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Capsular polysaccharides of encapsulated bacteria are weakly immunogenic T cell-independent type 2 (TI-2) Ags. Recent findings suggest that BAFF system molecules have a critical role in the development of Ab responses against TI-2 Ags. In this study, we investigated the effect of bacterial polysaccharides on B cell responses to BAFF and a proliferation-inducing ligand (APRIL). We determined that B cells exposed to meningococcal type C polysaccharide (MCPS) or group B Streptococcus serotype V (GBS-V) were unresponsive to BAFF- and APRIL-induced Ig secretion. Moreover, MCPS and GBS-V strongly downregulated transmembrane activator and calcium-modulator and cyclophilin ligand interactor, the BAFF and APRIL receptor that is responsible for Ab development against TI-2 Ags. Interestingly, (4-hydroxy-3-nitrophenyl)acetyl-Ficoll (NP-Ficoll), a prototype TI-2 Ag, did not manifest a suppressive effect on B cells. Paradoxically, whereas GBS-V and MCPS inhibited IFN-γ–induced BAFF production from dendritic cells, NP-Ficoll strongly increased BAFF secretion. TLR 9 agonist CpG deoxyoligonucleotide (ODN) was able to reverse the MCPS-mediated transmembrane activator and calcium-modulator and cyclophilin ligand interactor suppression but could not rescue the Ig secretion in BAFF- or APRIL-stimulated B cells. In support of these in vitro observations, it was observed that CpG ODN could help augment the Ab response against NP in mice immunized with a CpG ODN-containing NP-Ficoll vaccine but exhibited only marginal adjuvant activity for MCPS vaccine. Collectively, these results suggest a mechanism for the weak immunogenicity of bacterial polysaccharides and explain the previously observed differences between bacterial polysaccharide and NP-Ficoll immunogenicity. The Journal of Immunology, 2011, 186: 2430–2443.

Encapsulated Gram-positive and Gram-negative bacteria such as Neisseria meningitidis, group B Streptococcus, Streptococcus pneumoniae, and Haemophilus influenzae type b can become invasive and cause serious infections such as meningitis and pneumonia worldwide, particularly in infants and young children, leading to a huge global burden of morbidity and mortality (1). The capsule of these bacteria consists of repeating carbohydrate moieties and has been implicated in their virulence (2–5). The role of Ab against capsular polysaccharide (CPS) Ag in protection against encapsulated bacteria was first described in the early 1930s (6). However, CPSs are poorly immunogenic Ags, mostly because Ab responses against CPSs do not involve T cells and they are categorized as T cell-independent type 2 (TI-2) Ags (7, 8). TI-2 Ags are poor inducers of immunological memory but are known to induce rapid production of IgM (7, 9) and limited (10, 11). Newborns, however, do not respond to TI-2 Ags, and they are vulnerable to infections by encapsulated bacteria (12, 13). Engagement of B cell receptor by Ag is generally not sufficient to activate B cells. In the case of TI-2 Ags, a second signal is provided through the engagement of CD21/CR2 by complement C3d bound to CPS on B cells (14), although TI-2 Ags with greater antigenic strength, such as S. pneumoniae type 3 CPS, can induce IgG3 secretion in a CD21/CR2-dependent but C3d-independent fashion (15). In newborns and infants, the deficiency of CD21/CR2 in the splenic marginal zone (MZ) and low serum C3 levels have been implicated for the poor Ab response to CPS Ag (14).

Another important molecule that is essential for the development of Ab responses against TI-2 Ag is the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) (16). TACI is mostly expressed by B cells and is a receptor for the two TNF family member cytokines, BAFF and a proliferation-inducing ligand (APRIL) (17). In addition to binding to TACI, BAFF also binds to the BAFFR and the B cell maturation Ag (BCMA) on B cells, whereas APRIL binds only to TACI and BCMA. The ligands BAFF and APRIL and their receptors BAFFR, TACI, and BCMA of the “BAFF system” are important regulators of B cell survival and activation. BAFF maintains B cell maturation and survival through its interaction with BAFFR (18, 19). At the same time, both BAFF and APRIL can induce T cell-independent Ig isotype switch to IgA and IgG by binding to TACI (19). Engagement of TACI also promotes plasma cell generation (22–24), and BCMA is important for the maintenance of plasma cells (25, 26). Of the three receptors, TACI stands out as the key receptor for the generation of TI-2–specific Ab response because although TACI knockouts have normal B cell development, they are deficient in their ability to mount Ab responses to TI-2 Ag and they have reduced IgA levels (16, 27). Moreover, we have recently shown that despite expressing normal levels of BAFFR and BCMA, B cells from newborn mice express severely reduced levels of TACI, which may underlie the poor
ability of newborns to mount Ab response against CPS Ag (22). Both adult TACI knockout and newborn wild-type mice B cells fail to differentiate into IgG- or IgA-secreting plasma cells in response to BAFF or APRIL (22, 24).

These emerging data underscore the importance of BAFF family member molecules in the development of effective immune response against CPS Ag. Whether CPS Ags directly modulate the expression and function of BAFF family member molecules is not known. To address this question, in this study we analyzed BAFF- and APRIL-mediated B cell functions after the exposure of mouse B cells to CPS from meningococcal type C polysaccharide (MCPS) and group B Streptococcus serotype V (GBS-V). We found that B cells pretreated with MCPS or GBS-V were unable to develop into IgG-secreting plasma cells when stimulated with BAFF or APRIL. Unresponsiveness of MCPS- or GBS-V-exposed B cells to BAFF or APRIL appears to be due to a suppressive effect of CPS on B cells, because CPS-pretreated cells manifested increased cell death and they expressed significantly lower levels of TACI and BAFFR. Analysis of BAFF-mediated signaling indicated a blockage of both the classical and alternative NF-κB pathway. Thus, suppression of B cell functions by CPS emerges as an important underlying mechanism for the poor immunogenicity of CPS vaccines.

Materials and Methods

Mice

This study was approved by the Committee on the Ethics of Animal Experiments of the U.S. Food and Drug Administration/Center for Biologics Evaluation and Research Institutional Animal Care and Use Committee ( Permit No. 2002-31).

Cell culture and stimulation

Complete RPMI media RPMI 1640 (Cellgro, Manassas, VA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10,000 U/ml penicillin and streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, and 1 mM nonessential amino acids (Invitrogen, Carlsbad, CA) and 50 μM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) was used for all cell culture experiments. Splenic B cells were purified by negative selection using the B Cell Isolation Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions. Purity of isolated cells was >97% in all experiments, as determined by flow cytometry. MCPS that had been purified using a base extraction method (U.S. Patent 6 248 570) was obtained from Baxter (Beltsville, MD) (2). Purified GBS-V CPS was a kind gift of Dr. Dennis L. Kasper (Harvard Medical School, Boston, MA). The following reagents were used to stimulate cells at given concentrations, unless otherwise indicated: MCPS (40, 10, or 2.5 μg/ml), (4-hydroxy-3-nitrophenyl)acetate (NP-Ficoll) (40, 10, or 2.5 μg/ml) (Biosearch Technologies, Novato, CA), GBS-V (40 μg/ml), Escherichia coli LPS (10 μg/ml) (Sigma-Aldrich), F(ab′)2 fraction goat anti-mouse IgM (40 μg/ml) (Jackson ImmunoResearch Laboratories, West Grove, PA), IL-4 (50 ng/ml) (eBioscience, San Diego, CA), and TGF-β (50 ng/ml) (R&D Systems, Minneapolis, MN). CpG oligodeoxynucleotide (ODN) 1555 (1 μg/ml) (sequence: 5′-GCTAGACGTTAGCGT-3′) (28) was synthesized at the U.S. Food and Drug Administration, Center for Biologics Evaluation and Research Core Facility (Bethesda, MD). Recombinant BAFF (1 μg/ml) and APRIL (1 μg/ml) were purchased from Axxon (San Diego, CA).

Immunization of mice with MCPS and NP-Ficoll and detection of Abs

Groups of three BALB/c mice were injected i.p. with MCPS (50 μg) or NP-Ficoll (50 μg). The injection volumes were 400 μl. Mice were sacrificed for spleens after 24, 48, and 72 h, and splenic B cells were analyzed for receptor expression or for BAFF/APRIL stimulation. For MCPS- and NP-Ficoll-specific IgG response, five BALB/c mice per group were injected with 40 μg MCPS or NP Ficoll, with or without CpG ODN (100 μg) in total injection volumes of 400 μl. Four or five PBS-injected mice were included as controls. Another group of mice were immunized with 5 μg MCPS alone or 5 μg MCPS with CpG ODN (100 μg). Finally, mice were also immunized with a mixture of MCPS (40 μg) and NP-Ficoll (40 μg). Mice were tail bled for serum isolation at day 15. To measure serum anti-MCPS or anti-NP IgG Abs, Immulon 2HB ELISA plates (Thermo Electron Corporation, Waltham, MA) were coated with 10 μg/ml NP-KLH (Biosearch Technologies) or MCPS suspended in PBS. The next day, plates were blocked with 1.5% BSA, after which sera were added to wells at 1:20 dilution. Following 4 h of incubation, bound IgG Abs were detected using HRP-labeled goat anti-mouse IgG Abs (Bio-Rad Laboratories, Hercules, CA).

Measurement of Ig isotypes by ELISA

BAFF- and APRIL-induced Ig secretion was determined in purified B cells of BALB/c mice, as described previously (22, 28). Briefly, B cells from BALB/c mice were first incubated in vitro with media, MCPS, GBS-V, or NP-Ficoll for 24 h, and then equal numbers of B cells (0.5 × 10^6/ml) from each condition were restimulated with BAFF or APRIL along with IL-4. In some experiments, MCPS and NP-Ficoll preincubation conditions contained CpG ODN. B cells from BALB/c mice infected with MCPS or NP-Ficoll were directly stimulated with BAFF or APRIL along with IL-4. All experiments included B cells stimulated with LPS and IL-4 as control stimuli for IgG secretion, or LPS and TGF-β as control stimuli for IgA secretion. After 6 d of incubation, culture supernatant total IgG, IgA, and IgM concentrations were determined in ELISA using the Mouse mAb Isotyping Reagents Kit (Sigma-Aldrich) according to the manufacturer’s instructions.

Flow cytometry and Abs

Single-cell suspensions of splenocytes, B cells, or dendritic cells (DCs) were stained with Abs according to standard protocols. Abs against mouse cell markers and the isotype controls used in flow cytometry assay were TACI-PE, BCMA-FITC, BAFF-PE, Rat IgG2a-PE, Rat IgG1-FITC (R&D Systems), CD21-FITC, CD21-PE, CD23-APC-Cy7, CD23-Biotin, CD5-APC, IgM-APC, B220/CD45R-PE-Cy5, B220/CD45R-Pacific Blue, CD3-FITC, CD138-APC, CD19-FITC, CD40-APC, MHC-I-PE, MHC-II-FITC, CD80-FITC and CD86-PE, Rat IgG2b-PE, Rat IgG2a-Biotin, Rat IgG2a-APC, Rat IgG2a-PE-Cy5, Rat IgG2a-Pacific Blue, Southern-Bioin-APC-Cy7, CD11c-APC-Armstrong, IgG1, CD11c-APC, Armenian histiocyte factor, purified rat anti-mouse CD16/CD32 (BD Biosciences, Pharmingen, San Jose, CA), BAFFR-FITC, IgM-PECy7, Rat IgG1-FITC, Rat IgG2a-Cy7 (eBioscience), B220/CD45R Alexa Fluor 405, and IgG2a-Alexa Fluor 405 (Invitrogen). Propidium iodide (PI) and 7-aminoactinomycin D (7-AAD) was used to identify dead cells (BD Biosciences, Pharmingen).

Purified B cells were used in all in vitro stimulation studies for determination of expression of BAFF receptors TACI, BAFFR, and BCMA, B lymphocyte subsets, newly formed (B220+CD23-), MZ (B220+CD23+), follicular (FO) (B220+CD23+), and B1 (IgM+B220+CD5-), as described previously (22, 28) and for the determination of expression of B cell markers CD19, CD40, IgM, CD138, MHC-I, MHC-II, CD80, and CD86. In mice vaccinated with MCPS and NP-Ficoll, the expression of BAFF receptors TACI, BAFFR, and BCMA was assessed on splenocytes. All flow cytometric experiments were performed on a BD LSR II (BD Biosciences) machine, and analysis was done using FlowJo software (Tree Star, Ashland, OR).

Cell proliferation assays

B cells were labeled with 2 μM CFSE (Invitrogen) and cultured in complete RPMI 1640 media at 1 × 10^6 cells/ml in the presence of BAFF or APRIL, along with anti-IgM and IL-4, and costimulated with varying concentrations of MCPS. After 72 h, cells were harvested, stained with PI for dead cell exclusion, and assayed for proliferation by flow cytometry.

Cell survival measurement

B cells or DCs, stimulated with different concentrations of MCPS, GBS-V, or NP-Ficoll, and cultured for 24 h in complete RPMI 1640 media, were harvested, washed twice with 1× PBS, and resuspended in 1× Annexin-V Binding Buffer at a concentration of 1 × 10^6 cells/ml. Cells were stained with Annexin V and PI or 7-AAD (Caltag, Carlsbad, CA) according to the manufacturer’s recommendations and analyzed in flow cytometry.

Analysis of plasma cells

The percentages of plasma cells were determined by measuring CD138 expressing B220-positive cells in flow cytometry assay, as previously described (22, 28). Briefly, purified B cells were costimulated with MCPS, BAFF and anti-IgM, or APRIL and anti-IgM in complete RPMI 1640 media for 72 h. Cells were harvested, and percentages of plasma cells were assayed on live cells, as determined by PI or 7-AAD exclusion in flow cytometry.
**Immunoblotting**

For expression of NF-κB p100/p52 and RelB, purified splenic B cells from BALB/c mice were cultured in complete RPMI 1640 media, as mentioned above, and stimulated for 18 h with BAFF and MCPS (2.5–40 μg/ml concentrations), after which dead cells were removed using the Dead Cell Removal Kit (Miltenyi Biotec). Equal numbers of cells per condition were lysed in complete RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instructions.

For IκBα and Phospho-IκBα expression, B cells were cultured overnight in low serum media containing RPMI 1640 media supplemented with 2% heat-inactivated FBS, 2 mM L-glutamine, 10,000 U/ml penicillin/streptomycin, 10 mM HEPES, 1 mM sodium pyruvate, 1 mM nonessential amino acids, and 50 μM 2-mercaptoethanol stimulated with MCPS (2.5–40 μg/ml concentrations) or media alone. After 18 h, cells were harvested and dead cells removed using the Dead Cell Removal Kit (Miltenyi). Equal numbers of cells were resuspended in the low serum media and restimulated with BAFF for 10 min. Cells were washed twice with 1X PBS and lysed in complete RIPA Lysis Buffer.

Total protein was quantified by bicinchoninic acid assay, using the Pierce Protein Assay Kit (Pierce, Rockford, IL). Lysates were boiled for 5 min in NuPAGE loading buffer containing DTT (Invitrogen, Carlsbad, CA). A total of 30 μg from each lysate was separated by 10% NuPAGE, using a Novex Bis-Tris system (Invitrogen). Gels were blotted onto 0.45-μm nitrocellulose membrane (Invitrogen) and were stained with rabbit anti-nitrocellulose membrane (Invitrogen) and were stained with rabbit anti-NF-κB (C-5, sc-2398), anti-RelB (D-4, sc-4836), anti-α-tubulin (B-7sc-5286) (Santa Cruz Biotechnology), anti–p-IκBα (9246), and anti-IκBα (9242) (Cell Signaling Technology, Danvers, MA). Secondary Abs used were goat anti-mouse IgG-HRP conjugate and goat anti-rabbit IgG-HRP conjugate (Bio-Rad Laboratories). Blots were developed using the SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher, Rockford, IL) and visualized using the Fujifilm LAS-3000 Imaging System (Fujifilm Medical Systems USA, Stamford, CT).

**Measurement of BAFF production**

DCs were derived from BALB/c mouse bone marrow after flushing them with ice-cold PBS through a 70-μm wide-cut cell strainer. Differentiation of bone marrow precursors was achieved by incubating cells for 7 d in conditioned RPMI 1640 medium containing GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) (both from Peprotech, Rocky Hill, NJ). Differentiated cells were stimulated with MCPS, PBS-V, or NP-Ficoll alone or in the presence of IFN-γ (10 ng/ml) (R&D Systems). After 24 h, DCs were used for intracellular BAFF staining in flow cytometry, and culture supernatants were used for BAFF ELISA. Soluble BAFF was measured in the culture supernatants using the Quantikine BAFF ELISA Kit (R&D Systems) according to the manufacturer’s instructions. For intracellular staining of BAFF, harvested cells were initially blocked with anti-mouse Cd16/Cd32 Ab and then stained with Cd11c. After washing, cells were fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained for BAFF according to the manufacturer’s instructions.

**Statistical analysis**

Data were analyzed on the Microsoft Excel program. The Student t test was used to compare groups. A p value <0.05 was considered statistically significant.

**Results**

**MCPS-exposed B cells are impaired in their ability to secrete Igs in response to BAFF or APRIL stimulation**

We sought to determine the effect of MCPS on Ig secretion from B cells in response to BAFF or APRIL. B cells pretreated with 40, 10, or 2.5 μg/ml of MCPS or the prototype TI-2 Ag NP-Ficoll (29) for 24 h were restimulated with media alone, BAFF and IL-4, APRIL and IL-4, LPS and IL-4, or LPS and TGF-β. After 6 d of incubation, culture supernatant IgA, IgG, and IgM levels were measured by ELISA. Comparison of MCPS- or NP-Ficoll-pretreated B cell culture supernatant Ig levels with those of media-pretreated cells demonstrated that MCPS pretreatment led to a concentration-dependent impairment of Ig secretion in response to BAFF and APRIL even in the presence of a potent second signal, IL-4 (Fig. 1A). All three MCPS concentrations used for the preincubation of B cells led to statistically significantly lower (p < 0.05) IgG, IgA, and IgM secretion following BAFF or APRIL stimulation. NP-Ficoll did not yield any reduction of Ig secretion at any of the concentrations tested. Cells were also stimulated with either LPS and IL-4, or LPS and TGF-β, after pretreatment with MCPS or NP-Ficoll. These stimuli were found to induce IgG and IgA secretion from B cells, respectively, and therefore served as positive controls in the assay (20, 28). There was a concentration-dependent suppressive effect of MCPS on IgG and IgA secretion in response to LPS, but these differences did not reach statistically significant levels.

Previously, we and others have shown that BAFF and APRIL induced the generation of Ig-secreting plasma cells and that TACI signaling was essential in this process (22–24). Because MCPS-exposed B cells failed to secrete Igs in response to BAFF and APRIL stimulation, we therefore sought to understand whether the induction of plasma cells from B cells was inhibited by MCPS. Purified B cells were costimulated with MCPS at varying concentrations, along with BAFF and anti-IgM or APRIL and anti-IgM. Cells were harvested after 72 h and stained for CD138 (Syndecan-1) and B220. Indeed, flow cytometry analysis showed a statistically significant decrease in plasma cell development in response to BAFF at 40 μg/ml (p < 0.014) and 10 μg/ml (p < 0.028) MCPS concentrations (Fig. 1B, Table I). Similarly, 40 μg/ml (p < 0.032) and 10 μg/ml (p < 0.035) of MCPS inhibited APRIL-induced plasma cell development.

**MCPS downregulates TACI and BAFFR expression on B lymphocytes**

Our earlier research showed that newborn mouse B cells do not develop into plasma cells and do not secrete IgG and IgA Abs in response to BAFF or APRIL stimulation because they express low levels of TACI (22). Because we observed that MCPS-pretreated cells do not respond to BAFF- or APRIL-induced Ig secretion and fail to develop into plasma cells, we sought to determine whether an MCPS-mediated change in TACI expression level may account for the unresponsiveness of B cells to BAFF and APRIL. For this purpose, we measured the levels of BAFF and APRIL receptors on purified B cells 24 h following incubation with increasing concentrations of MCPS or NP-Ficoll. Flow cytometry analysis showed that B cells incubated with MCPS had a concentration-dependent decrease in TACI levels (Fig. 2A, Table II). The decrease in percentage of TACI-expressing cells was statistically significant at 40 μg/ml (p < 0.0001), 10 μg/ml (p < 0.0001), and 2.5 μg/ml (p < 0.002) concentrations, whereas NP-Ficoll stimulation did not affect TACI levels at any of the concentrations tested. The expression of BAFFR on B lymphocytes was also decreased after stimulation with 40 μg/ml (p < 0.0001) and 10 μg/ml (p < 0.0008) of MCPS concentrations (Fig. 2B, Table II). BAFFR levels did not change with MCPS (2.5 μg/ml) or with any of the NP-Ficoll concentrations. Expression of BCMA was not significantly altered by MCPS or NP-Ficoll stimulation (Fig. 2C, Table II).

To determine which subsets of B cells were most affected by MCPS, multicolor staining analysis of TACI and BAFFR on gated newly formed (B220⁺CD23negCD21neg), FO (B220⁺CD23hi CD21neg), MZ (B220⁺CD23negCD21hi), and B1 (B220⁺CD5⁺) cells was performed on splenic B cells. TACI was found to be downregulated on both B1 and B2 subsets tested, and the suppressive effect of MCPS was concentration dependent (Supplemental Fig. 1A, 1B). Similarly, BAFFR was also found to be downregulated on all four subsets. MCPS stimulation did not cause significant alterations in the number of MZ B cells, but a decrease in the number of FO cells was observed in MCPS-stimulated cells.

**Statistical analysis**

Data were analyzed on the Microsoft Excel program. The Student t test was used to compare groups. A p value <0.05 was considered statistically significant.
B cells from MCPS-injected mice express low levels of TACI and BAFFR, and respond poorly to BAFF or APRIL stimulation

We investigated whether MCPS maintained its B cell inhibitory activity in vivo as well. For this purpose, nine BALB/c mice were injected with MCPS (50 µg/mouse), and an equal number of mice were injected with NP-Ficoll (50 µg/mouse). Three mice from each group were sacrificed at 24, 48, and 72 h after injection. At each time point, the vaccine effect on B cell TACI, BAFFR and BCMA expression, and BAFF- or APRIL-induced Ig secretion was assessed. TACI-positive cell percentage (16.77 ± 3.94) in MCPS-injected mice was lower than that in NP-Ficoll–injected mice (20.73 ± 1.37) at 24 h, but this difference did not reach statistically significant levels (p, 0.087) (Fig. 3A, Table III). The decrease in MCPS-injected mice TACI levels progressed to statistically significant levels at 48 (p, 0.010) and 72 (p, 0.004) h time points. Similarly, in MCPS-injected mice BAFFR levels were significantly lower at the 48 (p < 0.013) and 72 (p < 0.041) h time points than those in NP-Ficoll–injected mice. The expression of NP-Ficoll–immunized mice TACI and BAFFR did not change during the three time points. MCPS-immunized mice TACI and BAFFR levels were also lower than in PBS-immunized mice, whereas PBS-immunized mice receptor levels were comparable to those immunized with NP-Ficoll at 72 h of immunization (Supplemental Fig. 2A). There was no significant change in BCMA levels between MCPS- and NP-Ficoll–injected mice at any of the time points tested.

The responses of MCPS-injected mice B cells to ex vivo stimulation with BAFF and APRIL corresponded to the gradual decrease in TACI levels observed in these mice. Compared with IgG levels in NP-Ficoll–injected mice, both BAFF- and APRIL-induced IgG levels in MCPS-injected mice were significantly lower at the 24-h time point (Fig. 3B). MCPS-injected mice IgG levels further decreased in BAFF- and APRIL-stimulated cells at 48 and 72 h. A similar trend was seen in IgA and IgM levels of MCPS-injected mice in which 72-h IgA and IgM levels were lower than those

Table I. Percent of CD138+ plasma cells in MCPS-treated B cells

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>CD138 (%)</th>
<th>p Value</th>
</tr>
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<tbody>
<tr>
<td>Media</td>
<td>11.15 ± 3.71</td>
<td></td>
</tr>
<tr>
<td>BAFF + IgM</td>
<td>21.93 ± 7.00</td>
<td>0.0132 versus media</td>
</tr>
<tr>
<td>BAFF + IgM and MCPS (40 µg/ml)</td>
<td>8.22 ± 0.75</td>
<td>0.0139 versus BAFF</td>
</tr>
<tr>
<td>BAFF + IgM and MCPS (10 µg/ml)</td>
<td>11.13 ± 0.59</td>
<td>0.0280 versus BAFF</td>
</tr>
<tr>
<td>BAFF + IgM and MCPS (2.5 µg/ml)</td>
<td>17.77 ± 3.23</td>
<td>0.2010 versus BAFF</td>
</tr>
<tr>
<td>APRIL + IgM</td>
<td>17.03 ± 5.52</td>
<td>0.0583 versus media</td>
</tr>
<tr>
<td>APRIL + IgM and MCPS (40 µg/ml)</td>
<td>8.38 ± 2.09</td>
<td>0.0320 versus APRIL</td>
</tr>
<tr>
<td>APRIL + IgM and MCPS (10 µg/ml)</td>
<td>9.10 ± 0.88</td>
<td>0.0349 versus APRIL</td>
</tr>
<tr>
<td>APRIL + IgM and MCPS (2.5 µg/ml)</td>
<td>14.47 ± 3.71</td>
<td>0.2702 versus APRIL</td>
</tr>
</tbody>
</table>

*Average values from three experiments.

*p < 0.05 is considered statistically significant and indicated in bold.
measured at 24 h. PBS- or NP-Ficoll–injected mice B cell responses to BAFF or APRIL did not change until the 72-h time point.

**MCPS impairs BAFF- and APRIL-induced B cell proliferation**

In addition to inducing plasma cell generation, and T cell-independent Ig isotype switch and secretion, BAFF (30, 31) and APRIL (32) are known to augment anti-IgM–induced B cell proliferation. Because MCPS treatment leads to impaired Ig secretion and plasma cell development in response to BAFF and APRIL signals in B cells, we therefore wanted to determine whether MCPS would also affect BAFF- and APRIL-mediated B cell proliferation. We first performed a pilot experiment to test the costimulatory molecule requirements for BAFF- and APRIL-induced B cell proliferation. As reported previously (33), the presence of anti-IgM receptors on splenic B cells after MCPS or NP-Ficoll treatment. Purified splenic B cells were cocultured with 2.5–40 μg/ml of MCPS-stimulated PBS- or NP-Ficoll–injected mice B cell responses to BAFF or APRIL did not change until the 72-h time point.

**MCPS increases B cell death**

The suppressive effect of MCPS on B cell response to BAFF or APRIL, and the downregulation of the two BAFF/APRIL receptors by MCPS, led us to investigate B cell survival in MCPS-exposed cells. For this purpose, we incubated purified B cells with 40, 10, or 2.5 μg/ml of MCPS or NP-Ficoll and determined cell survival and apoptosis by Annexin V and 7-AAD staining at 12-, 16-, 20-, and 24-h time points. Flow cytometry analysis demonstrated that MCPS- and NP-Ficoll–treated cells had similar live (Annexin V<sup>neg</sup> and 7-AAD<sup>neg</sup>), apoptotic (Annexin V<sup>pos</sup> and 7-AAD<sup>neg</sup>), and dead (Annexin V<sup>pos</sup> and 7-AAD<sup>pos</sup>) cell percentages, compared with cells left in media at 12- and 16-h incubation time points (Fig. S5, S6, Supplemental Fig. 4). The percentage of live cells significantly decreased with all three MCPS and NP-Ficoll concentrations at the 20-h time point, although the decrease in MCPS-treated cells was markedly greater (Supplemental Table 1). As expected, the decrease in live cell percentages led to increased dead cell percentages for MCPS- and NP-Ficoll–incubated cells at the 20-h time point. At the 24-h time point, the percentage of live cells further decreased in B cells incubated with all three MCPS and NP-Ficoll concentrations. At this time also, the decrease in MCPS-incubated cells was greater. Furthermore, the reduction in live cells led to an increase in the percentage of dead cells in MCPS-incubated cells at the 24-h time point. Although dead cell percentages increased at 24 h in NP-Ficoll–incubated B cells as well, this increase was not significantly greater than the percentage of dead cells in media-exposed cells. There was a trend for a decrease in the percentage of apoptotic cells in MCPS-exposed cells, starting from the 20-h time point, but only cells treated with 40 μg/ml of MCPS manifested a significant decrease. Overall, the results suggested that B cell death was accelerated by poly-
saccharides at the 20-h time point but the increase in cell death was significantly greater in MCPS-incubated cells.

**The effect of MCPS on B cell surface molecules other than BAFF/APRIL receptors**

To determine whether MCPS modulated the expression of B cell receptors other than TACI and BAFFR, we measured MHC-I, CD40, CD19, CD80, CD86, and MHC-II following the incubation of purified B cells with 40 μg/ml of MCPS or NP-Ficoll for 18 h. Two separate expression profiles emerged after FACS analysis (Fig. 6). The expression of MHC-I, CD40, and CD19 remained unchanged. Interestingly, the expression of CD86 ($p < 0.0029$) and MHC-II ($p < 0.0106$), which are involved in the Ag presentation machinery, was found to be significantly increased. The expression of another costimulatory molecule, CD80, also increased after MCPS treatment, although the difference did not reach statistical significance. NP-Ficoll did not change the expression of any of the molecules tested.

### Table III. Percent expression of receptors on splenocytes of mice injected with MCPS or NP-Ficoll

<table>
<thead>
<tr>
<th>Time after Injection</th>
<th>Injected with</th>
<th>TACI (%)$^a$</th>
<th>BAFFR (%)$^a$</th>
<th>BCMA (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>MCPS (40 μg/ml)</td>
<td>16.77 ± 3.94</td>
<td>19.37 ± 1.83</td>
<td>1.84 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>NP-Ficoll (40 μg/ml)</td>
<td>20.73 ± 1.37</td>
<td>19.83 ± 3.88</td>
<td>0.78 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>p value$^b$</td>
<td>0.087</td>
<td>0.430</td>
<td>0.064</td>
</tr>
<tr>
<td>48 h</td>
<td>MCPS</td>
<td>9.33 ± 1.59</td>
<td>16.53 ± 2.83</td>
<td>0.83 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>NP-Ficoll</td>
<td>16.20 ± 2.71</td>
<td>22.70 ± 1.25</td>
<td>0.77 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>p value$^b$</td>
<td>0.010</td>
<td>0.013</td>
<td>0.437</td>
</tr>
<tr>
<td>72 h</td>
<td>MCPS</td>
<td>11.02 ± 1.84</td>
<td>15.20 ± 2.88</td>
<td>2.98 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>NP-Ficoll</td>
<td>17.93 ± 1.50</td>
<td>20.33 ± 2.57</td>
<td>2.94 ± 0.27</td>
</tr>
<tr>
<td></td>
<td>p value$^b$</td>
<td>0.004</td>
<td>0.041</td>
<td>0.452</td>
</tr>
</tbody>
</table>

$^a$Average percentages from three mice in each group are listed.

$^b$The p value is calculated by comparing the MCPS and NP-Ficoll percentages at each time point. A p value <0.05 is considered statistically significant and indicated in bold.
were inhibited (Fig. 7). Suggesting an inhibition in the non-
RelB was decreased in MCPS-treated cells. Similarly, the ca-
transduce the canonical NF-
TACI- or BAFFR-mediated signaling may be blocked. In primary
Inhibition of TACI and BAFFR expression, as well as B cell
MCPS impairs BAFF-induced NF-
APRIL system is not a generalized phenomenon leading to a de-
concentrations tested (Supplemental Fig. 5). These results dem-
levels remained unchanged with all the MCPS and NP-Ficoll
splenocytes following the stimulation of cells with increasing
indicated concentrations of MCPS along with IL-4 + anti-IgM and BAFF
FIGURE 4. The effect of MCPS on B cell proliferation. Purified B cells
from BALB/c spleens were labeled with CFSE and costimulated with
nuine DC cultures. Surprisingly, NP-Ficoll alone increased
BAFF expression (Fig. 8
p
to a significant decrease (versus APRIL) 0.0920 (versus 40
APRIL stimulation (22, 28). We therefore asked whether CpG
ODN would enhance expression of TACI on MCPS-treated cells.
Mean percentages of TACI and BAFFR expression in MCPS-
stimulated B cells were determined by flow cytometry after co-
stimulation with CpG ODN for 24 h. Although TACI levels in
cells co-incubated with 40 g/ml (46.3 ± 3.40%) of MCPS and
CpG ODN did not reach those of cells exposed to CpG ODN only
(66.67 ± 1.08%), they were significantly higher (p < 0.0018)
than levels in cells that had been treated with 40 g/ml MCPS

Table IV. Percentage of CFSE-negative (proliferated) B cells

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>Average*</th>
<th>p Value*</th>
<th>p Value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>4.18 ± 1.19</td>
<td>0.0243 (versus media)</td>
<td>0.006 (versus media)</td>
</tr>
<tr>
<td>MCPS (40 μg/ml)</td>
<td>1.59 ± 1.08</td>
<td>0.0482 (versus media)</td>
<td>0.0243 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (10 μg/ml)</td>
<td>2.29 ± 0.94</td>
<td>0.0426 (versus media)</td>
<td>0.0018 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (2.5 μg/ml)</td>
<td>4.15 ± 2.04</td>
<td>0.0490 (versus media)</td>
<td>0.0000 (versus 40 μg/ml)</td>
</tr>
<tr>
<td>BAFF</td>
<td>64.70 ± 5.23</td>
<td>0.0024 (versus BAFF)</td>
<td>0.0048 (versus 40 μg/ml)</td>
</tr>
<tr>
<td>MCPS (40 μg/ml) + BAFF</td>
<td>15.00 ± 12.72</td>
<td>0.0020 (versus BAFF)</td>
<td>0.0048 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (10 μg/ml) + BAFF</td>
<td>52.80 ± 5.16</td>
<td>0.0024 (versus BAFF)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (2.5 μg/ml) + BAFF</td>
<td>58.63 ± 2.50</td>
<td>0.0720 (versus BAFF)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>APRIL</td>
<td>64.37 ± 4.38</td>
<td>0.0000 (versus APRIL)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (40 μg/ml) + APRIL</td>
<td>17.18 ± 17.45</td>
<td>0.0055 (versus APRIL)</td>
<td>0.0920 (versus 40 μg/ml)</td>
</tr>
<tr>
<td>MCPS (10 μg/ml) + APRIL</td>
<td>50.40 ± 7.68</td>
<td>0.0586 (versus APRIL)</td>
<td>0.0002 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (2.5 μg/ml) + APRIL</td>
<td>54.67 ± 7.27</td>
<td>0.0641 (versus APRIL)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>CpG</td>
<td>55.50 ± 6.15</td>
<td>0.0001 (versus media)</td>
<td>0.0006 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (40 μg/ml) + CpG</td>
<td>43.10 ± 10.07</td>
<td>0.1937 (versus CpG)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (10 μg/ml) + CpG</td>
<td>57.53 ± 5.37</td>
<td>0.3442 (versus CpG)</td>
<td>0.0000 (versus 2.5 μg/ml)</td>
</tr>
<tr>
<td>MCPS (2.5 μg/ml) + CpG</td>
<td>57.40 ± 12.00</td>
<td>0.4096 (versus CpG)</td>
<td>0.0008 (versus 2.5 μg/ml)</td>
</tr>
</tbody>
</table>

*Kp < 0.05 is considered statistically significant and indicated in bold.

molecule CD3 and the B cell marker B220 was measured in total
splenocytes following the stimulation of cells with increasing
concentrations of MCPS or NP-Ficoll. CD3 and B220 expression
levels remained unchanged with all the MCPS and NP-Ficoll
concentrations tested (Supplemental Fig. 5). These results demon-
strated that the suppressive effect of MCPS on the BAFF/
APRIL system is not a generalized phenomenon leading to a de-
creased expression of most B cell molecules.

MCPS impairs BAFF-induced NF-κB signaling by inhibiting both canonical and noncanonical pathways

Inhibition of TACI and BAFFR expression, as well as B cell
responses to BAFF or APRIL stimulation by MCPS, suggested that TACI- or BAFFR-mediated signaling may be blocked. In primary
B cells, BAFFR-mediated signals strongly activate the non-
canonical NF-κB pathway (34, 35) and weakly activate the ca-
onical NF-κB pathway (36). TACI, in contrast, is known to transduce the canonical NF-κB pathway signals (35). Analysis of
signaling in MCPS- and BAFF-stimulated B cells demonstrated that both the canonical and the noncanonical NF-κB pathways
were inhibited (Fig. 7). Suggesting an inhibition in the non-
canonical NF-κB pathway, the processing of p100 to p52 and RelB was decreased in MCPS-treated cells. Similarly, the ca-
onical NF-κB pathway was compromised because BAFF stim-
ulation led to decreased phosphorylation of IκB-α and degradation of IκB-α in the presence of MCPS.

BAFF expression in MCPS-treated DCs

Having shown that MCPS impairs BAFF-mediated B cell functions that are important in response to TI-2 Ag, we next sought to de-
termine whether MCPS had a modulatory effect on BAFF ex-
pression in myeloid cells. For this purpose, bone marrow-derived
murine DCs were incubated with MCPS or NP-Ficoll for 24 h, and
intracellular or culture supernatant BAFF levels were determined.
DCs were also incubated with MCPS and IFN-γ or NP-Ficoll and
IFN-γ to determine the effect of MCPS on NP-Ficoll on IFN-γ-
induced BAFF production. Analysis of cell culture supernatants
by ELISA showed that DCs left in media contained low levels of
BAFF and MCPS alone did not significantly change BAFF pro-
duction (Fig. 8A). In contrast, intracellular BAFF expression
was high in DCs incubated with media alone, and MCPS treatment led to a significant decrease (p < 0.048) in intracellular BAFF ex-
pression (Fig. 8B). As expected, IFN-γ treatment increased culture
supernatant and intracellular BAFF production (Fig. 8A, 8B). Both soluble (p < 0.008) and intracellular (p < 0.006) BAFF levels
were significantly lower when MCPS was included in the IFN-γ-
containing DC cultures. Surprisingly, NP-Ficoll alone increased
the soluble BAFF production significantly (p < 0.0004) (Fig. 8A).
Although intracellular BAFF levels were also consistently higher
after NP-Ficoll treatment, the increase in intracellular BAFF ex-
pression was modest (Fig. 8B).

The effect of CpG ODN on MCPS-mediated suppression of B cells

Earlier, we had shown that both in vitro stimulation of adult and newborn B cells with CpG ODN and in vivo injection of CpG
ODN upregulate TACI and BAFFR expression, which leads to
TACI-dependent augmentation of Ig secretion following BAFF or APRIL stimulation (22, 28). We therefore asked whether CpG
ODN would enhance expression of TACI on MCPS-treated cells.

Mean percentages of TACI and BAFFR expression in MCPS-
stimulated B cells were determined by flow cytometry after co-
stimulation with CpG ODN for 24 h. Although TACI levels in
cells co-incubated with 40 μg/ml (46.3 ± 3.40%) of MCPS and
CpG ODN did not reach those of cells exposed to CpG ODN only
(66.67 ± 1.08%), they were significantly higher (p < 0.0018)
than levels in cells that had been treated with 40 μg/ml MCPS.
alone (13.50 ± 3.72%) (Fig. 9A, Table V). The difference in TACI percentages was also significant between cells treated with 10 (p < 0.0021) or 2.5 μg/ml (p < 0.0023) of MCPS only and cells treated with the same amount of MCPS in the presence of CpG ODN. Inclusion of CpG ODN in the 40 μg/ml of MCPS-containing B cell cultures (46.33 ± 3.40%) resulted in a statistically significant increase (p < 0.0037) in TACI levels, compared with levels in those cells treated with media (34.53 ± 2.27%). The enhancement in BAFFR expression also reached statistically significant levels between wells containing 40 (p < 0.0105), 10 (p < 0.0061), or 2.5 μg/ml (p < 0.0109) of MCPS alone and those stimulated with the same amount of MCPS and CpG ODN (Fig. 9A, Table V). The addition of CpG ODN to 2.5 μg/ml of MCPS-containing cells led to significantly elevated levels of BAFFR expression when compared with media-exposed B cells (p < 0.0093), whereas no significant increase was observed in 40 or 10 μg/ml of MCPS-containing cells.

Next, we sought to understand whether the CpG ODN-mediated increase of TACI expression in MCPS-incubated B cells would also sensitize B cells to BAFF or APRIL stimulation. For this purpose, purified B cells were prestimulated with MCPS (40 μg/ml), alone or along with CpG ODN. After 24 h, cells were restimulated with BAFF and IL-4, APRIL and IL-4, LPS and IL-4, or LPS and TGF-β for 6 d. As shown in Fig. 9B, CpG ODN and MCPS costimulation, compared with pretreatment with MCPS alone, led to a statistically significant increase in the secretion of IgA and IgG in response to subsequent incubation with BAFF (Table VI). However, this rescue of Ig secretion was not in complete concordance with the increase in TACI expression after CpG ODN and MCPS stimulation, because CpG ODN- and MCPS-pretreated B cell responses to BAFF or APRIL were significantly lower than were responses of the media-pretreated cells (Fig. 9B, Table VI), whereas the percentage of cells expressing TACI in CpG ODN- and MCPS-pretreated cells was significantly higher than that left in media only (Fig. 9A). Furthermore, despite having a significantly higher percentage of TACI-expressing cells, when compared with MCPS-pretreated cells, the CpG ODN- and MCPS-pretreated B cells did not secrete significantly higher levels of IgG or IgA upon APRIL stimulation. In addition, these IgG and IgA levels remained significantly lower than those in cells pretreated with media only (Table VII), even though their TACI levels were markedly higher (Fig. 9A). IgM secretion in CpG ODN- and MCPS-pretreated cells also increased compared with secretion in those pretreated with MCPS only, but this difference did not reach statistical significance. Inclusion of CpG ODN and MCPS pretreatment conditions also led to a significant rise in LPS-induced IgG and IgA secretion, compared with that in cells pretreated with MCPS alone. However, as with BAFF- and APRIL-stimulated cells, IgG and IgA levels remained lower than those in media-pretreated cells. These data suggested that CpG ODN was not sufficiently potent to counteract the suppressive effect of MCPS on B cell response to BAFF or APRIL but was strong enough to upregulate TACI in MCPS-pretreated cells.

CpG ODN has an adjuvant effect for NP-Ficoll but not for MCPS

Having shown that MCPS inhibited TACI and BAFFR expression on B cells and rendered them less responsive to BAFF and APRIL
signals, even when costimulated with CpG ODN, we investigated the in vivo effect of CpG ODN when given as an adjuvant for MCPS or NP-Ficoll. For this purpose, groups of mice were immunized with PBS, MCPS (40 μg), NP-Ficoll (40 μg), MCPS (40 μg) with CpG ODN (100 μg), or NP-Ficoll (40 μg) with CpG ODN (100 μg), and serum IgG Ab levels were measured 15 d later. As shown in Fig. 10, compared with PBS-immunized mice, NP-Ficol–immunized mice developed significantly increased IgG Ab levels against NP (OD450 = 0.72 ± 0.08; p < 0.0023), whereas IgG Abs to MCPS in mice immunized with MCPS alone (OD450 = 0.24 ± 0.05; p < 0.3061) remained comparable to levels in those immunized with PBS. Although the CpG ODN-containing MCPS vaccine yielded a statistically significant increase (OD450 = 0.30 ± 0.04; p < 0.0213) in IgG Ab response against MCPS, compared with MCPS immunization alone, this difference was markedly lower than the adjuvant effect of CpG ODN on NP-Ficoll. Anti-NP serum Ab levels in mice immunized with CpG ODN-containing NP-Ficoll vaccine increased to OD450 = 1.24 ± 0.22 (p < 0.0006). To determine whether a smaller dose of MCPS would be more immunogenic, we also immunized groups of mice with 5 μg of MCPS alone or 5 μg of MCPS with CpG ODN (100 μg) and compared the results with PBS-immunized mice. Results demonstrated that no statistically significant difference (p < 0.1136) was found in Ab response among the mice that received 5 or 50 μg of MCPS (Supplemental Fig. 6A). Anti-MCPS IgG levels in both mice groups remained comparable to those immunized with PBS alone (p < 0.3973 and p < 0.14362, respectively). Moreover, inclusion of CpG ODN with 5 μg of MCPS did not induce significant increase in anti-IgG Ab levels, compared with immunization with MCPS alone (p < 0.0876) or PBS (p < 0.05833). These data support in vitro experiments in which inclusion of CpG ODN in MCPS-containing B cell cultures, as opposed to pretreatment with MCPS alone, resulted in a marginal increase in Ig secretion, which remained much lower than the increase observed with media-pretreated cells. Having determined that MCPS has a suppressive effect on the response of B cells to BAFF or APRIL, we sought to investigate whether MCPS would diminish Ab response against NP when MCPS and NP-Ficoll are injected together. Serum anti-NP IgG levels in mice immunized with MCPS and NP-Ficoll (40 μg each) were compared with levels in those immunized with NP-Ficoll alone. Results demonstrated that co-injection of MCPS and NP-Ficoll did not significantly decrease the Ab response against NP-Ficoll (p < 0.0624) (Supplemental Fig. 6B).

Analysis of the effect of GBS-V on the BAFF system

Results so far have suggested that the CPS from the Gram-negative bacterium N. meningitidis is suppressive on the BAFF system. We next sought to determine whether CPS from a Gram-positive bacterium would also modulate BAFF system biology and so compared the immunomodulatory effects of purified CPS from GBS-V and MCPS. Purified B cells were incubated with 40 μg/ml of GBS-V or MCPS, and the receptor expression was determined 24 h later. Although its effect was slightly less than that of MCPS, GBS-V also suppressed TACI (p < 0.014) and BAFFR (p < 0.0035) expression (Fig. 11A). Analysis of the responses of CPS–incubated B cells to BAFF and APRIL demonstrated that GBS-V also inhibited IgG (p < 0.04), IgA (p < 0.004), and IgM (p < 0.007) following BAFF stimulation (Fig. 11B), and IgG (p < 0.0003), IgA (p < 0.04), and IgM (p < 0.03) following APRIL stimulation. As with MCPS, GBS-V also induced cell death 24 h after incubation. The number of live cells decreased (p < 0.05) and dead cells increased (p < 0.034) in GBS-V–stimulated cells (Fig. 11C, 11D, Supplemental Table II). Finally, we compared the effect of GBS-V on BAFF secretion from DCs with that of MCPS. At 24 h after incubation with CPS, there was a significant suppression of IFN-γ–induced BAFF secretion by MCPS (p < 0.011) and GBS-V (p < 0.015) from DCs (Fig. 11E).

Discussion

In this study, we show that MCPS and GBS-V treatment inhibits BAFF expression by DCs and impairs B cell responses to BAFF and APRIL. B cells pre-exposed to MCPS or GBS-V were unable to secrete IgG, IgA, and IgM when stimulated with BAFF or APRIL. Similarly, MCPS- and GBS-V–treated B cells underwent apoptosis and failed to develop into plasma cells or proliferate in response to BAFF or APRIL stimulation. Decreased TACI and BAFFR ex-
pression and inhibition of TACI- and BAFFR-mediated signaling appear to be responsible for the unresponsiveness of MCPS-exposed B cells to BAFF or APRIL. Interestingly, the prototype TI-2 Ag, NP-Ficoll (29), did not manifest any of the suppressive properties of MCPS.

Studies in TACI knockout mice and newborn mice have convincingly shown the importance of TACI in the development of Ab response against TI-2 Ags (16, 22, 27). These mice do not respond to NP-Ficoll or bacterial polysaccharide vaccines. The function of TACI in response to TI-2 Ag is to mediate T cell-independent Ig isotype switch, plasma cell generation, and Ig secretion (20, 22, 24) in BAFF- or APRIL-stimulated B cells. Results with MCPS-treated B cells resemble those obtained in TACI-deficient and newborn mice. MCPS-exposed B cells also do not respond to BAFF or APRIL, and they express low levels of TACI. Both in vitro and in vivo experiments showed that NP-Ficoll, despite having been designated as TI-2 Ag–like MCPS, neither inhibited TACI expression nor blocked Ig secretion from BAFF- or APRIL-stimulated B cells.

Table V. Percentage of TACI- and BAFFR-expressing B cells following incubation with indicated conditions

<table>
<thead>
<tr>
<th>Culture Conditions</th>
<th>TACI (%)a</th>
<th>p Valuec</th>
<th>BAFF (%)a</th>
<th>p Valuec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>34.53 ± 2.27</td>
<td></td>
<td>44.53 ± 1.76</td>
<td></td>
</tr>
<tr>
<td>MCPS (40 μg/ml)</td>
<td>13.50 ± 3.72</td>
<td>0.0006</td>
<td>24.70 ± 10.50</td>
<td>0.0160</td>
</tr>
<tr>
<td>MCPS (10 μg/ml)</td>
<td>21.37 ± 3.61</td>
<td>0.0029</td>
<td>37.10 ± 8.84</td>
<td>0.1131</td>
</tr>
<tr>
<td>MCPS (2.5 μg/ml)</td>
<td>26.93 ± 2.07</td>
<td>0.0064</td>
<td>42.07 ± 6.10</td>
<td>0.2680</td>
</tr>
<tr>
<td>Cpg</td>
<td>66.67 ± 1.08</td>
<td>0.0000</td>
<td>69.63 ± 5.94</td>
<td>0.0011</td>
</tr>
<tr>
<td>Cpg + MCPS (40 μg/ml)</td>
<td>46.33 ± 3.40</td>
<td>0.0037</td>
<td>48.20 ± 3.40</td>
<td>0.0864</td>
</tr>
<tr>
<td>Cpg + MCPS (10 μg/ml)</td>
<td>59.80 ± 10.79</td>
<td>0.0083</td>
<td>62.60 ± 4.97</td>
<td>0.0020</td>
</tr>
<tr>
<td>Cpg + MCPS (2.5 μg/ml)</td>
<td>62.10 ± 10.54</td>
<td>0.0057</td>
<td>65.70 ± 9.42</td>
<td>0.0093</td>
</tr>
</tbody>
</table>

*p < 0.05 is considered statistically significant and indicated in bold.

aAverage values from three experiments.
Table VI. Culture supernatant mean IgG, IgA, and IgM concentrations (ng/ml) following the stimulation of pretreated B cells with BAFF or APRIL

<table>
<thead>
<tr>
<th>Preincubation</th>
<th>Mean SD</th>
<th>BAFF + IL-4</th>
<th>APRIL + IL-4</th>
<th>LPS + IL-4</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean SD</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Mean SD</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Comparison of Groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG Media</td>
<td>0.34 0.59</td>
<td>38.73 16.54</td>
<td>21.24 16.30</td>
<td>84.91 14.73</td>
<td>0.000</td>
<td>0.39 0.68</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>MCPS</td>
<td>1.13 1.96</td>
<td>0.267 1.49</td>
<td>0.008 0.39</td>
<td>0.084 0.68</td>
<td>0.000</td>
<td>0.39 0.68</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>Cpg</td>
<td>14.67 10.85</td>
<td>0.042 23.98</td>
<td>0.011 0.035</td>
<td>0.53 16.74</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCPS + Cpg</td>
<td>12.45 12.78</td>
<td>0.088 4.29</td>
<td>0.048 5.01</td>
<td>0.094 6.91</td>
<td>0.010</td>
<td>0.157 0.046</td>
<td>0.000 0.000</td>
<td>0.000 0.000</td>
<td>0.002 Versus MCPS</td>
<td></td>
</tr>
<tr>
<td>IgA Media</td>
<td>1.39 0.96</td>
<td>31.44 5.23</td>
<td>14.25 2.88</td>
<td>30.08 4.17</td>
<td>0.000</td>
<td>0.56 0.69</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>MCPS</td>
<td>0.12 0.23</td>
<td>0.020 0.17</td>
<td>0.000 0.17</td>
<td>0.000 0.17</td>
<td>0.000</td>
<td>0.56 0.69</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>Cpg</td>
<td>7.40 4.58</td>
<td>0.021 18.82</td>
<td>0.021 25.50</td>
<td>0.049 11.11</td>
<td>0.000</td>
<td>0.56 0.69</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>MCPS + Cpg</td>
<td>2.55 2.02</td>
<td>0.171 2.39</td>
<td>0.000 4.31</td>
<td>0.010 5.66</td>
<td>0.010</td>
<td>0.56 0.69</td>
<td>0.046</td>
<td>26.09 22.95</td>
<td>0.010 Versus media</td>
<td></td>
</tr>
<tr>
<td>IgM Media</td>
<td>2.58 5.17</td>
<td>90.91 34.32</td>
<td>53.62 40.76</td>
<td>0.000 0.021</td>
<td>0.043 77.45</td>
<td>0.000 0.021</td>
<td>0.043 77.45</td>
<td>0.000 0.021</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCPS</td>
<td>0.33 0.66</td>
<td>0.210 10.54</td>
<td>0.002 13.89</td>
<td>0.074 24.96</td>
<td>0.000 0.021</td>
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<sup>a</sup>Statistically significant p values are indicated in bold.
stimulated B cells. These results suggested that MCPS-mediated suppression of TACI expression may be responsible for the unresponsiveness of B cells to BAFF or APRIL. However, even though CpG ODN was able to relieve the downregulation of TACI by MCPS, it was not able to restore B cell responses to BAFF or APRIL stimulation. Ig levels in the culture supernatants of CpG ODN- and MCPS-exposed cells were significantly lower than those of cells incubated in media only and were only slightly higher than those of cells preincubated with MCPS alone. The unresponsiveness of B cells to BAFF or APRIL in cells with normal or increased TACI levels points to a blockage of signaling. Indeed, analysis of the effect of MCPS on BAFF-mediated signaling showed that both TACI (canonical) (35) and BAFFR (noncanonical) (34, 35) mediated NF-κB activation was inhibited with MCPS. These findings suggest that MCPS has dual effect on B cells; it suppresses TACI expression and blocks BAFF- or APRIL-mediated signaling. In addition, it appears that CpG ODN could restore the MCPS-mediated TACI suppression, but not the signaling blockade on TACI and BAFFFR because the Ig secretion was still impaired in cells costimulated by MCPS and CpG.

FIGURE 10. Ag-specific IgG response in MCPS- and NP-Ficoll-injected BALB/c mice. MCPS (dose) or NP-Ficoll (dose) with or without CpG ODN (dose) was used to vaccinate mice. PBS-injected mice were included as controls. Day 15 serum anti-NP-Ficoll and anti-MCPS IgG Ab levels were plotted. Other than the PBS-injected mice group, which contained four mice, each vaccine group contained five mice. Data shown represent average OD values from one experiment. *p < 0.05, **p < 0.01, ***p < 0.001.

FIGURE 11. The effect of GBS-V on the BAFF system. A. Purified mouse splenic B cells were incubated with 40 μg/ml MCPS or GBS-V for 24 h and stained with Abs against TACI and BAFFR along with B220. Data shown represent one of three experiments with similar results. B, MCPS or GBS-V, each at 40 μg/ml concentration, was used to prestimulate B cells. At 24 h, cells were restimulated with BAFF and IL-4 or APRIL, and IL-4; LPSs with IL-4 or TGF-β were included as positive controls for the IgG and IgA switch respectively. Culture supernatants were harvested after 6 d, and Ig levels were measured by ELISA. Data shown represent an average of three experiments. The p values are derived from the comparison of polysaccharide-pretreated cells with media-pretreated cells. *p < 0.001, **p < 0.01, ***p < 0.05. C, MCPS or GBS-V (both at 40 μg/ml concentration) was used to stimulate splenic B cells for 24 h, and cell death was determined by Annexin V/7-AAD staining in flow cytometry. Data from a representative experiment of three experiments with similar results are shown. D, Percent live (Annexin V−/7-AAD−) and dead cells (Annexin V+/7-AAD−) following MCPS and GBS-V incubation. Average percentages from three experiments are plotted. *p < 0.05 compared with media-treated cells; **p < 0.05, media-treated live cells. E, Concentration of BAFF in the culture supernatants of bone marrow-derived DCs stimulated for 24 h with indicated CPS, with or without IFN-γ. *p < 0.05.
B Streptococcus serotype III CPS is composed of sialic acid residues (Siaα2-3Galβ1-4GlcNAc), and it interacts with Siglec-9 in neutrophils to inhibit neutrophil functions (42). So far, B cells are shown to express two members of the siglec family proteins with inhibitory and apoptotic properties. These are CD22 and Siglec-G. Duong and colleagues (43) have shown that ligands for CD22 and Siglec-G are attached to the TI-2 Ag polyacrylamide-conjugated NP. Ab response against NP is strongly reduced. Although α2–3 linked sialic acid residues also bind to CD22, α2–6 linked sialic acid residues show superior binding. Siglec-G may have broader sialic acid specificity, but it is restricted to B1 cells. Thus, both these sialic acid binding proteins and as yet undefined others can be receptors for bacterial CPSs. Interestingly, Duong and colleagues (43) have shown that conjugation of sialic acid residues to NP-polyacrylamide was much more effective in inhibiting anti-NP Abs than was injecting NP-polyacrylamide and sialic acid residues together. Similarly, we observed that coinjection of mice with MCPS and NP-Ficoll did not lead to a diminished Ab response against NP, suggesting that the B cell suppressive effect of MCPS does not extend to a co-administered TI-2 Ag.

Unlike purified CPS, protein-conjugated forms of CPS vaccines are able to induce high levels of Ab responses (7), suggesting that CPS is unable to inhibit B cells when conjugated to protein carriers. A key difference between unconjugated CPS from the conjugate vaccine is that the conjugate vaccine induces a T cell-dependent immune response to CPS Ags (39, 45). Therefore, it is likely that in conjugate MCPS vaccines T cell interaction with B cells through the costimulatory molecules and possibly cytokines ablates the suppressive effect of MCPS on B cells. Studies in our laboratory are currently underway to understand the role of T cells in blocking the MCPS-mediated inhibition of B cell responses to BAF or APRIL.

Analysis of the effect of MCPS on B cell molecules other than BAF and APRIL receptors revealed that although the expression of MHC-I, CD40, and CD19 did not change, CD80, CD86, and MHC-II were increased following MCPS treatment. Because these molecules are involved in Ag presentation, MCPS may enhance the immune response to protein Ags. Indeed, Lees and colleagues (46) have previously shown in mice that conjugation of BSA with dextran elicited higher BSA Abs than in mice immunized with protein alone. Dextran-induced upregulation of costimulatory molecules may be responsible for the increased Ab response to protein Ag.

Measurement of DC BAFF levels revealed that MCPS and NP-Ficoll differed in their ability to modulate BAFF expression and secretion. Surprisingly, NP-Ficoll was as potent as IFN-g. MCPS and NP-Ficoll differed in their ability to modulate BAFF expression and protein Ag.

At this point, we do not know whether bacterial polysaccharides other than MCPS and GBS-V also have B and DC suppressive properties similar to those of MCPS. Others have reported that increasing concentrations of free CPS in protein-conjugated types 4, 6B, and 14 pneumococcal vaccines result in decreased anti-pneumococcal Ab response in immunized mice (50, 51). Interestingly, although underlying mechanisms were not identified, previous studies with other bacterial CPS vaccines have also shown that CpG ODN was ineffective in enhancing Ab response against polysaccharides from Haemophilus influenzae (52) and S. pneumoniae (53). Furthermore, in similarity to our findings with MCPS and NP-Ficoll, Kovarik and colleagues (46) have shown that whereas CpG ODN enhanced Ab response against trinitrophenylaminoethyl-carboxymethyl-Ficoll it had no adjuvant effect when given with S. pneumoniae CPS Ags (53). Our findings suggest that the suppression of B cells, and possibly DCs, by CPS may be the reason why CpG ODN fails to manifest an adjuvant effect on bacterial polysaccharides. The suppression of B cell responses to BAFF or APRIL through downregulation of TACI and BAFFR expression and by blocking TACI- and BAFFR-mediated signaling is clearly an advantage for microorganisms bearing a polysaccharide capsule because TACI-mediated Ig isotype switch and secretion is an essential mechanism of host resistance to encapsulated bacteria. A clinical observation supporting our hypothesis was recently reported by Xia and colleagues (54), who have detected significantly decreased B cell TACI expression and reduced serum IgG Abs in Cryptococcus meningitis patients. Although a fungus, Cryptococcus is also coated with a polysaccharide capsule that has immunologic and virulence properties similar to those of bacterial CPS (55, 56). Thus, pathogens with a polysaccharide capsule may have evolved to modulate TACI-mediated B cell functions to block humoral immune response. Further studies will reveal whether CPSs from other encapsulated bacteria also manifest a suppressive effect on the BAFF/APRIL system. Nevertheless, these data collectively suggest that weak immunogenicity of MCPS and GBS-V vaccines appears to be due to their suppressive effect on BAFF and B cell responses to BAFF and APRIL stimulation.

Acknowledgments

This article is in memory of Milan S. Blake, who recently died. We thank Manuel Osorio and Roberto De Pascalis (Food and Drug Administration, Center for Biologics Evaluation and Research) for critical reading of the manuscript, Dennis L. Kasper (Harvard Medical School, Boston, MA) for kindly providing purified GBS-V, and Scott Stibitz (Food and Drug Administration, Center for Biologics Evaluation and Research) for the use of the LAS 3000 imaging system.

Disclosures

The authors have no financial conflicts of interest.

References


Supplemental figure 1B

B2 Cells

FO    MZ    NF

CD21

FO    MZ    NF

FO    MZ    NF

FO    MZ    NF

FO    MZ    NF

FO    MZ    NF

FO    MZ    NF

MCPS-40

MCPS-10

MCPS-2.5
Supplemental figure 2

A) **TACI**  **BAFF-R**  **BCMA**

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B) **IgG (ng/ml)**

- **Media**
- **BAFF+IL-4**
- **APRIL+IL-4**
- **LPS+IL-4**

- **IgA (ng/ml)**

- **IgM (ng/ml)**

Legend:
- **PBS**
- **MCPS**
- **NP-Ficoll**

Significance levels:
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Supplemental figure 3
Supplemental figure 4

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Supplemental figure 5

Media   MCPS-40   MCPS-10   MCPS-2.5   NP-40   NP-10   NP-2.5

CD3

B220
Supplemental figure 6

A) Anti-MCPS

B) Anti-NP-Ficoll
Supplemental Table I. Percentages of live (Annexin-V^{neg} and 7-AAD^{neg}) and dead/late apoptotic (Annexin-V^{pos} and 7-AAD^{pos}) B cells following MCPS or NP-Ficoll incubation.

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* p<0.05 was considered as statistically significant and indicated in bold
Each data point is average of three samples.
Supplemental Table II. Percentages of live (Annexin-V \textsuperscript{neg} and 7-AAD \textsuperscript{neg}) and dead/late apoptotic (Annexin-V \textsuperscript{pos} and 7-AAD \textsuperscript{pos}) B cells following MCPS or GBS-V incubation.

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\textsuperscript{I}Average percentages from three samples are indicated.

\textsuperscript{p} \textless 0.05 was considered as statistically significant and indicated in bold.
Supplemental Figure 1. Concentration dependent effect of MCPS on TACI and BAFF-R expression in splenic B cell subsets. MCPS or NP- Ficoll, ranging from 2.5 to 40 µg/ml concentrations, were used to stimulate purified splenic B cells and stained for TACI and BAFF-receptor on B1 (A) and B2 (B) subsets after 24 hrs (open histograms). Equal numbers of cells from each condition were pooled together to stain for isotype control (shaded histogram). One representative experiment out of three independent experiments with similar results is shown.

Supplemental Figure 2. BAFF/APRIL receptor expression and B cell response to BAFF/APRIL 72 hrs after PBS, MCPS or NP-Ficoll injection of mice. Groups of mice were immunized with PBS, MCPS (50 µg) or NP-Ficoll (50 µg). Seventy-two hrs later mice were sacrifice and splenocytes were analyzed. (A) Expression of TACI, BAFF-R and BCMA were determined in flow cytometry on splenic B cells (B220+). Data from one mouse out of 3 mice from each group is shown. (B) Purified B cells were exposed to media, BAFF+IL-4, APRIL+IL-4, LPS+TGF-β or LPS+IL-4 for six days. Culture supernatant IgG, IgA, and IgM were quantified in ELISA. Average data from 3 mice are plotted. *p < 0.05, **p < 0.01, ***p < 0.001.

Supplemental Figure 3. Co-stimulatory molecule requirements for BAFF or APRIL induced B cell proliferation. B cells from Balb/c mice were labeled with CFSE and stimulated with IL-4, anti-IgM and/or BAFF /APRIL as shown above, and analyzed for cell proliferation on day 3. Dead cells were excluded from analysis by PI staining. CFSE Unlabelled cells were used as control for autoflourescence and are shown as shaded histogram. Results from one of two experiments with similar results is shown.

Supplemental Figure 4. Concentration dependent effect of MCPS and NP-Ficoll on B cell survival. Increasing concentrations of (µg/ml) MCPS or NP-Ficoll was used to stimulate splenic B cells for 12, 16, 20, and 24 hrs. Live (Annexin-Vneg and 7-AA-Dneg), apoptotic (Annexin-Vpos and 7-AA-Dneg), and dead (Annexin-Vpos and 7-AA-Dpos) cell percentages were determined by Annexin-V/7-AA-D staining in flow cytometry. Data from a representative experiment out of three experiments with similar results are shown.

Supplemental Figure 5. The effect of MCPS and NP-Ficoll on CD3 and B220 expression. Splenic B cells were stimulated with indicated concentrations of (µg/ml) MCPS or NP-Ficoll for 24 hrs. Percentages of CD3 or B220 expressing cells are gated. Result from one of two experiments with similar results is shown.

Supplemental Figure 6. Antibody response against MCPS or NP-Ficoll in immunized mice. (A) Groups of five Balb/c mice were intraperitoneously immunized with 5 or 40 µg of MCPS alone. Another group of five mice were also immunized with 5 µg of MCPS and 40 µg of CpG ODN. PBS immunized mice (n=5) served as control. Day fifteen serum anti-MCPS IgG antibodies were measured in ELISA. (B) Groups of five mice were immunized with NP-Ficoll (40 µg) alone or NP-Ficoll and MCPS (40 µg each). PBS immunized mice (n=5) served as control. Day fifteen serum anti-NP IgG antibodies were measured in ELISA.