Hyporesponsiveness Following Booster Immunization With Bacterial Polysaccharides Is Caused by Apoptosis of Memory B Cells

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Background. Repeated immunizations with polysaccharide (PS) vaccines cause hyporesponsiveness through undefined mechanisms. We assessed the effects of a PS booster on immune responses, frequency, and survival of PS-specific B-cell subpopulations in spleen and bone marrow.

Methods. Neonatal mice were primed with meningococcus serotype C (MenC) conjugate MenC-CRM197+CpG1826, boosted with MenC-CRM197, MenC-PS, or saline; subsequently, bromodeoxyuridine (BrdU) was injected daily intraperitoneally. MenC-PS–specific cells were labeled with fluorescent MenC-PS and phenotyped by flow cytometry.

Results. After MenC-PS booster, proliferating (BrdU+ MenC-PS–specific naive B cells (CD138−/B220+; P ≤ .0003) and plasma cells (CD138+/B220−; P ≤ .0002) in spleen were fewer than after saline booster. BrdU+ MenC-PS–specific plasma cells were also reduced in bone marrow (P ≤ .0308). Compared to saline, MenC-PS booster reduced BrdU+ IgG+ MenC-PS–specific B cells in spleen (P ≤ .0002). Twelve hours after the MenC-PS booster, an increased frequency of apoptotic (AnnexinV+) MenC-PS–specific B cells in spleen was observed compared with MenC-CRM197 (P = .0286) or saline (P = .001) boosters.

Conclusions. We demonstrated that the MenC-PS booster significantly reduced the frequency of newly activated MenC-PS–specific B cells—mostly switched IgG+ memory cells—by driving them into apoptosis. It shows directly that apoptosis of PS-specific memory cells is the cause of PS-induced hyporesponsiveness. These results should be taken into account prior to consideration of the use of PS vaccines.

Polysaccharide (PS) vaccines have been available against some serogroups of Neisseria meningitidis (meningococcus) since the 1960s; however, outbreaks still occur, the last in 2009 [1]. With improved treatment, the case fatality rate of meningococcal disease has decreased from 70%–90% to approximately 10% [2] if patients are treated with the appropriate antibiotics.

In 1975 it was observed that repeated immunizations with a capsular PS of meningococcus serotype C (MenC) induced hyporesponsiveness in infants [3]. This was confirmed in Gambian children who received plain MenC-PS boosters [4]. The effect was long-lasting. Children who were primed as infants with a meningococcal C conjugate MenC-CRM197 booster experienced, at 5 years of age, a reduced MenC-PS–specific antibody response and serum bactericidal activity (SBA) following a MenC-PS booster at 2 years of age, compared with children who were given MenC-CRM197 or saline [5]. MenC-PS–induced hyporesponsiveness has been observed in additional studies in infants [6–8] and adults [9–11]. Hyporesponsiveness has also been observed for MenA [12], and recent data indicate that serogroups W-135 and Y may induce hyporesponsiveness (reviewed in [13]).

Although PS-induced hyporesponsiveness has been known for decades, the mechanisms that cause it are...
poorly defined. A common theory is that PSs deplete the memory B-cell pool [5, 14, 15]. Understanding the mechanisms that cause PS-induced hyporesponsiveness is important because PS vaccines are recommended for children at risk of invasive bacterial diseases, and PS boosters are used to demonstrate that conjugate vaccines have induced memory. Polysaccharides are also given to elderly people at increased risk of infectious diseases [16], which may not have the desired effects if the PS has detrimental effects on immune memory.

The aim of this study was to evaluate the effects of MenC-PS booster on the immune responses of mice primed with the MenC-CRM197 as neonates and to assess the survival of MenC-PS–specific memory B cells, in spleen and bone marrow, by using a fluorochrome-labeled MenC-PS, markers identifying B-cell subpopulations, and Annexin V to label apoptotic cells.

MATERIALS AND METHODS

Animals
NMRI mice (M&B) were kept in microisolator cages with free access to food pellets and water and housed under standard conditions with regulated daylight, humidity, and temperature. Breeding cages were checked daily, and pups were kept with their mothers until weaning. The study was authorized by the Animal Experimental Committee of Iceland.

Vaccines and Adjuvants
Meningococcal oligosaccharide C conjugated to CRM197 [17] (MenC-CRM197) and MenC-PS were provided by Novartis Vaccines and Diagnostics. CpG2006 and CpG1826 were purchased from Oligos, Etc.

Experimental Setup
In the first set of experiments (3 independent experiments), neonatal mice (1 week old, 8–9 per group) were immunized subcutaneously in the scapular girdle with 2 primary doses of monovalent MenC-CRM197 (2.5 µg/dose) and CpG2006 (100 µg/dose, except for the first dose of 20 µg/dose [18]), at 1 and 3 weeks of age. The mice were boosted at 5 weeks of age with MenC-CRM197+CpG2006, saline+CpG2006, or MenC-PS (10 µg/dose)+CpG2006. Mice that received saline or MenC-CRM197 without CpG2006 were used as controls. A second MenC-PS+CpG2006 booster was administered to all groups (13 weeks of age). Mice were bled from the tail vein weekly from 3 weeks of age; serum samples were isolated and stored at −20°C until use. In the second set of experiments, mice were immunized as in the first, except that for priming, CpG1826 was used instead of CpG2006, and no adjuvant was given with the boosters. To label proliferating cells, the mice received intraperitoneal injections of BrdU (1.5 ug) at the booster day and daily, until they were killed 8 hours, 12 hours, 24 hours, 48 hours, and 5 days later, when spleen and bone marrow were removed for enumeration and phenotyping of MenC-PS–specific B cells.

Antibody Measurements
MenC-PS–specific immunoglobulin G (IgG) was measured by enzyme-linked immunosorbent assay (ELISA) [19]. ELISA plates (MaxiSorp, Nunc) were coated with 5 µg/mL MenC-PS (Novartis Vaccines and Diagnostics) in phosphate-buffered saline (PBS) with methylated human serum albumin (5 µg/mL) overnight (4°C), blocked with 1% gelatin (BDH Chemicals) in PBS for 3 hours at 37°C, and fixed with 10% saccharose (Merck) and 4% polyvinylpyrrolidone (Sigma) for 2 hours. Serial dilutions of samples and standards in PBS-Tween-1% BSA (Sigma) were incubated overnight at 4°C, followed by horseradish peroxidase–conjugated goat anti-mouse IgG (Southern Biotechnology) and developed by 3.3’-5.5’-tetramethylbenzidine substrate (Kirkegaard and Perry Laboratories). The reaction was stopped with 0.18 mol/L H2SO4 and absorbance read at 450 nm with an ELISA spectrophotometer (Original Multiscan Ex, Thermo Electron Corporation). Results were calculated from standard (hyperimmunized serum) and assigned a titer of the inverse dilution, which gave an optical density (OD) of 1.0.

Avidity of MenC-PS–specific IgG was measured by the same ELISA, including KSCN incubation (serial dilutions: 7.5–0.117 mol/L) or PBS-Tween (100% binding) for 15 minutes after the serum incubation. Bound antibodies were detected with alkaline phosphatase–conjugated goat antimouse IgG (Southern Biotechnology) and p-NPP (Sigma) and absorbance read at 405 nm. Results are expressed as avidity index (AI) = [mol/L] KSCN that displaces 50% of antibodies.

Serum Bactericidal Activity
SBA was measured in serum pools from each group (equal volumes per mouse) [20]. N. meningitidis strain C11 was grown overnight at 37°C on chocolate agar plates with 5% CO2 colonies inoculated into 7 mL Mueller-Hinton broth containing 0.25% glucose to reach an OD at 600 nm of 0.05–0.06; it was then incubated 1.5 hours at 37°C with 5% CO2 until the OD at 600 nm reached 0.23–0.24. Bacteria were diluted in Grey’s salt solution (Sigma) and 1% BSA (Sigma) at 107 colony-forming units/mL. The reaction mixture contained 25 µL of serum dilution, 12.5 µL of bacteria, and 12.5 µL of baby rabbit complement. Controls included 1) bacteria with complement, 2) bacteria with immune sera and complement inactivate at 56°C for 30 minutes. Immediately after complement addition, controls were plated on Mueller-Hinton agar plates using the tilt method (time 0) and then incubated for 1 hour at 37°C with 5% CO2. Samples were transferred to Mueller-Hinton agar plates as spots and controls transferred using the tilt method (time 1) were incubated.
for 18 hours at 37°C with 5% CO₂. Colonies corresponding to
time 0 and time 1 were counted, and reciprocal serum dilu-
tions killing 50% of bacteria were calculated.

### Cell Isolation and Flow Cytometry

Single-cell suspensions were prepared from spleen and bone
marrow by filtering through a 70-μm cell strainer (BD Bio-
sciences) and Histopaque-1077 (Sigma) gradient centrifugation.
Viability was >98%. Washed cells were incubated (30 minutes
on ice) with fluorocrome-labeled MenC-PS (provided by Sanofi Pasteur) and antibodies (BD Biosciences) in PBS/0.5% BSA (Sigma)/4 mmol/L EDTA (Sigma): anti-CD138 (clone
281-2, used for labeling plasmablasts/memory cells and plasma
cells), anti-B220 (clone RA3-6B2, used for labeling naive B cells,
plasmablasts, and memory B cells), anti-IgG1 (clone A85-1),
anti–immunoglobulin M (IgM; clone R6-60.2), AnnexinV, 7AAD,
and anti–BrdU (clone 3D4, used for labeling proliferating cells).
For intracellular staining, cells were fixed in 4% formaldehyde,
washed, resuspended, and permeabilized in 0.5% saponin
(Sigma) in PBS/BSA. Fluorochrome-labeled MenC-PS and Ig-
isotype specific monoclonal antibodies or 7AAD were added
and incubated on ice for 30 minutes. Flow cytometry was
performed using FACSCalibur, and analysis was performed by
CellQuest (Becton Dickinson) and FlowJo (Tree Star).

### Statistical Analysis

Nonparametric Mann–Whitney U test was used, and a P value
of <.05 was considered statistically significant.

### RESULTS

**A MenC-PS Booster Elicited Lower Antibody Levels, Affinity,
and SBA Than MenC-CRM₁₉₇ and Reduced Response to
a Second MenC-PS Booster**

Neonatal mice (1 week old) were immunized with 2 primary
dooses of MenC-CRM₁₉₇ together with CpG2006 as an adjuvant
and boosted as adult mice (5 weeks old) with MenC-CRM₁₉₇,
saline, or MenC-PS, all with CpG2006. A second MenC-
PS+CpG2006 booster was given to all groups at 13 weeks of
age.

The first MenC-CRM₁₉₇ booster induced higher MenC-PS–
specific IgG levels than did MenC-PS (P = .0006) or saline
(P = .0061); the levels persisted after the second booster. Anti-
body levels were comparable in mice that received saline or
MenC-PS booster; these persisted after the second booster
(Table 1). The MenC-CRM₁₉₇ booster induced a 16-fold increase
in SBA (Table 1), but MenC-PS induced a 2-fold increase. SBA
decayed until the second MenC-PS+CpG2006 booster at week
13. The first MenC-CRM₁₉₇ booster induced higher antibody
AI than did MenC-PS (P = .009) or saline (P = .031). After the
second MenC-PS+CpG2006 booster, the AI increased in mice
that received MenC-CRM₁₉₇ or saline at 5 weeks. In contrast,

**Table 1. MenC-PS–Specific Immunoglobulin G Antibody Levels, Avidity, and Serum Bactericidal Activity Before and After First and Second Booster**

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The results are representative for 1 of 3 independent experiments with 7–9 mice per group.
Abbreviations: GI, immunoglobulin G; MenC, meningococcus serotype C; PS, polysaccharide; SBA, serum bactericidal activity; SD, standard deviation.
A Higher than MenC-PS, P < .05.
B Higher than PS and saline, P < .05.
the AI did not increase in mice boosted with MenC-PS at 5 weeks suggesting that high-affinity MenC-PS–specific memory B cells generated by MenC-CRM197 priming had been depleted by the MenC-PS booster at week 5 (Table 1). We therefore analyzed the fate of MenC-PS–specific memory B cells activated by the different boosters.

Activation of MenC-PS–Specific B Cells to Proliferate Was Reduced More by MenC-PS Booster Than by MenC-CRM197 and Saline

The fate of MenC-PS–specific B cells after boosters with MenC-PS, MenC-CRM197, or saline (week 5) was assessed by labeling the proliferating cells with daily intraperitoneal injections of BrdU from the time of booster. Five days later, MenC-PS–specific B cells in spleen and bone marrow were enumerated by direct labeling with fluorescent MenC-PS and flow cytometry. The frequencies of MenC-PS–specific B cells that had been activated to proliferate (BrdU$^+$) were higher in mice that received MenC-CRM197 than in mice that received MenC-PS or saline booster in both bone marrow ($P = .0031$ and $P = .0216$, respectively) and spleen ($P < .001$ and $P = .002$, respectively). Mice that received MenC-PS booster had lower frequency of BrdU$^+$ MenC-PS–specific B cells in spleen than did mice that received saline ($P < .0012$), whereas there was no difference in the bone marrow (Figure 1). We therefore evaluated which MenC-PS–specific B-cell populations were reduced by the MenC-PS booster.

MenC-PS Booster Did Not Induce Full Differentiation of MenC-PS–Specific Memory B Cells Into Plasma Cells

MenC-PS–specific B-cell subpopulations in spleen and bone marrow were studied after MenC-PS, MenC-CRM197, and saline boosters. B220 was used as a B-cell marker, which is expressed on early B cells and memory B cells but not on naive B cells. BrdU was used to label proliferating cells.

In the spleen the total frequencies of naive B220$^+$CD138$^-$ MenC-PS–specific B cells were comparable in mice that received MenC-CRM197, MenC-PS, or saline. The percentages of naive B220$^+$CD138$^-$ MenC-PS–specific B cells were 84.6% ± 3.5%, 87.3% ± 3.4%, and 85.0% ± 2.9% in MenC-CRM197, MenC-PS, and saline booster groups, respectively. In the bone marrow the total percentages of naive B220$^+$CD138$^-$ MenC-PS–specific B cells were 85.3% ± 3.2%, 87.1% ± 3.1%, and 86.2% ± 2.9% in MenC-CRM197, MenC-PS, and saline booster groups, respectively.

MenC-PS Causes Apoptosis of Memory Cells

Figure 1. Percentage of BrdU$^+$ MenC-PS–specific B cells out of the total MenC-PS–specific B cells in spleen and bone marrow, 5 days after booster with MenC-CRM197, MenC-PS, or saline. Neonatal mice were mice primed with 2 doses of MenC-CRM197+CpG1826 and boosted 2 weeks later with MenC-CRM197, MenC-PS, or saline without adjuvant. The percentage of BrdU$^+$ MenC-PS–specific B cells out of total MenC-PS–specific B cells in the spleen (left panel) and in the bone marrow (right panel) is shown. The results are presented as mean (SD) for each group (n = 8–9 mice per group; $^*P < .05$ compared with mice that received MenC-PS or saline). Abbreviations: BrdU, bromodeoxyuridine; MenC, meningococcus serotype C; PS, polysaccharide.

Figure 2. Frequency of MenC-PS–specific B-cell subpopulations in spleen and bone marrow 5 days after booster with MenC-CRM197, MenC-PS, or saline. Neonatal mice were primed with 2 doses of MenC-CRM197+CpG1826 and boosted 2 weeks later with MenC-CRM197, MenC-PS, or saline without adjuvant. The frequency of MenC-PS–specific B-cell subpopulations is shown as percentage of lymphocytes in spleen (upper panel) and bone marrow (lower panel) for naive B cells (MenC$^+$B220$^+$CD138$^-$), memory B cells and plasmablasts (MenC$^+$B220$^+$CD138$^+$), and plasma cells (MenC$^+$B220$^-$CD138$^+$). The results are presented as mean (SD) for each group (8–9 mice per group) in 1 of 3 representative independent experiments. $^*P < .05$ compared with mice that received MenC-PS or saline. Abbreviations: MenC, meningococcus serotype C; PS, polysaccharide.
MenC-PS, MenC-CRM197, or saline 5 days after booster (Figure 2). However, among B cells that had been activated by the booster to proliferate (BrdU\(^{+}\)), the frequencies of naive MenC-PS–specific B cells (BrdU\(^{+}\)MenC\(^{+}\)B220\(^{-}\)CD138\(^{-}\)) were higher in mice that received the MenC-CRM197 booster than in mice that received MenC-PS or saline (\(P < .001\) and \(P = .0003\), respectively) (Figure 3) and was lower in mice boosted with MenC-PS than saline (\(P < .0003\)). These results suggest that the MenC-PS booster depleted naive MenC-PS–specific B cells in the spleen.

Mice that received MenC-CRM197 booster showed higher total frequencies of MenC\(^{+}\)B220\(^{-}\)CD138\(^{-}\) memory B cells/plasmablasts than did mice that received MenC-PS (\(P = .011\)) or saline (\(P = .024\)), and there were no differences between the MenC-PS and saline groups (Figure 2). The same was true for newly activated BrdU\(^{+}\) MenC-PS–specific B220\(^{-}\)CD138\(^{-}\) memory B cells/plasmablasts; mice that received MenC-CRM197 had higher frequencies than did mice that received MenC-PS (\(P < .0001\)) or saline (\(P = .0002\)) (Figure 3). However, MenC-PS booster induced higher frequencies of newly activated BrdU\(^{+}\) MenC-PS–specific B220\(^{-}\)CD138\(^{-}\) memory B cells/plasmablasts than saline (\(P = .0002\)), demonstrating that MenC-PS can activate MenC-PS–specific memory cells/plasmablasts to proliferate, although less efficiently than MenC-CRM197.

Examination of the B220\(^{-}\)CD138\(^{-}\) plasma cell compartment showed that mice that received MenC-CRM197 booster had a higher frequency of MenC-PS–specific plasma cells than did mice that received MenC-PS booster (\(P = .0031\)) or saline (\(P = .016\)) (Figure 2); however, there was no difference between the MenC-PS and saline groups. The frequencies of newly activated BrdU\(^{+}\) MenC-PS–specific B220\(^{-}\)CD138\(^{-}\) plasma cells were lower in mice that received MenC-PS booster than in mice that received MenC-CRM197 booster (\(P < .0001\)) or saline (\(P = .0002\)) (Figure 3), but MenC-CRM197 booster induced higher frequencies than saline (\(P = .0006\)). These results indicate that the MenC-PS booster is able to activate memory B

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**Figure 3.** Frequency (%) of BrdU\(^{+}\) MenC-PS–specific B-cell subpopulations in spleen and bone marrow, 5 days after booster with MenC-CRM197, MenC-PS, or saline. Neonatal mice were primed with 2 doses of MenC-CRM197+CpG1826 and boosted 2 weeks later with MenC-CRM197, MenC-PS, or saline without adjuvant. The frequency of BrdU\(^{+}\) cells as percentage of MenC-PS–specific B cells within the B-cell subpopulations in spleen (upper panel) and bone marrow (lower panel) are shown for naive B cells (MenC\(^{+}\)B220\(^{-}\)CD138\(^{-}\)), memory B cells and plasmablasts (MenC\(^{+}\)B220\(^{-}\)CD138\(^{-}\)), and plasma cells (MenC\(^{+}\)B220\(^{-}\)CD138\(^{-}\)). The results are presented as mean (SD) for each group (\(n = 8–9\) mice per group) in 1 of 3 representative independent experiments. *\(P < .05\) compared with mice that received MenC-PS or saline. Abbreviations: BrdU, bromodeoxyuridine; MenC, meningococcus serotype C; PS, polysaccharide.
cells/plasmablasts, but they are unable to fully differentiate into plasma cells.

In bone marrow, frequencies of MenC⁺B220⁻CD138⁺ memory B cells/plasmablasts were lower in mice that received MenC-CRM197 booster than in mice that received a MenC-PS booster (P = .0004) or saline (P = .0004), whereas there was no difference between the MenC-PS and saline groups (Figure 2). However, the frequency of newly activated BrdU⁺ MenC⁺B220⁻CD138⁺ memory B cells/plasmablasts was lower in mice that received MenC-PS booster than in mice that received saline (P = .0008) (Figure 3).

MenC-CRM197 booster induced higher frequencies of MenC-PS–specific MenC⁺B220⁻CD138⁺ plasma cells in bone marrow than did the MenC-PS booster (P = .0027) or saline (P = .0004) (Figure 2), whereas no difference was found between the MenC-PS and saline groups. Mice that received MenC-CRM197 booster also had higher frequencies of newly activated BrdU⁺MenC⁺B220⁻CD138⁺ plasma cells than did mice that received MenC-PS booster (P = .0004) or saline (P = .0019) (Figure 3). As in the spleen, the frequencies of newly activated BrdU⁺ MenC⁺B220⁻CD138⁺ plasma cells were lower in the bone marrow of mice that received MenC-PS booster than in mice that received saline (P = .0308). Together, these data suggest that MenC-PS booster does not induce full differentiation of MenC-PS–specific memory B cells into plasma cells. Therefore, we investigated whether MenC-PS had similar or different effects on unswitched IgM⁺ and class-switched IgG⁺ MenC-PS–specific B cells.

**MenC-PS Depleted Class-Switched IgG⁺ MenC-PS–Specific B Cells**

In the spleen there were no differences in frequencies of IgM⁺ or BrdU⁺ IgM⁺ MenC-PS–specific B cells between mice that received the different boosters (Figure 4). MenC-CRM197 booster induced higher frequencies of IgG⁺ MenC-PS–specific B cells in spleen than did MenC-PS (P = .032) or saline (P = .015) (Figure 4), but there was no difference between the MenC-PS and saline groups. In contrast, mice that received MenC-PS booster had lower frequencies of newly activated BrdU⁺ IgG⁺ MenC-PS–specific B cells than did mice that received saline (P = .0002). Mice that received MenC-CRM197 booster showed higher frequencies of BrdU⁺ IgG⁺ MenC-PS–specific cells than did mice that received MenC-PS (P = .0002) or saline (P = .021). These results indicate that the MenC-PS booster has depleted class-switched IgG⁺ memory B cells in the spleen.

In the bone marrow there were no differences in frequencies of IgM⁺, IgG⁺, or BrdU⁺ IgG⁺ MenC-PS–specific B cells between mice that received the different boosters (Figure 4). However, the frequencies of BrdU⁺ IgG⁺ MenC-PS–specific cells were lower in mice that received saline than in mice that received MenC-PS (P = .0001) or MenC-CRM197 (P = .0004) boosters (Figure 4), but there was no difference between the MenC-PS and MenC-CRM197 groups. This indicates that the depletion of MenC-PS–specific memory observed in the spleen is not yet reflected in the bone marrow at day 5, but possibly some short-lived plasmablasts have reached the bone marrow.

Next we analyzed whether the MenC-PS–induced depletion of IgG⁺ memory B cells in the spleen was due to apoptosis.

**MenC-PS Booster Induced Apoptosis of MenC-PS–Specific B Cells Generated by MenC-CRM197 Priming**

To explain the differences in frequencies of MenC-PS–specific B cells after different boosters, AnnexinV staining was performed to study apoptosis. The frequencies of MenC⁺B220⁻ AnnexinV⁺ B cells were determined in spleen at 8 hours, 12 hours, 24 hours,
Figure 5. Apoptosis of MenC-PS–specific B cells in spleen 12 h after booster with MenC-CRM$_{197}$, MenC-PS, or saline. Neonatal mice were primed with 2 doses of MenC-CRM$_{197}$+CpG1826 and boosted 2 weeks later with MenC-CRM$_{197}$, MenC-PS, or saline without adjuvant. The percentage of Annexin V$^+$ MenC-PS–specific B cells in spleen is shown as mean (SD) for each group ($n=8–9$ mice per group) for 1 of 3 representative independent experiments. Abbreviations: MenC, meningococcus serotype C; PS, polysaccharide.

and 48 hours after booster. Mice that received MenC-PS showed increased apoptosis of MenC-PS–specific B cells after 8 hours and 12 hours compared with mice that received MenC-CRM$_{197}$ (8 hours, $P=.005$; 12 hours, $P=.0286$) or saline (8 hours, $P=.0286$; 12 hours, $P=.001$) (Figure 5 and Supplementary Figure 1), whereas at 24 and 48 hours the differences had disappeared (Supplementary Figure 2). These results indicate that the differences in frequencies of MenC-PS–specific B cells 5 days after the different boosters were caused by MenC-PS–induced apoptosis of the MenC-PS–specific cells during the first 12 hours.

**DISCUSSION**

We have demonstrated that a booster with plain MenC-PS had a detrimental effect on MenC-PS–specific antibody responses. Accordingly, SBA was lower in mice that received MenC-PS as a second booster than in mice that received saline or MenC-CRM$_{197}$. The increase in affinity and SBA after the second MenC-PS booster in the MenC-PS and saline groups may be due to the effect of the adjuvant CpG given concomitantly with the vaccines, as recent studies showed that TLR9 stimulation induces differentiation memory and transitional B cells into plasma cells [21].

MenC-PS–induced hyporesponsiveness has been repeatedly demonstrated by serological measurements in humans and mice. The effects of MenC-PS on antibody levels, affinity, and SBA demonstrated in the current study are in agreement with the effects of MenC-PS in human infants, teenagers, and adults showing decrease in MenC-PS–specific IgG levels [3, 5–7, 22, 23], affinity [24], and SBA [6, 7, 22–24].

It has been hypothesized that a MenC-PS booster depletes the memory cell pool [5, 14, 15]. We demonstrated the direct effect of MenC-PS on frequency, activation, and fate of MenC-PS–specific B cells. The results indicate that the hyporesponsiveness is caused by MenC-PS driving the MenC-PS–specific memory B cells into apoptosis, thus causing a reduction of BrdU$^+$ MenC-PS–specific B-cell frequencies in the spleen 5 days after booster. By analyzing major B-cell subpopulations, we showed that compared with saline, MenC-PS booster caused reduction in newly activated MenC-PS–specific B cells within the naive and plasma cell pools in spleen and bone marrow. In bone marrow the frequencies of recently activated BrdU$^+$ MenC-PS–specific B cells of memory/plasmablast phenotype were also reduced in mice that received the MenC-PS booster. These results indicate that the memory/plasmablasts in spleen fail to differentiate into plasma cells that migrate to niches in the bone marrow and survive as long-lived plasma cells. This could not, however, explain the marked reduction of recently activated BrdU$^+$ MenC-PS–specific B cells in the spleen. We therefore studied apoptosis of MenC-PS–specific B cells as a possible mechanism for depletion of BrdU$^+$ MenC-PS–specific B cells. The reduction of BrdU$^+$ IgG$^+$ MenC-PS–specific B cells in spleen indicates that, primarily, class-switched IgG$^+$ MenC-PS–specific B cells became apoptotic. The decrease in MenC-PS–specific plasma cells in spleen and bone marrow, the reduced frequency of IgG$^+$ MenC-PS–specific B cells and the reduced affinity of MenC-PS–specific IgG further supports that MenC-PS booster caused depletion of switched memory B cells by apoptosis, without inducing hypermutations and maturation of new B cells. The increased frequency of activated BrdU$^+$ MenC-PS–specific memory B cells/plasmablasts and the switched BrdU$^+$ IgG$^+$ MenC-PS–specific B cells following PS booster suggests that MenC-PS induces short-lived low-affinity plasmablasts that move to the bone marrow but do not survive [25]. AnnexinV staining of early apoptotic B cells showed that MenC-PS–specific B cells underwent increased apoptosis within the first 8–12 hours after MenC-PS booster.

Polysaccharide-induced hyporesponsiveness is not limited to meningococcal serogroup C but has been observed in other serogroups [12] (reviewed in [13]) and pneumococcal serotypes in infants [26, 27], adults [28, 29], and elderly persons [30]. Live pneumococci can also induce serotype-specific hyporesponsiveness in infants who are colonized before pneumococcal conjugate immunization, showing lower antibody responses to the colonizing serotypes than noncarriers [31, 32].

It is conceivable that apoptosis as a cause of MenC-PS–specific memory-cell depletion may contribute to hyporesponsiveness induced by other PS, in mice and humans, although the extent may vary. Immunization with a conjugate vaccine may partially overcome the MenC-PS–induced hyporesponsiveness,
although the quality of the antibody response to the conjugate vaccine is reduced in individuals who have received MenC-PS [9–11, 19, 33].

Taken together, we have demonstrated directly that MenC-PS reduces the frequency of MenC-PS–specific B cells in spleen and bone marrow—in particular, the newly activated BrdU$^+$ IgG$^+$ B cells within the memory cell and plasmablast subpopulations. Furthermore, we demonstrated increased apoptosis of PS-specific B cells in the spleen within 12 hours after PS booster, which strongly suggests that PSs drive the specific memory B cells into apoptosis. This provides direct evidence for apoptosis being at least one major mechanism of PS-induced hyporesponsiveness.

The recommendation of meningococcal polysaccharide vaccines for children (2–10 years) with underlying diseases, which increases their risk of invasive bacterial diseases [34], was recently replaced by the recommendation of a quadrivalent meningococcal conjugate vaccine to all adolescents and individuals aged 2–55 years who are at increased risk of meningococcal disease [35]. The clinical relevance of PS-induced hyporesponsiveness needs to be further studied, but until it is better understood, the results presented here should be considered when designing PS-based vaccine trials and vaccination schedules.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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