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# Polysaccharide Capsules Equip the Human Symbiont *Bacteroides thetaiotaomicron* to Modulate Immune Responses to a Dominant Antigen in the Intestine

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Bacteria express multiple diverse capsular polysaccharides (CPSs) for protection against environmental and host factors, including the host immune system. Using a mouse TCR transgenic CD4<sup>+</sup> T cell, B $\theta$ OM, that is specific for *B. thetaiotaomicron* and a complete set of single CPS-expressing *B. thetaiotaomicron* strains, we ask whether CPSs can modify the immune responses to specific bacterial Ags. Acapsular *B. thetaiotaomicron*, which lacks all *B. thetaiotaomicron* CPSs, stimulated B $\theta$ OM T cells more strongly than wild-type *B. thetaiotaomicron*. Despite similar levels of B $\theta$ OM Ag expression, many single CPS-expressing *B. thetaiotaomicron* strains were antistimulatory and weakly activated B $\theta$ OM T cells, but a few strains were prostimulatory and strongly activated B $\theta$ OM T cells just as well or better than an acapsular strain. *B. thetaiotaomicron* strains that expressed an antistimulatory CPS blocked Ag delivery to the immune system, which could be rescued by Fc receptor-dependent Ab opsonization. All single CPS-expressing *B. thetaiotaomicron* strains stimulated the innate immune system to skew toward M1 macrophages and release inflammatory cytokines in an MyD88-dependent manner, with antistimulatory CPS activating the innate immune system in a weaker manner than prostimulatory CPS. The expression of antistimulatory versus prostimulatory CPSs on outer membrane vesicles also regulated immune responses. Moreover, antistimulatory and prostimulatory single CPS-expressing *B. thetaiotaomicron* strains regulated the activation of Ag-specific and polyclonal T cells as well as clearance of dominant Ag in vivo. These studies establish that the immune responses to specific bacterial Ags can be modulated by a diverse set of CPSs. *The Journal of Immunology*, 2020, 204: 000–000.

Many human gut bacteria synthesize phase variant capsular polysaccharides (CPSs) on their surface (1). Although many functions of CPSs remain poorly understood, CPSs are known to be immunomodulatory (2). The best-studied immunoregulatory CPS is the polysaccharide A capsule (PSA), which is one of eight CPSs expressed in *Bacteroides fragilis* (3). PSA is zwitterionic, and purified PSA has been shown to modulate host cytokine levels and induce CD4<sup>+</sup> Foxp3<sup>+</sup> regulatory T cells (T<sub>regs</sub>) that protect against a variety of models of autoimmune inflammatory diseases (4–7). In support of these findings, other zwitterionic polysaccharide-encoding bacteria from a diverse taxa induced more IL-10 and T<sub>regs</sub> in human mononuclear cells than those without the zwitterionic polysaccharide motif (8).

Nonzwitterionic capsules have also been shown to exhibit anti-inflammatory properties. For example, neutrally charged cell surface  $\beta$ -glucan/galactan polysaccharides that were purified from *Bifidobacterium bifidum* induced T<sub>regs</sub> in the intestine and suppressed inflammation in a T cell transfer model of colitis (9). Bacterial capsules also enable bacteria to evade adaptive immune responses, as a *Bifidobacterium breve* strain that expressed exopolysaccharide persisted longer than an acapsular strain in wild-type (WT) mice but not in B cell-deficient mice (10). Specifically, capsules can mask bacterial surface Ags from exposure to Ab responses, leading to weaker Ab responses against an exopolysaccharide-coated versus acapsular *B. breve* strain (10). In addition, capsules can promote a mutually beneficial state of

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S. Hsieh designed the project, performed experiments, and wrote the paper. N.T.P. generated the single CPS-expressing *B. thetaiotaomicron* mutants with and without GFP. D.L.D. generated the *B. thetaiotaomicron* Abs. S. Horvath processed the fecal colonization samples. G.S. performed the quick-freeze, deep-etch scanning electron microscopy. N.Z. assisted with peritoneal experiments. B.T.S. and B.Z. captured the india ink images. E.C.M., T.S.S., and P.M.A. guided the overall project design and assisted in data interpretation and writing of the manuscript.

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Abbreviations used in this article: Acap, acapsular *B. thetaiotaomicron* strain; BHI, brain heart infusion; BMDC, bone marrow-derived dendritic cell; BMDM, bone marrow-derived macrophage; cdLN, colon-draining lymph node; CPS, capsular polysaccharide; DC, dendritic cell; iNOS, inducible NO synthase; OM, outer membrane; OMV, OM vesicle; PSA, polysaccharide A capsule; RT, room temperature; T<sub>reg</sub>, regulatory T cell; WT, wild-type.

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tolerance with the innate immune system in the intestine. Both exopolysaccharide from *Bacillus subtilis* and a large soluble polysaccharide released by *Helicobacter hepaticus* have been shown to induce anti-inflammatory M2 macrophages (11, 12). CPSs from other pathogens, such as *Streptococcus pneumoniae*, can also subvert innate immune recognition by impeding complement deposition and bacteria phagocytosis (13, 14). Collectively, these studies reveal the importance of CPSs in microbiota-immune interactions.

A limitation of current studies is that they examined a small subset of capsules expressed by a given bacterium. One recent study interrogated the roles of a complete set of bacterial capsules in a single species by individually expressing all eight capsules in *Bacteroides thetaiotaomicron* (15). The Gram-negative anaerobe *B. thetaiotaomicron* is a model gut symbiont that is a prominent member of the adult human gut microbiota and degrades a variety of diet, host, and microbial glycans (16). *B. thetaiotaomicron* dedicates a substantial portion of its genome—182 genes in eight distinct genomic loci—to CPS production and expresses eight distinct CPSs (CPS1–CPS8), which can be several hundred nanometers thick (17). The WT *B. thetaiotaomicron* strain outcompeted all of the single CPS-expressing strains in C57BL/6 germ-free mice, demonstrating that the ability to express multiple CPSs is advantageous in vivo (15). Higher levels of IgA production correlated with increased abundance of the CPS5-expressing strain, suggesting that CPS5 may promote evasion of adaptive immune responses (15). This study provided key insights into the roles of CPSs in competitive intestinal colonization but only examined how the presence of an immune system influences colonization among single CPS-expressing *B. thetaiotaomicron*. The mechanisms by which a complete set of CPSs affects immune responses remain poorly understood.

Another limitation of current studies is that these studies only investigated how the CPS itself interacts with the immune system. It is unknown if CPSs on bacteria can modulate the immune response to dominant bacterial Ags. Progress in this area has been hampered by the lack of a model system in which a CD4<sup>+</sup> T cell response can be examined for a specific gut symbiont. With our B $\theta$ OM T cell system, which consists of a TCR transgenic CD4<sup>+</sup> T cell that is specific for an outer membrane (OM) protein in *B. thetaiotaomicron*, we are able to control expression of a dominant *B. thetaiotaomicron* Ag. We have previously characterized the B $\theta$ OM T cell system and shown that B $\theta$ OM T cells are specific for BT4295, a SusE/SusF OM lipoprotein contained in one of *B. thetaiotaomicron*'s many polysaccharide utilization loci (18). We have also demonstrated that B $\theta$ OM T cells are functional in vivo as adoptively transferred B $\theta$ OM T cells proliferate and differentiate into T<sub>regs</sub> and effector T cells in the colon, colon-draining lymph node (cdLN), and spleen in healthy mice colonized with *B. thetaiotaomicron* (18).

Using our B $\theta$ OM T cell system (18) and a complete set of single CPS-expressing *B. thetaiotaomicron* (15), we investigated if all CPSs on *B. thetaiotaomicron* modulate the immune responses to a dominant *B. thetaiotaomicron* Ag. An acapsular *B. thetaiotaomicron* strain (Acap) that lacks all *B. thetaiotaomicron* CPSs stimulated B $\theta$ OM T cells more strongly than WT *B. thetaiotaomicron*, suggesting that CPSs may regulate the interactions between specific bacterial Ags and the immune system. We then performed, to our knowledge, the first unbiased examination of how a complete set of CPSs modulate the host's immune responses to dominant Ag using a defined symbiont-specific T cell model system. Although many single CPS-expressing *B. thetaiotaomicron* strains were antistimulatory and suppressed the immune response to a dominant Ag, we identified a few prostimulatory strains that activated

the immune system just as well or better than Acap. We reveal that by altering CPS expression, even at the level of OM vesicles (OMVs), bacteria can control the delivery of dominant Ags and the innate and adaptive immune responses. We show that by directing Ag accessibility, CPSs also enable bacteria to regulate immune responses in vivo, including activation of Ag-specific and polyclonal T cells as well as clearance of dominant Ag. These results demonstrate that the ability to dynamically express diverse CPSs equips bacteria to modulate immune responses to dominant Ags.

## Materials and Methods

### Strains and culture conditions

The single CPS-expressing *B. thetaiotaomicron* strains used in this study have been generated and characterized previously (15, 19). Strains were routinely grown in TYG medium (10 g/l tryptone, 5 g/l yeast extract, 4 g/l D-glucose, 100 mM KH<sub>2</sub>PO<sub>4</sub>, 8.5 mM [NH<sub>4</sub>]<sub>2</sub>SO<sub>4</sub>, 15 mM NaCl, 10  $\mu$ M vitamin K<sub>3</sub>, 2.63  $\mu$ M FeSO<sub>4</sub>·7H<sub>2</sub>O, 0.1 mM MgCl<sub>2</sub>, 1.9  $\mu$ M hematin, 0.2 mM L-histidine, 3.69 nM vitamin B<sub>12</sub>, 413  $\mu$ M L-cysteine, and 7.2  $\mu$ M CaCl<sub>2</sub>·2H<sub>2</sub>O), minimal medium (20 g/l tryptone, 10 g/l yeast extract, 5 g/l D-glucose, 8.25 mM L-cysteine, 78  $\mu$ M MgSO<sub>4</sub>·7H<sub>2</sub>O, 294  $\mu$ M KH<sub>2</sub>PO<sub>4</sub>, 230  $\mu$ M K<sub>2</sub>HPO<sub>4</sub>, 1.4 mM NaCl, 7.9  $\mu$ M hemin [hematin], 4  $\mu$ M resazurin, 24  $\mu$ M NaHCO<sub>3</sub>, 68  $\mu$ M CaCl<sub>2</sub>·2H<sub>2</sub>O), or on brain heart infusion (BHI) agar plates containing 10% horse blood (Quad Five, Rygate, MT) with gentamicin (200  $\mu$ g/ml) (BHI-blood-gentamicin plates). Bacteria were routinely grown at 37°C in a Bactron IV Anaerobic Chamber (Sheldon Manufacturing) or in a BD GasPak EZ Small Incubation Container with BD GasPak EZ Anaerobe Container System Sachets with Indicator. *B. thetaiotaomicron* has a different core oligosaccharide in place of the O Ag-repeating unit found in LPS and makes lipooligosaccharide, which can also stimulate innate immunity. Each of the single CPS-expressing strains have been examined for lipooligosaccharide expression, and they have been shown to express the same levels (20).

For GFP labeling of the CPS-producing strains, a plasmid encoding for GFP expression was integrated into the chromosome of each strain as previously described (21, 22). Briefly, *Escherichia coli* S17-1  $\lambda$  pir containing the pWW3452 vector (22) was grown to midlog phase in Luria-Bertani medium containing ampicillin (300  $\mu$ g/ml), and CPS-producing strains were grown anaerobically to midlog phase in TYG medium. Cells were washed and combined at an ~1:1 ratio before plating on a BHI agar plate containing 10% horse blood (BHI-blood plate). After 1 d of aerobic incubation at 37°C, the cell mass was scraped off the plate, and dilutions were plated on BHI-blood plates containing 200  $\mu$ g/ml gentamicin and 25  $\mu$ g/ml erythromycin. After 2 d of anaerobic growth at 37°C, colonies were restreaked on the same medium and grown anaerobically for an additional 2 d. Isolated colonies were inoculated into TYG medium for overnight growth, and resulting cultures were confirmed to fluoresce when exposed to UV light.

### Mouse experiments

All experimental procedures were performed under approval by Washington University's Animal Studies Committee. All non-germ-free mice were housed in an enhanced specific pathogen-free facility. B $\theta$ OM transgenic mice on the Rag1<sup>-/-</sup> background were maintained by breeding to a nontransgenic Rag1<sup>-/-</sup> mouse (18). Mice were randomly assigned into groups. Animal numbers for each experiment were chosen based on the minimum numbers required in previous studies to observe significant changes in the readouts measured in each experiment. Mice aged 6–12 wk old of various genders were used, dependent on availability, and none of these were involved in any previous experiments. Groups within an experiment were age and gender matched to the greatest extent possible.

### Functional in vitro T cell stimulation assay

The B $\theta$ OM T cell stimulation assay was performed as previously described (18). Bone marrow cells isolated from the tibia and femurs of C57BL/6, MyD88<sup>-/-</sup>, or FeR $\gamma$ <sup>-/-</sup> mice were cultured in M-CSF for 7 d to differentiate into bone marrow-derived macrophages (BMDM). BMDM were stimulated with IFN- $\gamma$  at 2000 U/ml in I-10 media (IMDM, 10% FBS, glutamine, and gentamicin) and plated on a 96-well plate at 1  $\times$  10<sup>5</sup> cells per well. The cells were washed with PBS 24 h later and kept in 100  $\mu$ l of fresh I-10 medium without IFN- $\gamma$ . For bone marrow-derived dendritic cells (BMDCs), bone marrow cells were cultured in 10% FBS tyrosine kinase 3 (Flt3L) ligand supernatant and 5 ng/ml GM-CSF for 7 d and plated on a 96-well plate at 1  $\times$  10<sup>5</sup> cells per well. When splenic



CD11c<sup>+</sup> dendritic cells (DCs) were used, splenocytes were enriched for CD11c<sup>+</sup> DCs using MACS CD11c Microbeads and plated on a 96-well plate at  $1 \times 10^5$  cells per well. A total of  $5 \times 10^5$  cells isolated from the spleen and lymph nodes of B0OM transgenic mice or  $1 \times 10^7$  B0OM T cell hybridomas or B0OM T cell blasts with or without 10  $\mu\text{g}/\text{ml}$  of *B. thetaiotaomicron* Ab were then added per well in 50  $\mu\text{l}$  plus 50  $\mu\text{l}$  of half log dilutions of *B. thetaiotaomicron* strains or OMVs. Single CPS-expressing *B. thetaiotaomicron* strains were grown in a 5 ml TYG culture at 37°C overnight and then reinoculated at 1:50 and grown to midlog phase. Cultures were washed once and resuspended in PBS prior to adding to the assay. Twenty-four hours later, the supernatant containing the T cells was transferred to a fresh 96-well plate and spun down at 1200 rpm. The cells were washed with FACS buffer and stained for CD4, CD45.1, and CD69. All samples were run on a BD FACSCanto and analyzed using FlowJo.

#### Generation of B0OM T cell hybridomas

B0OM T cell hybridomas were generated as previously described (18). A 2-ml *B. thetaiotaomicron* (BT5482 strain) overnight culture was spun down and pelleted, washed one time in PBS, resuspended in 1 ml PBS, and used as an Ag source for immunization. An equal volume of *B. thetaiotaomicron*/PBS mixture was emulsified with IFA, and 50  $\mu\text{l}$  was injected into the rear footpads of C57BL/6 mice (male). Ten days later the draining popliteal lymph nodes were removed, and a single-cell suspension of  $2 \times 10^7$  cells was cultured in 7 ml of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM HEPES, 1 mM Sodium Pyruvate,  $5 \times 10^{-5}$  M 2-ME, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 14  $\mu\text{l}$  of the *B. thetaiotaomicron*/PBS Ag source (1:500 dilution) for 3 d. Blasting B0OM T cells were harvested by centrifugation and fused to the BW5147 $\alpha$ - $\beta$  T cell hybridoma fusion partner using a standard protocol (23).

#### Generation of B0OM T cell blasts

BMDM were stimulated with IFN- $\gamma$  at 2000 U/ml in I-10 media (see above) and plated on a 24-well plate at  $5 \times 10^5$  cells per well. The cells were washed with PBS 24 h later and kept in 1 ml I-10 media. One micromolar B0OM peptide and  $2 \times 10^5$  isolated CD4<sup>+</sup> B0OM T cells were added per well in 0.5 ml with 50 U IL-2 and incubated for 6 d at 37°C. B0OM T cell blasts were then harvested from the supernatant and added to the in vitro macrophage cell assay.

#### Quantitative ELISA for BT4295

BT4295 protein levels in the single CPS-expressing *B. thetaiotaomicron* strains were measured by quantitative ELISA as previously described (18). Single CPS-expressing *B. thetaiotaomicron* strains were grown in a 5-ml TYG culture at 37°C overnight and then reinoculated at 1:50 and grown to midlog phase. Samples were obtained from equivalent numbers of *B. thetaiotaomicron* from OD<sub>600</sub>-measured cultures. Bacteria were lysed in 100 mM CHAPS detergent and incubated with agitation for 1 h at room temperature (RT) and stored at 4°C overnight. An Immulon T20 ELISA Plate was coated overnight with purified anti-BT4295 Ab ERC11 in carbonate coating buffer (5  $\mu\text{g}/\text{ml}$  [pH value of 9.6]) at 4°C, washed, blocked with buffer (PBS with 0.5% BSA and 0.1% Tween 20) for 1 h at RT. Plates were washed and samples were added for 2 h at RT, washed again, and then the anti-BT4295 Ab biotin-4E9 (5  $\mu\text{g}/\text{ml}$ ) was added for 1 h at RT. Plates were washed again, and a 1:5000 dilution for streptavidin HRP was added for 30 min at RT. Plates were washed, and 100  $\mu\text{l}$  of One-Step Ultra TMB Substrate was added to each well and A<sub>450</sub> was determined. Unknown sample concentrations were quantified by comparison with a standard curve of rBT4295 protein performed in the same ELISA using GraphPad Prism Software.

#### Live-cell phagocytosis assay

BMDM were plated on a 96-well glass bottom plate with no. 1.5 cover glass and a black frame (Cellvis) at  $1 \times 10^5$  cells per well in I-10 media overnight. The cells were stained with 5  $\mu\text{M}$  CellTracker Orange CMTMR Dye at 37°C for 30 min, then washed. Cells were washed and incubated in 150  $\mu\text{l}$  I-10 media. Fifty microliters of a 1:31 dilution of single CPS-expressing *B. thetaiotaomicron* strains that endogenously expressed GFP with or without 10  $\mu\text{g}/\text{ml}$  of *B. thetaiotaomicron* Ab was added to each well. *B. thetaiotaomicron* strains expressing a single CPS and GFP were grown in a 2-ml TYG culture at 37°C overnight to mid log phase. Cultures were washed once and resuspended in PBS prior to adding to the assay. Four hours later, images were acquired in the same z-plane using an Olympus IX70 microscope with a 100 $\times$ /1.4NA oil objective, a Yokogawa spinning-disk confocal scanning unit, and a Hamamatsu Orca Flash4 CMOS Camera. Cells were maintained at 37°C with 5.0% CO<sub>2</sub> in

a Tokai Hit humidified chamber. Images were acquired with NIS-Elements Advanced Research Software, and the number of bacteria uptaken per BMDM was measured manually using Imaris Software. A total of 150 individual cells were measured for each bacterial strain analyzed with 50 cells measured per experiment in three experiments.

#### Microscopic imaging of capsules by india ink staining

*B. thetaiotaomicron* capsule thickness was measured by india ink staining as described previously (17). Samples were taken from fresh cultures of *B. thetaiotaomicron* strains grown to late exponential phase in TYG media. A 10  $\mu\text{l}$  aliquot of the fresh culture was mixed on a slide with an equivalent amount of india ink, and a cover glass was added. Cells were imaged with a Leica SP8 Confocal Laser Scanning Fluorescent Microscope, and capsule thickness was measured using Imaris and Fiji Software. A total of 200 individual cells were measured for each bacterial strain analyzed. Four measurements were made per cell and averaged to generate a single average capsule width.

#### Microscopic imaging of capsules by scanning electron microscopy

Samples for quick-freeze, deep-etch scanning electron microscopy were obtained as described previously (17). Briefly, 5 ml cultures of *B. thetaiotaomicron* strains grown to late exponential phase in TYG media were gently centrifuged. A 3- $\mu\text{l}$  droplet of the resulting bacterial pellet was layered onto a thin slice of aldehyde-fixed boiled egg white (used for support during freezing) and frozen by abrupt application of the sample against a liquid helium cooled copper block with a Cryopress freezing machine. Frozen samples were transferred to a liquid nitrogen-cooled Leica Ace 900 Freeze Etching Unit, fractured, and “deep-etched” for 2 min at  $-104^\circ\text{C}$ . Specimens were then rotary replicated with  $\sim 3.5$  nm of platinum applied from an angle of 20° above the horizontal, and “backed” with an  $\sim 8.5$  nm film of pure carbon deposited from an 85° angle. Replicas were picked up on hexagonal 100-mesh Formvar-carbon coated microscope grids and imaged in a JEOL 1400 Microscope with an attached Advanced Microscopy Techniques digital camera.

#### Generation of *B. thetaiotaomicron*-specific Abs

C57BL/6 mice were immunized with killed CPS1 and boosted, then splenic B cells were fused with P3Ag8.6.5.3 myeloma cells to create hybridomas (24).

#### In vitro macrophage skewing and cytokine release assay

BMDM or BMDCs were plated on a 96-well plate at  $1 \times 10^5$  cells per well in I-10 media for 1 h at 37°C. Fifty microliters of half log dilutions of *B. thetaiotaomicron* strains or OMVs were added. Single CPS-expressing *B. thetaiotaomicron* strains were grown in a 5-ml TYG culture at 37°C overnight and then reinoculated at 1:50 and grown to midlog phase. Cultures were washed once and resuspended in PBS prior to adding to the assay. Twenty hours later, the supernatant was transferred to a fresh 96-well plate and stained for IL-2, IL-4, IL-6, IL-10, TNF, IFN- $\gamma$ , and IL-17A using a BD Cytometric Bead Array Mouse T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 Cytokine Kit. The BMDMs were washed once with PBS, treated with trypsin, transferred to a fresh 96-well plate, surface stained with CD11b, CD86, PD-L1 for 20 min at 4°C, fixed and permeabilized with a Foxp3 transcription factor staining buffer set for 1 h at 4°C, and intracellularly stained with inducible NO synthase (iNOS) for 30 min 4°C. All samples were run on a BD FACSCanto and analyzed using FlowJo.

#### Generation of OMVs

Two-liter *B. thetaiotaomicron*-TYG cultures were grown overnight at 37°C in an anaerobic chamber and spun down at 6000 rpm for 15 min in a Beckman floor centrifuge (10.1 rotor) (25). The supernatant was filtered through a 0.45- $\mu\text{m}$  filter. The filtered supernatant was then spun down in a Beckman centrifuge (25.5 rotor) at  $38,500 \times g$  for 1 h in polypropylene 40-ml tubes. The supernatant was poured off, and the pellet was washed once with PBS by spinning in a Beckman centrifuge (25.5 rotor) at  $38,500 \times g$  for 1 h. The OMV pellets were suspended in PBS and pooled. The OMV concentration of OMVs was determined using the Bradford protein assay.

#### Extraction of purified CPS

Purified CPS1 was extracted by the hot water phenol method (17). Five milliliters of overnight cultures grown in TYG medium were inoculated into 250 ml of minimal medium with 0.5% glucose for 2 d, centrifuged, flash frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Cells were resuspended in 100 ml of hot water and then an equal volume of phenol was added.

The mixture was stirred for 1 h at 65°C, cooled at 4°C overnight, and centrifuged. The aqueous phase was dialyzed against deionized distilled water (12–14 kDa cutoff) and then lyophilized to dryness. Preparations were resuspended in a buffer containing 20 mM Tris HCl (pH 7.4), 2.5 mM MgCl<sub>2</sub>, and 0.5 mM CaCl<sub>2</sub>, and RNaseA and DNase I were added before samples were rotated gently overnight at 37°C. Proteinase K was then added and samples were incubated overnight at 65°C. After the addition of phenol, samples were vortexed and centrifuged. The aqueous layer was again dialyzed and then lyophilized to dryness.

#### B $\theta$ OM and polyclonal T cell stimulation in vivo

Rag1<sup>-/-</sup> mice were placed on 0.66 mg/ml ciprofloxacin, 2.5 mg/ml metronidazole, and 20 mg/ml sugar-sweetened grape Kool-Aid Mix in the drinking water at 3 wk of age for 2–3 wk. Mice were then taken off the antibiotic and given regular water for 4 d before gavage with 100  $\mu$ l of *B. thetaiotaomicron* strains at  $1 \times 10^8$  CFUs per ml. *B. thetaiotaomicron* strains had been grown anaerobically from single isolates in TYG medium at 37°C for 24 h (26). Each culture was mixed with sterile PBS and glycerol to a final concentration of 20% glycerol and frozen at -80°C in single-use aliquots. Three days after gavage, Rag1<sup>-/-</sup> mice were injected retro-orbitally with  $2 \times 10^5$  B $\theta$ OM T cells isolated from the peripheral lymph nodes (axially, brachial, inguinal, superficial and deep cervical, lumbar, and caudal), mesenteric lymph nodes, and spleen. Cells were enriched for CD4<sup>+</sup> T cells by negative selection using a homemade mixture of Abs (anti-mouse Ter-119, CD11c, CD11b, CD8 $\alpha$ , CD19, CD45R/B220, CD49b, and CD24) and antibiotin microbeads. Fecal pellets were obtained on days 0, 8, and 9 to determine colonization. Seven days after T cell transfer, mice were euthanized, and leukocytes were isolated from the lamina propria. For germ-free experiments, C57BL/6 germ-free mice were gavaged with 100  $\mu$ l of *B. thetaiotaomicron* strains at  $1 \times 10^8$  CFUs per ml, and leukocytes were also isolated from the lamina propria 10 d after gavage. Colons and cecums were flushed with PBS to remove colonic contents, and fat tissue was pulled off. Intestines were cut longitudinally and laterally into freshly made 20 ml of predigestion solution (1 $\times$  HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup>, 10 mM HEPES, 5 mM EDTA, 5% FBS, and 1 mM DTT). Samples were incubated for 20 min at 37°C while shaking at 220 rpm and then vortexed for 10 s. Tissue was transferred to 20 ml of fresh predigestion solution and incubated while shaking for another 20 min. After vortexing for 10 s, tissue was transferred to a fresh tube containing 20 ml of 1 $\times$  HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> and 10 mM HEPES and incubated while shaking for 20 min. Tissue was cut into small pieces using fine scissors and digested for 30 min in freshly made digestion solution (1 $\times$  HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup>, 10 mM HEPES, 5% FBS, 10 mg/ml of DNase, and 5 mg/ml of collagenase) while shaking at 200 rpm. Tissue was mechanically disrupted using a gentleMACS Octo Dissociator (Miltenyi) using the program m\_intestine\_01. Samples were filtered through a 100- $\mu$ m filter. The cdLN and spleen were removed and processed into single-cell suspensions using Eppendorf tube lids and filtered through a 70- $\mu$ m filter. All samples were counted, stained with anti-mouse CD4, CD45.1, CD44, CD62L, Foxp3, IFN- $\gamma$ , and/or IL-17A, and run on a BD FACSCanto.

#### Bacterial clearance in vivo

A total of  $1 \times 10^6$  CPS1 or CPS8 was injected i.p into C57BL/6 mice. Peritoneal fluid was collected 0, 1, 2, 4, 6, or 8 h after injection. Peritoneal fluid was plated on BHI-blood plates with 200  $\mu$ g/ml gentamicin at 37°C in an anaerobic chamber for 2 d to determine the CFU.

#### Quantification and statistical analysis

None of the investigators were blinded to sample identity during any of the described experiments, and no samples or measurements were excluded from analysis. For each experiment, the total number of biological replicates (*n*) is indicated in the corresponding legend along with a description of what is considered a replicate (mice, replicate cultures, Ab titers, etc.) as well as the number of experiments conducted. FlowJo Software v10.4.2 (FlowJo, LLC, Ashland, OR) was used to analyze all flow cytometry experiments. Statistical analyses were performed in Prism Software v7 (GraphPad Software, La Jolla, CA). In the live-cell imaging experiments, NIS-Elements Advanced Research Software (Nikon, Tokyo, Japan) was used to capture images, and uptake of *B. thetaiotaomicron* was counted manually in Imaris Software v8.4 (Oxford Instruments, Zurich, Switzerland). In the confocal imaging experiments, Leica Application Suite X Software (Leica Microsystems, Wetzlar, Germany) was used to capture images, and the thickness of the *B. thetaiotaomicron* capsule was measured in Fiji (Image J). When data did not generally follow a normal distribution, nonparametric tests (Mann–Whitney) were used to determine statistical significance. Unless otherwise indicated, statistical significance

is indicated as follows: \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001, \*\*\*\**p* < 0.0001. Statistical tests used are indicated in each figure legend.

## Results

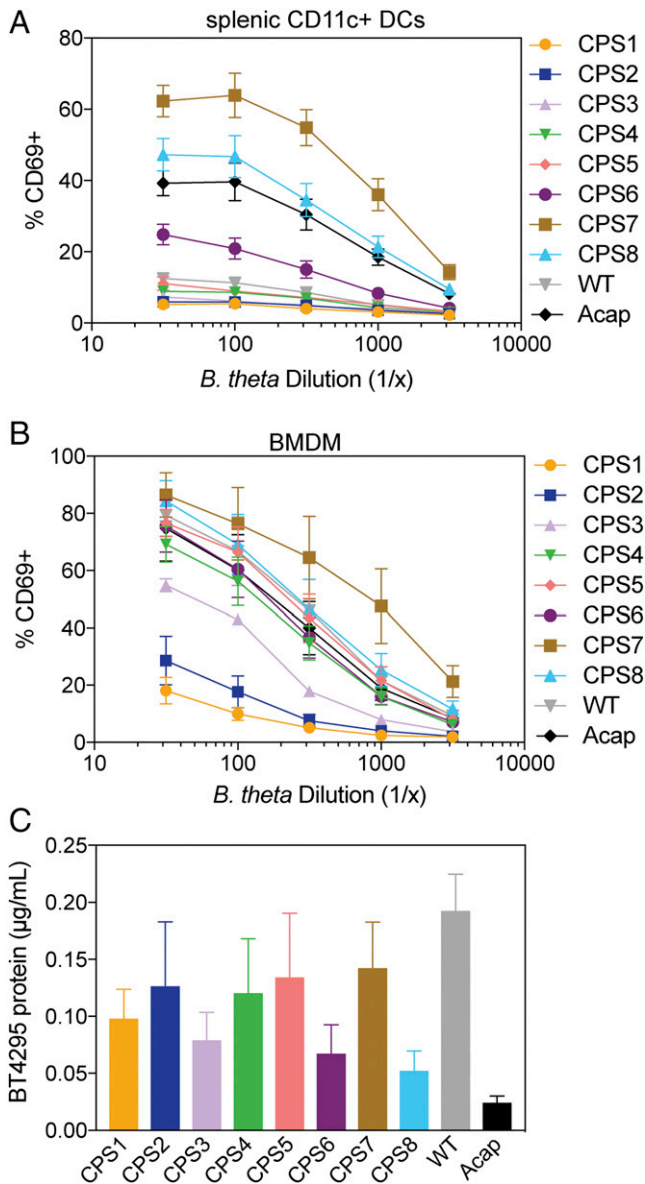
### *Most, but not all, single CPS-expressing B. thetaiotaomicron strains are antistimulatory and suppress Ag-specific adaptive immune responses*

To determine if CPSs can regulate the immune response to dominant Ags, we first compared the ability of *B. thetaiotaomicron* WT and Acap, which lacks all *B. thetaiotaomicron* CPSs, to stimulate B $\theta$ OM CD4<sup>+</sup> T cells. Using primary CD11c<sup>+</sup> splenic DCs as the APCs, we found that Acap stimulated B $\theta$ OM T cells more strongly than WT (Fig. 1A). We hypothesized that all CPSs on *B. thetaiotaomicron* suppress the immune response to a dominant Ag and examined the ability of a complete set of eight single CPS-expressing *B. thetaiotaomicron* strains to stimulate B $\theta$ OM T cells. Most single CPS-expressing *B. thetaiotaomicron* strains were antistimulatory and weakly activated B $\theta$ OM T cells, but one single CPS-expressing *B. thetaiotaomicron* strain, CPS8, was prostimulatory and activated B $\theta$ OM T cells similarly or even better than Acap in the presence of primary CD11c<sup>+</sup> splenic DCs (Fig. 1A). The CPS7 strain was also strongly stimulated, but previously, it has been shown to very weakly express a capsule, and thus acted essentially as an Acap (15). To interrogate further the roles of CPSs on *B. thetaiotaomicron* to modulate the immune response to specific bacterial Ags, we chose to focus on one antistimulatory CPS, CPS1, and one prostimulatory CPS, CPS8. Using these two model single CPS-expressing *B. thetaiotaomicron* strains, we found that these differences in B $\theta$ OM T cell stimulation were also present when BMDMs were used as the APCs (Fig. 1B), extending our finding that CPSs on *B. thetaiotaomicron* differentially alter T cell responses to dominant Ags. Protein levels of the B $\theta$ OM T cell Ag BT4295 were not statistically different between all of the single CPS-expressing *B. thetaiotaomicron* strains, ruling out the possibility that the differences in B $\theta$ OM T cell stimulation were due to differences in Ag expression (Fig. 1C). These findings demonstrate that bacteria can modulate adaptive immune responses to their dominant Ags by altering CPS expression.

### *Antistimulatory CPSs on B. thetaiotaomicron block the immune system's access to a dominant Ag*

We hypothesized that antistimulatory single CPS-expressing *B. thetaiotaomicron* strains suppress Ag-specific CD4<sup>+</sup> T cell responses because the CPSs either block Ag uptake, Ag processing, or Ag presentation. We first used B $\theta$ OM T cell blasts, which are B $\theta$ OM T cells that have been previously activated in vitro, to examine if antistimulatory CPSs on *B. thetaiotaomicron* impede Ag presentation. B $\theta$ OM T cell blasts have already upregulated costimulatory molecules, enabling us to test if CPSs on *B. thetaiotaomicron* block Ag presentation by preventing critical costimulatory molecules from being expressed on the APC surface. T stimulation assays with B $\theta$ OM T cell blasts recapitulated our results with primary B $\theta$ OM T cells. Prostimulatory single CPS-expressing *B. thetaiotaomicron*, including CPS8 and Acap, strongly activated B $\theta$ OM T cell blasts. Antistimulatory single CPS-expressing *B. thetaiotaomicron*, including CPS1, weakly activated B $\theta$ OM T cell blasts, suggesting that antistimulatory CPSs on *B. thetaiotaomicron* do not inhibit the Ag presentation pathway (Fig. 2A, Supplemental Fig. 1A). Using B $\theta$ OM T cell hybridomas, which only require TCR stimulation to be activated, we next investigated if *B. thetaiotaomicron* expressing antistimulatory CPSs block Ag processing. B $\theta$ OM T cell hybridomas only recognize MHC class II loaded with peptide and do not need any costimulation, enabling us to test if CPSs on *B. thetaiotaomicron* prevent peptide–MHC class II complexes from being processed and





**FIGURE 1.** Most *B. thetaiotaomicron* (*B. theta*) CPSs are antistimulatory and prevent B0OM T cell recognition of a dominant Ag in *B. thetaiotaomicron*. (A) Percentage of CD69-expressing B0OM T cells after a 24 h culture with CD11c<sup>+</sup> splenic DCs loaded with *B. thetaiotaomicron* CPS1–8, WT, or Acap ( $n = 6$ , three experiments). (B) Percentage of CD69-expressing B0OM T cells after a 24 h culture with CPS1–8, WT, or Acap loaded onto BMDM ( $n = 6$ , three experiments). (C) Concentration in microgram per milliliter of BT4295 protein expressed in CPS–8, WT, and Acap ( $n = 4$ , four experiments).

expressed on the APC surface. B0OM T cell hybridomas were also strongly activated by *B. thetaiotaomicron* expressing prostimulatory CPSs but weakly activated by *B. thetaiotaomicron* with antistimulatory CPSs (Fig. 2B, Supplemental Fig. 1B), demonstrating that antistimulatory CPSs on *B. thetaiotaomicron* do not block the Ag processing pathway. To determine if antistimulatory single CPS-expressing *B. thetaiotaomicron* strains inhibit the uptake of dominant Ags by APCs, we cultured labeled BMDMs with *B. thetaiotaomicron* strains that expressed a single CPS and GFP and performed live-cell imaging. *B. thetaiotaomicron* strains with prostimulatory CPSs, such as CPS8 and Acap, were highly phagocytosed, whereas *B. thetaiotaomicron* strains with antistimulatory CPSs, such as CPS1, were poorly phagocytosed (Fig. 2C, 2D, Supplemental Fig. 1C, 1D),

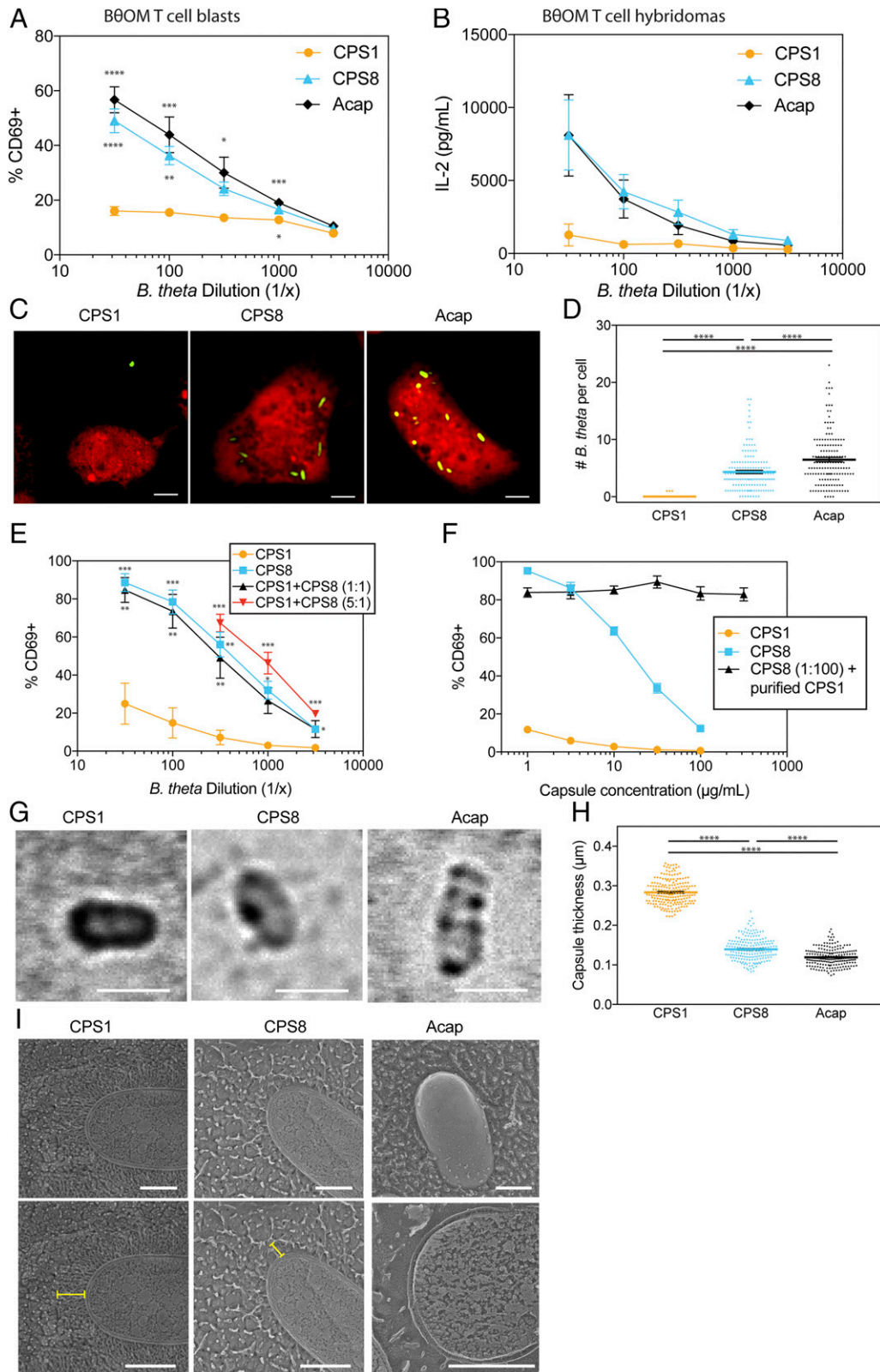
revealing that *B. thetaiotaomicron* can control the delivery of dominant Ag to the immune system.

Given that CPSs are phase variant and can be shed into the environment (2), we wondered if single CPS-expressing *B. thetaiotaomicron* strains can also act in *trans*. Mixing the prostimulatory CPS8-expressing *B. thetaiotaomicron* strain with either the antistimulatory CPS1-expressing *B. thetaiotaomicron* strain or purified CPS1 capsule failed to decrease B0OM T cell stimulation (Fig. 2E, 2F), suggesting that CPS1 cannot inhibit CPS8 on *B. thetaiotaomicron* in *trans*. Live-cell imaging of BMDM treated with unlabeled CPS1-expressing *B. thetaiotaomicron* as well as *B. thetaiotaomicron* that expressed CPS8 and GFP revealed that CPS8-expressing *B. thetaiotaomicron* was still readily uptaken by BMDM in the presence of CPS1-expressing *B. thetaiotaomicron* (data not shown). To rule out the possibility that CPS8-expressing *B. thetaiotaomicron* could be enhancing the uptake and B0OM T cell stimulation of CPS1-expressing *B. thetaiotaomicron*, we also incubated BMDMs with unlabeled CPS8-expressing *B. thetaiotaomicron* and *B. thetaiotaomicron* that expressed CPS1 and GFP and found no increase in uptake of CPS1-expressing *B. thetaiotaomicron* (data not shown). These findings definitively demonstrate that the antistimulatory CPS1 and prostimulatory CPS8 do not function in *trans* to alter *B. thetaiotaomicron* uptake or B0OM T cell stimulation.

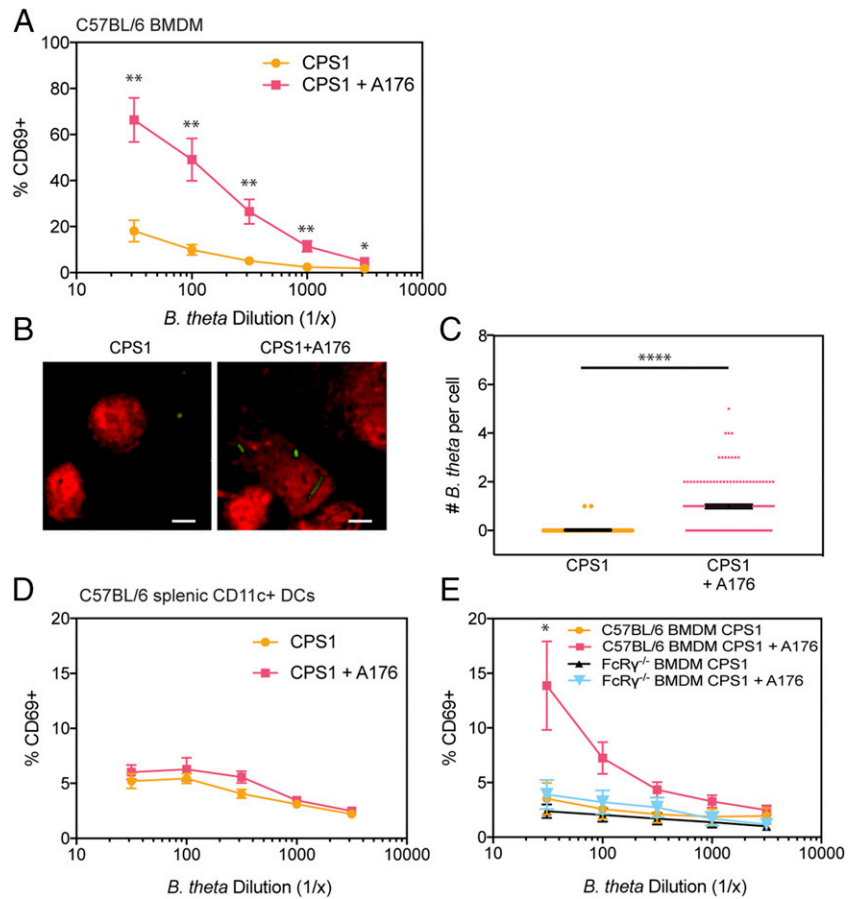
We then used india ink staining and quick-freeze, deep-etch scanning electron microscopy to examine if the thicknesses of the CPSs contribute to their anti- and prostimulatory properties. Both india ink staining and scanning electron microscopy showed that CPS1 is thicker than CPS8, suggesting that thicker CPSs, along with known differences in sugar composition (15), may play a role in impeding APC phagocytosis (Fig. 2G–I, Supplemental Fig. 1E–G). Taken together, these studies demonstrate that by changing the CPS expressed, bacteria can control the ability of the innate and adaptive system to access dominant Ags.

#### *Fc receptor–dependent Ab optimization can rescue immune access to dominant Ag on B. thetaiotaomicron with antistimulatory CPSs*

Because Ab opsonization has been shown to increase bacterial uptake (13), we hypothesized that Ab opsonization could overcome the ability of antistimulatory CPSs to block access to dominant Ags. We generated the A176 Ab, which recognize *B. thetaiotaomicron* and is IgG2a,  $\kappa$  (Supplemental Fig. 2A). Because A176 binds to all single CPS-expressing *B. thetaiotaomicron*, A176 recognizes an Ag that is present in all *B. thetaiotaomicron* strains and is not unique to the CPS1 capsule. Adding A176 to our B0OM T cell stimulation assay allowed CPS1-expressing *B. thetaiotaomicron* to now strongly activate B0OM T cells (Fig. 3A, Supplemental Fig. 2B). Live-cell imaging demonstrated that CPS1-expressing *B. thetaiotaomicron*'s enhanced B0OM T cell activation in the presence of A176 was due to A176's ability to increase the uptake of CPS1-expressing *B. thetaiotaomicron* in BMDMs (Fig. 3B, 3C). The addition of A176 had no effect on B0OM T cell activation by CPS1-expressing *B. thetaiotaomicron* when splenic CD11c<sup>+</sup> DCs were used as the APCs, suggesting that A176 likely increased CPS1 uptake through a receptor present on BMDM but not splenic CD11c<sup>+</sup> DCs (Fig. 3D). Because Abs have been shown to increase bacterial uptake through Fc receptors, we tested the mechanism of A176's enhanced bacterial uptake with Fc $\gamma$ <sup>-/-</sup> BMDM, which lack the Fc receptor common  $\gamma$ -chain and do not express any of the activating mouse Fc $\gamma$ Rs (Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\gamma$ RIV). A176 no longer increased CPS1-expressing *B. thetaiotaomicron*'s B0OM T cell stimulation in the presence of Fc $\gamma$ <sup>-/-</sup> BMDM, demonstrating that A176 increases CPS1 uptake and B0OM T cell stimulation by Ab opsonization through



**FIGURE 2.** Antistimulatory CPSs block uptake of a dominant Ag in *B. thetaiotaomicron* (*B. theta*), partly because of capsule thickness. **(A)** Percentage of CD69-expressing B $\theta$ OM T blasts after a 24 h culture with BMDM loaded with CPS1, CPS8, or Acap ( $n = 6$ , three experiments). **(B)** IL-2 levels in picogram per milliliter produced by B $\theta$ OM T cell hybridomas after a 24-h culture with BMDM loaded with CPS1, CPS8, or Acap ( $n = 6$ , three experiments). **(C)** Representative live-cell images and **(D)** quantification of uptaken GFP-labeled CPS1, CPS8, or Acap per BMDM after a 4 h culture ( $n = 150$ , three experiments). Scale bar, 5  $\mu\text{m}$ . **(E)** Percentage of CD69-expressing B $\theta$ OM T cells after a 24-h culture with BMDM loaded with CPS1, CPS8, or CPS1 and CPS8 in a 1:1 or 5:1 ratio ( $n = 6$ , three experiments).  $***p = 0.0207$ . **(F)** Percentage of CD69-expressing B $\theta$ OM T cells after a 24 h culture with BMDM loaded with CPS1, CPS8, or a 1:100 dilution of CPS8 and varying concentrations of purified CPS1 ( $n = 3$ , three experiments). **(G)** Representative images and **(H)** quantification of capsule thickness of CPS1, CPS8, and Acap stained with india ink ( $n = 200$ , three experiments). Scale bar, 2  $\mu\text{m}$ . **(I)** Quick-freeze, deep-etch scanning electron micrographs of CPS1, CPS8, and Acap *B. thetaiotaomicron*. The yellow line indicates the width of each capsule. Scale bar, 0.5  $\mu\text{m}$ . Data represent mean  $\pm$  SEM. One-way ANOVA analysis.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ .



**FIGURE 3.** Blockade of dominant Ag delivery by antistimulatory CPSs can be overcome by Ab opsonization through FcR $\gamma$ . **(A)** Percentage of CD69-expressing B $\theta$ OM T cells after a 24 h culture with CPS1 only or CPS1 + A176 Ab loaded onto BMDM ( $n = 6$ , three experiments). **(B)** Representative live-cell images and **(C)** quantification of uptaken GFP-labeled CPS1 per BMDM after a 4-h culture with or without A176 Ab ( $n = 150$ , three experiments). Scale bar, 5  $\mu$ m. **(D and E)** Percentage of CD69-expressing B $\theta$ OM T cells after a 24-h culture with CPS1 and A176 Ab loaded onto **(D)** CD11c<sup>+</sup> splenic DCs ( $n = 6$ , three experiments) or **(E)** C57BL/6 or FcR $\gamma$ <sup>-/-</sup> BMDM ( $n = 6$ , three experiments). Data represent mean  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ , Student  $t$  test.

Fc receptors (Fig. 3E). Thus, the immune system can overcome antistimulatory CPSs and restore access to dominant Ags through Fc receptor-dependent Ab opsonization.

*All single CPS-expressing B. thetaiotaomicron stimulate the innate immune system through MyD88, but antistimulatory CPSs on B. thetaiotaomicron decrease innate immune system activation*

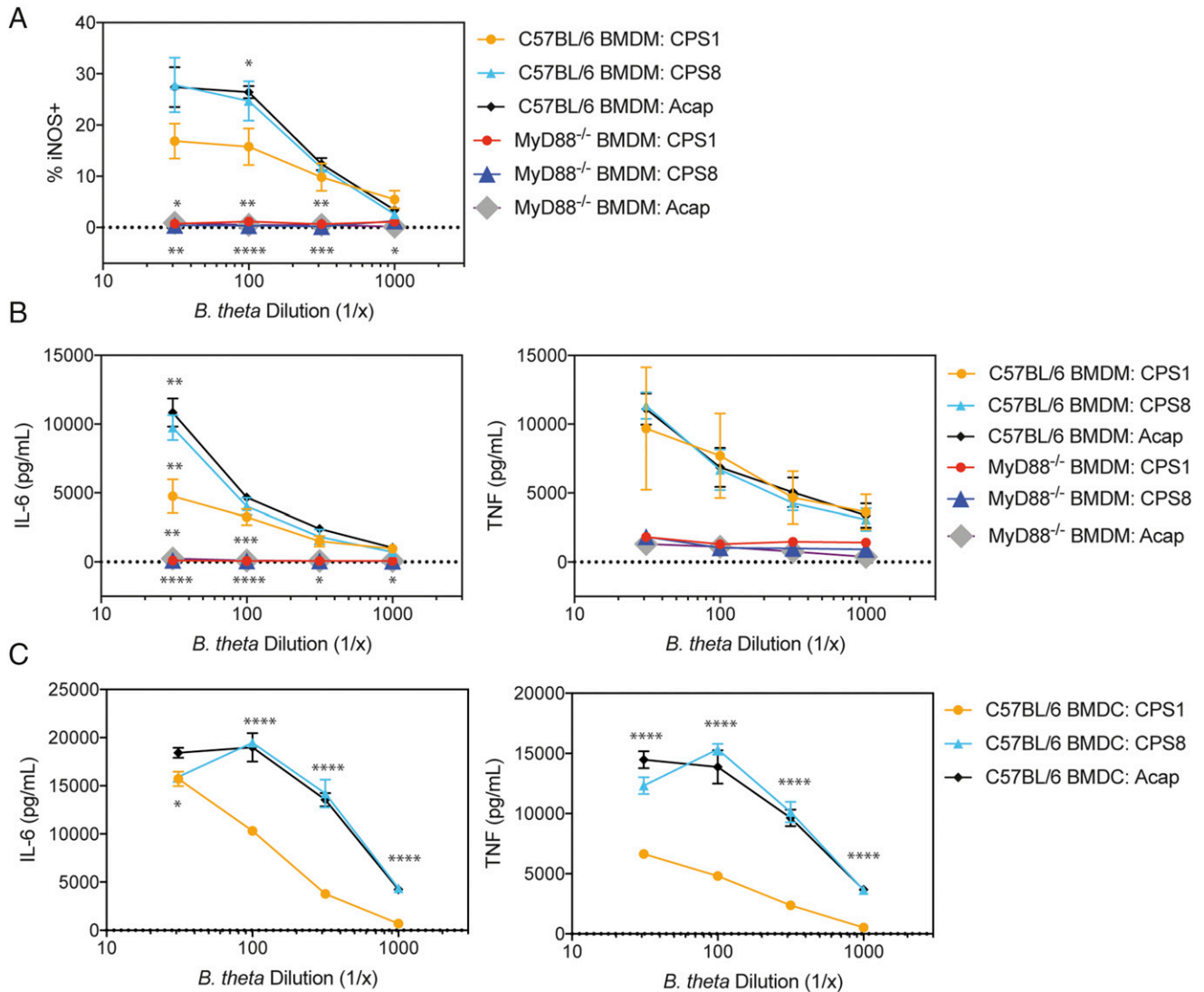
We next sought to determine if the single CPS-expressing *B. thetaiotaomicron* strains activate the innate immune system. None of the single CPS-expressing *B. thetaiotaomicron* upregulated expression of the costimulatory molecules PD-L1 and CD86 in BMDM (Supplemental Fig. 3A). Despite differences in APC uptake, all single CPS-expressing *B. thetaiotaomicron* upregulated the expression of iNOS in BMDM, which is an inflammatory M1 marker (Fig. 4A, Supplemental Fig. 3B) (27). All single CPS-expressing *B. thetaiotaomicron* also induced both BMDMs and BMDCs to release the inflammatory cytokines IL-6 and TNF (Fig. 4B, 4C, Supplemental Fig. 3C, 3D). Activation of the innate immune system by single CPS-expressing *B. thetaiotaomicron* was dependent on the MyD88 pathway, as MyD88<sup>-/-</sup> BMDMs did not skew to an M1 phenotype or release IL-6 and TNF (Fig. 4B, 4C, Supplemental Fig. 3B, 3C). Although *B. thetaiotaomicron* strains with antistimulatory CPSs activated the innate immune system, they stimulated the innate immune system in a weaker manner than *B. thetaiotaomicron* strains with prostimulatory CPSs and induced fewer M1 macrophages and inflammatory cytokines (Fig. 4A–C). Adding the A176 Ab had no effect on BMDM skewing to M1 macrophages or IL-6 and TNF cytokine release in the presence of CPS1-expressing *B. thetaiotaomicron* (Supplemental Fig. 2C, 2D). To explore what innate receptor may be involved through the MyD88 pathway, we tested TLR4-deficient

BMDMs. The TLR4-deficient BMDM responded identically to the WT BMDM (data not shown), indicating that TLR4 was not involved. These results demonstrate that although all single CPS-expressing *B. thetaiotaomicron* strains stimulate the innate immune system through MyD88, antistimulatory CPSs may help shield *B. thetaiotaomicron* from fully activating the innate immune system.

*Single CPS-expressing OMVs also regulate immune responses to dominant Ags*

Although our above-mentioned findings used whole *B. thetaiotaomicron*, *B. thetaiotaomicron* also releases OMVs, which can deliver *B. thetaiotaomicron* Ags to immune cells in the host intestine (19). OMVs also contain a CPS outer layer, but the roles of CPSs on OMVs remain poorly understood. The most well-studied CPS on OMVs is PSA. OMVs containing PSA have been shown to be immunomodulatory because WT OMVs, but not OMVs that lack PSA ( $\Delta$ PSA OMVs), induce CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T<sub>reg</sub>s and prevent experimental colitis (28). A limitation of this study is that only  $\Delta$ PSA OMVs that lack PSA but still express other CPSs were tested. Because our B $\theta$ OM T cell Ag is also present on OMVs, we examined if single CPS-expressing OMVs can regulate the immune response to dominant Ags. In accordance with our findings, using single CPS-expressing *B. thetaiotaomicron*, OMVs with antistimulatory CPSs, such as CPS1, weakly activated B $\theta$ OM T cells, whereas OMVs with prostimulatory CPSs, such as CPS8 and Acap, strongly activated B $\theta$ OM T cells (Fig. 5A). Although OMVs with antistimulatory CPS1 or prostimulatory CPS8 did not upregulate the costimulatory molecules CD86 and PD-L1 in BMDM (data not shown), they both activated the innate immune system to skew to M1 macrophages and release IL-6 and TNF inflammatory cytokines in a MyD88-dependent manner





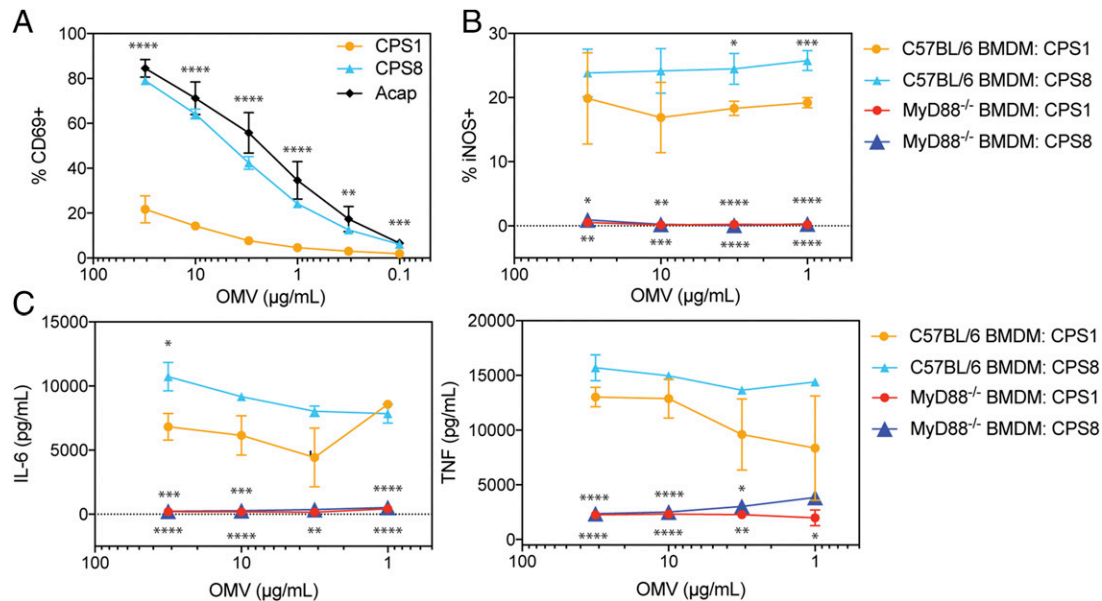
**FIGURE 4.** All *B. thetaiotaomicron* (*B. theta*) CPSs stimulate the innate immune system in a MyD88-dependent manner, but antistimulatory CPSs activate the innate immune system in a weaker manner than prostimulatory CPSs. **(A)** Percentage of iNOS-expressing C57BL/6 or MyD88<sup>-/-</sup> BMDM after a 24-h culture with CPS1, CPS8, or Acap ( $n = 4$ , three experiments). **(B and C)** IL-6 and TNF levels in picogram per milliliter produced by **(B)** C57BL/6 or MyD88<sup>-/-</sup> BMDM and **(C)** C57BL/6 BMDC after a 24-h culture with CPS1, CPS8, or Acap ( $n = 4-6$ , three experiments). Data represent mean  $\pm$  SEM. One-way ANOVA analysis (A and B). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

(Fig. 5B, 5C). Similar to whole *B. thetaiotaomicron* expressing CPS1, OMVs with antistimulatory CPS1 activated the innate immune system in a weaker manner than OMVs with prostimulatory CPS8 (Fig. 5B, 5C). Because A176 also binds to CPS1 OMVs, we added A176 to our B $\theta$ OM T cell stimulation assay with OMVs containing antistimulatory CPS1 but saw no increase in B $\theta$ OM T cell stimulation. These studies, to our knowledge, are the first time the adaptive and innate immune responses of a complete set of CPS on OMVs have been reported and demonstrate that CPSs at the level of OMVs can also modulate innate and adaptive immune responses to dominant Ag.

#### Single CPS-expressing *B. thetaiotaomicron* modulate Ag-specific and polyclonal immune responses in vivo

We then evaluated the ability of CPSs on *B. thetaiotaomicron* to regulate the immune responses to dominant Ags in vivo using our previously published B $\theta$ OM T cell transfer model (18). Rag1<sup>-/-</sup> mice were pretreated with antibiotics for 2 wk, gavaged with antistimulatory CPS1-expressing *B. thetaiotaomicron* or prostimulatory CPS8-expressing *B. thetaiotaomicron* on day 0, adoptively transferred

with B $\theta$ OM T cells on day 3, and then harvested on day 10 (Supplemental Fig. 4A). Although *B. thetaiotaomicron* expressing CPS1 or CPS8 colonized mice equally (Supplemental Fig. 4B), we recovered fewer B $\theta$ OM T cells in the colon lamina propria of mice colonized with CPS1-expressing *B. thetaiotaomicron* than with CPS8-expressing *B. thetaiotaomicron* (Fig. 6A). These findings are consistent with our in vitro results and further demonstrate that antistimulatory CPS1-expressing *B. thetaiotaomicron* weakly activate B $\theta$ OM T cells, whereas prostimulatory CPS8-expressing *B. thetaiotaomicron* strongly activate B $\theta$ OM T cells in vivo. Although there were no differences in the percentage of naive CD44<sup>+</sup> CD62L<sup>-</sup> or activated CD44<sup>+</sup> CD62L<sup>-</sup> B $\theta$ OM T cells (Supplemental Fig. 4C, 4D), CPSs on *B. thetaiotaomicron* also directed the differentiation of Ag-specific T cells as B $\theta$ OM T cells in the colon differentiated into more IFN- $\gamma$ <sup>+</sup> IL-17A<sup>+</sup> T cells in the presence of *B. thetaiotaomicron* containing prostimulatory CPS8 (Fig. 6B). In addition, CPS8-expressing *B. thetaiotaomicron* induced more effector IFN- $\gamma$ <sup>+</sup> IL-17A<sup>-</sup> T<sub>H1</sub> and IFN- $\gamma$ <sup>-</sup> IL-17A<sup>+</sup> T<sub>H17</sub> B $\theta$ OM T cells, whereas CPS1-expressing *B. thetaiotaomicron* induced more Foxp3<sup>+</sup> B $\theta$ OM T<sub>regs</sub> in the colon, although these trends did



**FIGURE 5.** OMV CPSs have similar immunomodulatory effects on a *B. thetaiotaomicron*-dominant Ag as CPSs on whole bacteria. **(A)** Percentage of CD69-expressing B00M T cells with CPS1, CPS8, and Acap OMVs loaded onto BMDM (CPS1 and CPS8:  $n = 7$ , three experiments; Acap:  $n = 3$ , two experiments). **(B)** Percentage of iNOS-expressing C57BL/6 or MyD88<sup>-/-</sup> BMDM after a 24 h culture with CPS1 and CPS8 OMVs ( $n = 6$ , three experiments). **(C)** IL-6 and TNF levels in picogram per milliliter produced by C57BL/6 or MyD88<sup>-/-</sup> BMDM and after a 24-h culture with CPS1 and CPS8 OMVs ( $n = 6$ , three experiments). Data represent mean  $\pm$  SEM. (B and C) The asterisks above the CPS8 line are comparing CPS1 versus CPS8. The asterisks above the red/blue triangles are comparing CPS1 versus MyD88<sup>-/-</sup> CPS1 and CPS8 versus MyD88<sup>-/-</sup> CPS8. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.000$ , one-way ANOVA.

not reach statistical significance (Supplemental Fig. 4E–G). These studies demonstrate that CPSs on *B. thetaiotaomicron* can control the delivery of specific bacterial Ags and manipulate Ag-specific immune responses in vivo.

Because B00M T cells are specific for a single Ag in *B. thetaiotaomicron*, we next sought to determine if single CPS-expressing *B. thetaiotaomicron* could also modulate polyclonal immune responses. We colonized germ-free C57BL/6 mice with *B. thetaiotaomicron* expressing CPS1 or CPS8. Ten days later, we harvested T cells from the colon lamina propria, cdLN, and spleen. Although there were no differences in T cell differentiation (Supplemental Fig. 4H–M), antistimulatory CPS1-expressing *B. thetaiotaomicron* weakly activated polyclonal T cells, whereas prostimulatory CPS8-expressing *B. thetaiotaomicron* strongly activated polyclonal T cells in vivo in the colon lamina propria (Fig. 6C). These findings reveal that by altering CPS expression, bacteria can also regulate polyclonal T cell responses in vivo.

#### CPSs on *B. thetaiotaomicron* control clearance of *B. thetaiotaomicron* in vivo

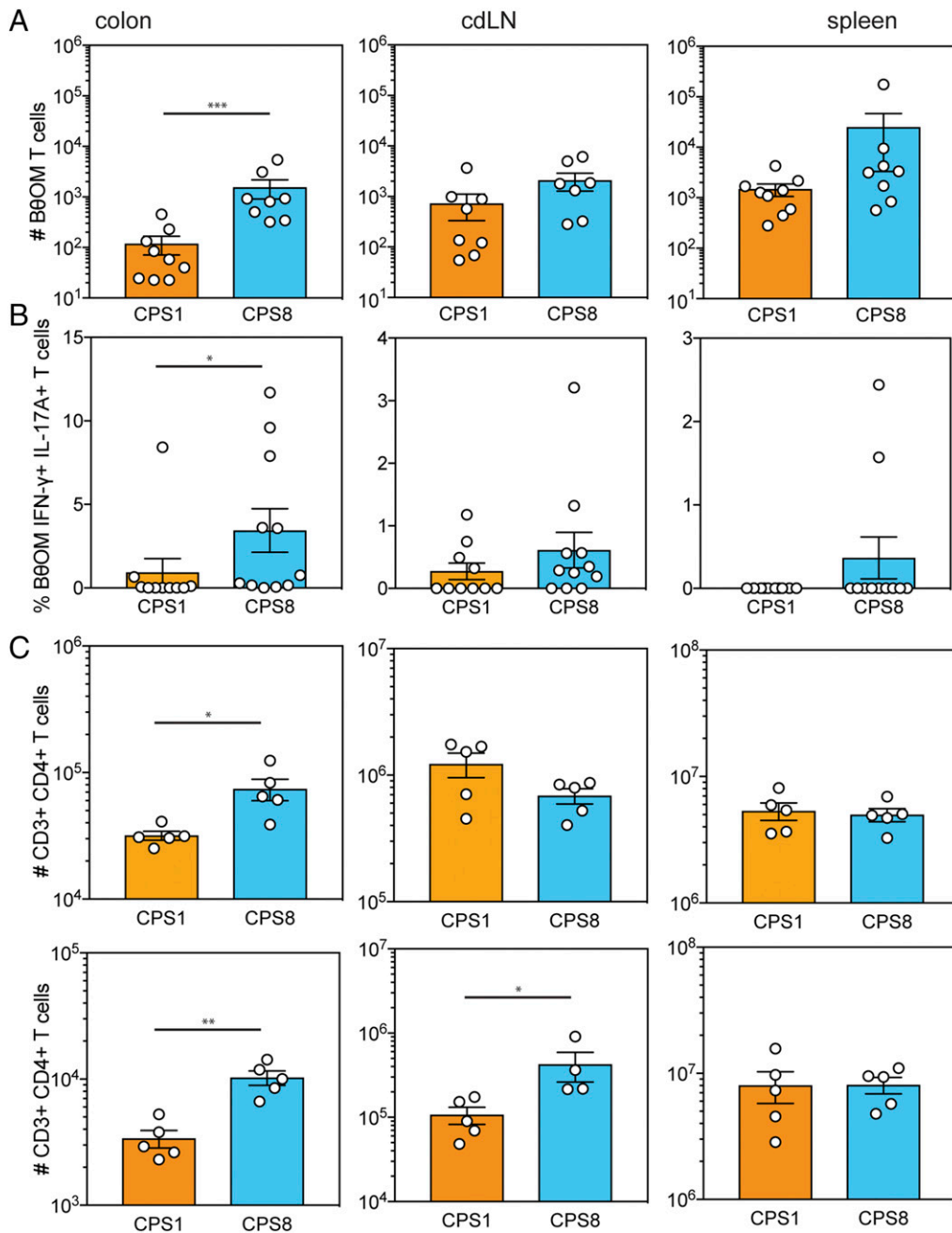
When the intestine is perforated, intestinal bacteria can migrate into the peritoneal cavity and disseminate in the bloodstream (29). Because *B. thetaiotaomicron* has been reported to cause sepsis and bacteremia (30, 31), we next examined the functional roles of CPSs on *B. thetaiotaomicron* in vivo in the peritoneum. We injected *B. thetaiotaomicron* expressing CPS1 or CPS8 i.p., harvested the peritoneal fluid 4 h later, and measured the *B. thetaiotaomicron* remaining in the peritoneal fluid. CPS8-expressing *B. thetaiotaomicron* exhibited enhanced bacterial clearance from the peritoneal fluid compared with antistimulatory CPS1-expressing *B. thetaiotaomicron*, likely because antistimulatory CPS1-expressing *B. thetaiotaomicron* is poorly uptaken by immune cells (Fig. 7A). We then performed a time course of bacterial clearance in the peritoneal fluid and found substantial differences in *B. thetaiotaomicron* clearance as early as 1 h postinjection. These results confirmed that readily phagocytosed

CPS8-expressing *B. thetaiotaomicron* is cleared much more rapidly than the poorly uptaken CPS1-expressing *B. thetaiotaomicron* (Fig. 7A). Thus, CPSs on *B. thetaiotaomicron* also modulate the immune responses to dominant Ag by controlling clearance of specific bacterial Ags in vivo.

## Discussion

Bacteria express a variety of different surface CPSs, but the functional consequences of the bacteria phase variance remain poorly understood. CPSs are known to be immunomodulatory, but current studies have only investigated the immune effects of a handful of CPSs, such as the zwitterionic PSA produced by *B. fragilis* (32). Our recent study explored the roles of a complete set of CPSs in competitive intestinal colonization, but we did not study how CPSs on *B. thetaiotaomicron* influence the immune response (15). In addition, current studies have focused on how the CPS itself interacts with the immune system (5, 28, 33). It remains unknown if CPSs on bacteria can regulate the immune responses to specific bacterial Ags.

Our symbiont-specific T cell system combined with a complete set of single CPS-expressing *B. thetaiotaomicron* strains provide an opportunity to interrogate if the immune responses to dominant Ags can be modulated by CPSs on bacteria. Our findings reveal a critical role for CPSs on *B. thetaiotaomicron* to regulate Ag accessibility and the innate and adaptive immune responses to specific bacterial Ags. Although many single CPS-expressing *B. thetaiotaomicron* strains were antistimulatory, a few strains were prostimulatory and activated B00M T cells just as well or better than an Acap that lacked all *B. thetaiotaomicron* CPSs. *B. thetaiotaomicron* strains expressing antistimulatory CPSs suppressed B00M T cell stimulation by blocking the uptake of dominant Ag. Poor uptake of *B. thetaiotaomicron* with antistimulatory CPSs may be partly explained by their thicker capsules but could also be due to differences in CPS composition. Prostimulatory CPS8 consists of 19% N-acetyl-glucosamine, 2% galactose, 63%



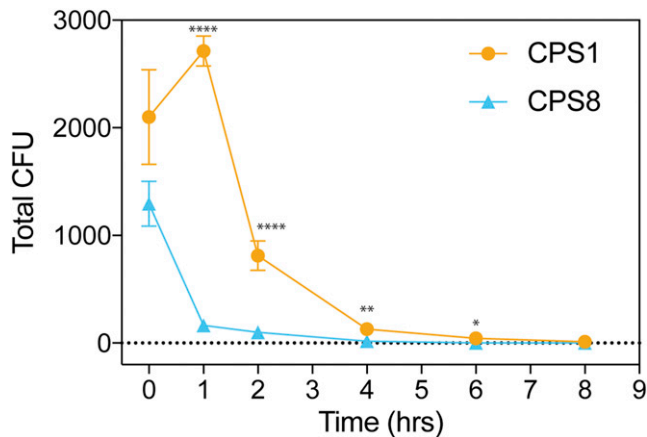
**FIGURE 6.** CPSs modulate Ag-specific and polyclonal T cell responses in vivo. **(A)** Number of live CD4<sup>+</sup> CD45.1<sup>+</sup> B0OM T cells and **(B)** the percentage of B0OM IFN- $\gamma$ <sup>+</sup> IL-17A<sup>+</sup> T cells in the colon, cdLN, and spleen of specific pathogen-free Rag1<sup>-/-</sup> mice gavaged with CPS1 or CPS8 and adoptively transferred with B0OM T cells 3 d later ( $n = 24$  mice, three experiments). **(C)** Number of T cells among live leukocytes that are CD3<sup>+</sup> CD4<sup>+</sup> in the colon, cdLN, and spleen of germ-free mice gavaged with CPS1 or CPS8 ( $n = 20$  mice, two experiments). Data represent mean  $\pm$  SEM. \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$ . Mann-Whitney  $U$  test for nonnormally distributed data.

glucose, 14% mannose, and 1% glucuronic acid (15). Antistimulatory CPS1 consists of 22% N-acetyl-glucosamine, 33% glucose, 9% mannose, and 36% galacturonic acid (15). Although CPS1 may be more negatively charged because of the presence of galacturonic acid, which could play a role in CPS1's poor uptake, this compositional analysis is limited to the monosaccharides present in the purified *B. thetaiotaomicron* capsules. CPSs contain many complex carbohydrates, and more studies will be needed to decipher why some CPSs are poorly phagocytosed. Taken together, these findings demonstrate that CPSs on bacteria control the delivery of dominant Ags to the immune system.

Poor uptake and B0OM T cell stimulation with CPS1-expressing *B. thetaiotaomicron* could be rescued by Fc receptor-dependent Ab

opsonization through the A176 Ab. The A176 Ab binds to *B. thetaiotaomicron* but is not specific to CPS1, suggesting that it recognizes an epitope that is either conserved throughout all eight *B. thetaiotaomicron* capsules or is not present on the capsule surface. A176 may increase immune recognition of CPS1-expressing *B. thetaiotaomicron* by several mechanisms. For example, A176 could mask a surface molecule that inhibits phagocytosis or stimulate a surface molecule to trigger uptake. Given the literature showing that Ab conformation is more important for Ab neutralization in viruses than the Ab epitope or binding affinity (34), we hypothesize that A176 may increase phagocytosis by binding to CPS1-expressing *B. thetaiotaomicron* in a particular geometrical configuration that allows CPS1-expressing *B. thetaiotaomicron* to





**FIGURE 7.** CPSs control clearance of a dominant *B. thetaiotaomicron* Ag in vivo. Total CFU in the peritoneal interstitial fluid in C57BL/6 mice injected with  $1 \times 10^6$  CFUs of CPS1 or CPS8 i.p. and lavaged 0, 1, 2, 4, 6, or 8 h later.  $n = 90$  mice, three experiments. Data represent mean  $\pm$  SEM. \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , Student  $t$  test.

be amenable to APC uptake. This may also explain the inability of A176 to upregulate B $\theta$ OM T cell stimulation in the presence of OMVs expressing CPS1. OMVs are 20–250 nm in diameter and are much smaller than whole bacteria (35), so A176 may not be able to form the optimal Ab configuration to opsonize CPS1-expressing OMVs. These results show that the immune system can overcome the ability of CPSs on *B. thetaiotaomicron* to block phagocytosis by generating Abs that restore immune access to dominant Ags through Fc receptor–dependent Ab opsonization.

In addition to regulating adaptive immune responses, all single CPS–expressing *B. thetaiotaomicron* activated the innate immune system in a MyD88-dependent manner. Because MyD88 is downstream of many TLRs and *B. thetaiotaomicron* expressing antistimulatory CPSs are poorly uptaken, we suggest that these innate immune responses are primarily due to interactions between *B. thetaiotaomicron* and surface pattern recognition receptors and may represent immune adaptations to overcome CPS phase variance, especially to detect antiphagocytic bacteria. Although all single CPS–expressing *B. thetaiotaomicron* activated the innate immune system, *B. thetaiotaomicron* expressing antistimulatory CPSs activated the innate immune system in a weaker manner than *B. thetaiotaomicron* expressing prostimulatory CPSs. These differences in innate immune activation may be due to variations in CPS composition, especially in the immunomodulatory molecules present, or the ability of antistimulatory CPSs to shield immunostimulatory molecules from activating the innate immune system.

Many Gram-negative bacteria release OMVs, which can deliver bacterial Ags to host immune cells in the intestine (19). CPSs on OMVs, such as PSA on *B. fragilis* OMVs, have been shown to regulate the immune system (28), but the roles of a complete set of CPSs on OMVs have not yet been interrogated. We show that a complete set of CPSs on *B. thetaiotaomicron* OMVs have similar immunomodulatory roles as CPSs on whole *B. thetaiotaomicron*. All single CPS–expressing OMVs activated the innate immune system to skew to M1 macrophages and release inflammatory cytokines. OMVs with antistimulatory CPSs activated B $\theta$ OM T cells and APCs in a weaker manner compared with OMVs with prostimulatory CPSs. These findings establish that a complete set of CPSs on OMVs can also regulate adaptive and innate immune responses to dominant Ags.

Finally, we reveal that CPSs on *B. thetaiotaomicron* can also modulate immune responses to dominant Ags in vivo. In accordance

with our in vitro findings, antistimulatory CPS1 poorly stimulated B $\theta$ OM T cells, whereas prostimulatory CPS8 strongly stimulated B $\theta$ OM T cells in our B $\theta$ OM T cell transfer mouse model. CPS expression on *B. thetaiotaomicron* also directed B $\theta$ OM T cell differentiation, as B $\theta$ OM T cells in the colon converted to more IFN- $\gamma^+$  IL-17A $^+$  T cells in the presence of *B. thetaiotaomicron* expressing prostimulatory CPS8. The roles of these T $H$ 1-like T $H$ 17 cells are poorly understood, but they have been shown to play important roles in the pathogenesis of autoimmune diseases, including inflammatory bowel disease (36). CPSs on *B. thetaiotaomicron* also regulated polyclonal immune responses as germ-free mice colonized with antistimulatory CPS1-expressing *B. thetaiotaomicron* weakly activated polyclonal T cells, whereas prostimulatory CPS8-expressing *B. thetaiotaomicron* strongly activated polyclonal T cells. Because bacteria can disseminate into the peritoneal cavity when the intestine is perforated, we then examined the roles of CPSs on *B. thetaiotaomicron* in a model of peritoneal infection. Readily phagocytosed *B. thetaiotaomicron* expressing CPS8 was cleared more rapidly from the peritoneal cavity compared with poorly phagocytosed *B. thetaiotaomicron* expressing CPS1, suggesting that CPSs on *B. thetaiotaomicron* may also regulate immune responses during peritoneal infection. Furthermore, antistimulatory and prostimulatory CPSs have been found to be expressed on *B. thetaiotaomicron* in human gut samples, demonstrating that CPSs on *B. thetaiotaomicron* may also modulate immune responses to specific bacterial Ags in the human intestine (15). Given the vast, unexplored universe of gut bacterial CPS structures, we believe that future studies will expand the known lexicon of bacterial polysaccharide-immune system interactions and may even lead to the discovery of new, bioactive CPS for potential use as therapeutics.

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## Disclosures

The authors have no financial conflicts of interest.

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