- or CD35/CD21-dependent. In view of the high expression and proposed role for CD35/CD21 in matured MZ B but not in B1 cells, our results suggest that MZ B cells are not the source of adaptive CPS-specific IgM antibodies. However, MZ B cells are critically involved in the generation of IgG to CPS and DNP–Ficoll as well as anti-group B *Streptococcus* type III CPS antibodies (this study and [30], respectively).

Our findings are consistent with the mechanism proposed for the activation of MZ B cells by whole bacteria and model TI-2 antigens including DNP–Ficoll. In this model, MZ B cells receive signals to migrate to the outer-T cell zones where they differentiate into plasmablasts and proliferate at bridging channels in the MZ between the white and red pulp. Plasmablasts differentiate into plasma cells leading to rapid anti-hapten or anti-polysaccharide antibody production. The latter step requires survival signals from dendritic cells [58,64]. Although able to cross-link CPS-specific BCR, purified CPS, unlike whole bacteria, do not contain TLR-stimulating activities that provide co-stimulatory signals for MZ B cells. In contrast DNP–Ficoll is known to activate CD35/CD21 high MZ B cells and induce the formation of extrafollicular plasmablasts. Why IgM and IgG anti-type 4 CPS responses are more similar (in restricted specificity and complement dependency) to those for DNP–Ficoll than the other 11 *Sp* CPS (this study and [65]) is not clear. One common feature between type 4 CPS and DNP–Ficoll is the presence of a small size immunodominant epitope, cyclic pyruvate and DNP, respectively (this study and [29,33]). In effect cyclic pyruvate in type 4 CPS could be considered as a hapten. We speculate that haptenated polysaccharides engage their specific BCR more strongly than other *Sp* CPS–BCR interactions and overcome the requirement of co-stimulatory signals for MZ B cell activation. We found that IgM responses to PC of CWPS, another small size epitope on a polysaccharide, were also critically dependent on C3 (data not shown). It has also been noted in humans that the type 4 CPS of the 7-valent *Sp* CPS conjugate vaccine behaves differently to the other polysaccharides suggesting that B cells recognizing type 4 are different from those of B cells specific for other *Sp* serotypes [66]. Further investigations are required to determine whether or not MZ B cells are selectively activated by type 4 CPS structure. In addition to identifying the source of CPS-specific antibodies, it will also be important to demonstrate the functionality of antibodies induced by purified *Sp* CPS.

Together our results on the contribution of natural IgM, cross-reactivity and complement to anti-CPS antibody responses provide support for the concept of “division of labour” among B cell subsets [12] with B1a cells being the main source of natural (spontaneously induced) *Sp* CPS-specific IgM antibodies, B1b cells producing most of the adaptive IgM anti-*Sp* CPS antibodies that are triggered by pure CPS immunogens, and splenic MZ B cells producing IgG anti-CPS antibodies when stimulated by complement-opsonized CPS and co-stimulatory signals such as those provided by TLR activation. Using a relatively large panel of *Sp* CPS enabled us to identify CPS with distinct properties such as types 4 and 14 and to provide evidence against the sole use of model TI-2 antigens, such as DNP–Ficoll to understand antibody responses to CPS as it mim-

ics one (i.e. type 4) out of 12 CPS tested. Grouping of CPS based on cross-reactions and special features in CPS such as net charge and epitope size or conformation as well as strain and species differences in BCR repertoire should be taken into consideration in future CPS analysis and vaccine design. Studies investigating in more detail CPS cross-reactions, the repertoire of *Sp* CPS-specific B cells and the mechanisms of B cell activation by purified CPS, in particular for B1 subsets in mice and their counterparts in humans, would be required to provide further insights and additional ways to overcome non-responsiveness to CPS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.07.063.

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