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


J Immunol 2020; 204:1035-1046; Prepublished online 3 January 2020; doi: 10.4049/jimmunol.1901206
http://www.jimmunol.org/content/204/4/1035

Supplementary Material
http://www.jimmunol.org/content/suppl/2020/01/02/jimmunol.1901206.DCSupplemental

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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+ T cell, B00M, 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 B00M T cells more strongly than wild-type *B. thetaiotaomicron*. Despite similar levels of B00M Ag expression, many single CPS–expressing *B. thetaiotaomicron* strains were antistimulatory and weakly activated B00M T cells, but a few strains were prostimulatory and strongly activated B00M 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: 1035–1046.

Any 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+ Foxp3+ regulatory T cells (Tregs) 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 Tregs 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 β-glucan/galactan polysaccharides that were purified from *Bifidobacterium bifidum* induced Tregs 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...
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 dictates a substantial portion of its genome—182 genes in eight distinct genomic loci—to CPS production. The single CPS–expressing B. thetaiotaomicron strain 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 KH2PO4, 8.5 mM [NH4]2SO4, 15 mM NaCl, 10 µM vitamin K2, 2.63 µM FeSO4·7H2O, 0.11 mM MgCl2, 1.9 µM hematin, 0.2 mM l-histidine, 3.69 mM vitamin B12, 413 µM l-cysteine, and 7.2 µM CaCl2·2H2O) minimal medium (20 g/l tryptone, 10 g/l yeast extract, 5 g/l d-glucose, 8.25 g/l t-cysteine, 78 µM MgSO4·7H2O, 294 µM KH2PO4, 230 µM K3HPO4, 1.4 mM NaCl, 7.9 µM hematin [hematin], 4 µM resazurin, 24 µM NaHCO3, 68 µM CaCl2·2H2O) or on brain heart infusion (BHI) agar plates containing 10% horse blood (Quad Five, Rygate, MT) with gentamicin (200 µg/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 Satchets 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 λ pir containing the pWW3452 vector (22) was grown to midlog phase in Luria-Bertani medium containing ampicillin (300 µg/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 µg/ml gentamicin and 25 µg/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. B6OM transgenic mice on the Rag1−/− background were maintained by breeding to a nontransgenic Rag1−/− (S17-1 λ pir) mouse. 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 B6OM T cell stimulation assay was performed as previously described (18). Bone marrow cells isolated from the tibia and femurs of C57BL/6, MyD88−/−, or FcRγ−/− mice were cultured in M-CSF for 7 d to differentiate into bone marrow–derived macrophages (BMDM). BMDM were stimulated with IFN-γ at 2000 U/ml in 1-10 media (IMDM, 10% FBS, glutamine, and gentamicin) and plated on a 96-well plate at 1 × 105 cells per well. The cells were washed with PBS 24 h later and kept in 100 µl of fresh 1-10 medium without IFN-γ. 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 × 105 cells per well. When spleenic
CD11c+ dendritic cells (DCs) were used, splenocytes were enriched for CD11c+ DCs using MACS CD11c Microbeads and plated on a 96-well plate at 1 × 10^5 cells per well. A total of 5 × 10^5 cells isolated from the spleen and lymph nodes of B70L6 transgenic mice or 1 × 10^5 B8OM T cell hybridomas or B8OM T cell blasts with or without 10 μg/ml of B. thetaiotaomicron Ab were then added per well in 50 μl plus 50 μl 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 FACSCan and analyzed using FlowJo.

**Generation of B8OM T cell hybridomas**

B8OM 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 μl 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 × 10^5 cells was cultured in 7 ml of RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM Sodium Pyruvate, 5 × 10^{-5} M 2-ME, 50 μg/ml gentamicin, and 14 μl of the B. thetaiotaomicron/PBS Ag source (1:500 dilution) for 3 d. Blasting B8OM T cells were harvested by centrifugation and fused to the BW5147+T-7 T cell hybridoma fusion partner using a standard protocol (23).

**Generation of B8OM T cell blasts**

BMDM were stimulated with IFN-γ at 2000 U/ml in I-10 media (see above) and plated on a 24-well plate at 5 × 10^5 cells per well. The cells were washed with PBS 24 h later and kept in 1 ml I-10 media. One micromolar B8OM peptide and 2 × 10^5 isolated CD4+ B8OM T cells were added per well in 0.5 ml with 50 μl IL-2 and incubated for 6 d at 37˚C. B8OM T cell blasts were then harvested from the supernatant and added to the in vitro macrophage cell assay.

**Quantitative ELISA for BT2495**

BT2495 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 to midlog phase in a 2-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_{600}-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 Two ELISA Plate was coated overnight with purified anti-BT2495 Ab ERC11 in carbonate coating buffer (10 mM carbonate, pH 9.6) at 4˚C and washed, blocked, and incubated 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-BT2495 Ab biotin-4E9 (5 μg/ml) was added for 1 h at RT. Plates were washed again, and a 1:5000 dilution of streptavidin HRP was added for 30 min at RT. Plates were washed, and 100 μl of One-Step Ultra TMB Substrate was added and incubated well and A450 was determined. Unknown sample concentrations were quantified by comparison with a standard curve of BT2495 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 × 10^5 cells per well in I-10 media overnight. The cells were stained with 2 μM CellTracker Orange CMTMR Dye at 37˚C for 1 h, then washed, blocked, and incubated 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-BT2495 Ab biotin-4E9 (5 μg/ml) was added for 1 h at RT. Plates were washed again, and a 1:5000 dilution of streptavidin HRP was added for 30 min at RT. Plates were washed, and 100 μl of One-Step Ultra TMB Substrate was added and incubated well and A450 was determined. Unknown sample concentrations were quantified by comparison with a standard curve of BT2495 protein performed in the same ELISA using GraphPad Prism Software.

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

BMDM or BMDCs were plated on a 96-well plate at 1 × 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 with IL-2, IL-4, IL-6, IL-10, TNF, IFN-γ, and IL-17A using a BD Cytometric Bead Array Mouse T cell Inflamed Kit. The BMDCs 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-μm filter. The filtered supernatant was then spun down in a Beckman centrifuge (25.5 rotor) at 38,500 × 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 × 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 CPS 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˚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 MgCl2, and 0.5 mM CaCl2, 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. thetaiotaomicron single-use aliquots. Three days after gavage, Rag1−/− mice were injected retro-orbitally with 2 × 108 B00M T cells isolated from the peripheral lymph nodes (axially, brachial, inguinal, superficial and deep cervical, lumbar, and candal), mesenteric lymph nodes, and spleen. Cells were cultured for CD4+ T cells by negative selection using a homemade mixture of Abs (anti-mouse Ter-119, CD11c, CD11b, CD8α, CD19, CD45R/B220, CD49b, and CD24) and antibiotic 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 μl of B. thetaiotaomicron strains at 1 × 108 CFUs per ml, and leukocytes were also isolated from the lamina propria 10 d after gavage. Colon and cecum were flushed with PBS to remove colonic contents, and fat tissue was pulsed off. Intestines were cut longitudinally and laterally into freshly made 20 ml of predigestion solution (1× HBSS without Ca2+ and Mg2+, 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× HBSS without Ca2+ and Mg2+ 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× HBSS with Ca2+ and Mg2+, 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-μm filter. The cdLN and spleen were removed and processed into single-cell suspensions using Eppendorf tube lids and filtered through a 70-μm filter. All samples were counted, stained with anti-mouse CD4, CD45.1, CD4, CD62L, Foxp3, IFN-γ, and IFN-β, and run on a BD FACSCanto.

Bacterial clearance in vivo

A total of 1 × 108 CPS1 or CPS8 was injected i.p into C57BL/6 mice. Peritoneal fluid was collected 0, 1, 2, 4, 6, or 8 h after ingestion. Peritoneal fluid was plated on BHI-blood plates with 200 μg/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 uptake of B. thetaiotaomicron 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 B00M CD4+ T cells. Using primary CD11c+ splenic DCs as the APCs, we found that Acap stimulated B00M 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 B00M T cells. Most single CPS–expressing B. thetaiotaomicron strains were antistimulatory and weakly activated B00M T cells, but one single CPS–expressing B. thetaiotaomicron strain, CPS8, was prostimulatory and activated B00M T cells similarly or even better than Acap in the presence of primary CD11c+ 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 anti-stimulatory CPS, CPS1, and one prostimulatory CPS, CPS8. Using these two model single CPS–expressing B. thetaiotaomicron strains, we found that these differences in B00M 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 B00M 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 B00M 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+ T cell responses because the CPSs either block Ag uptake, Ag processing, or Ag presentation. We first used B00M T cell blasts, which are B00M T cells that have been previously activated in vitro, to examine if antistimulatory CPSs on B. thetaiotaomicron impede Ag presentation. B00M 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 B00M T cell blasts recapitulated our results with primary B00M T cells. Prostimulatory single CPS–expressing B. thetaiotaomicron, including CPS8 and Acap, strongly activated B00M T cell blasts. Antistimulatory single CPS–expressing B. thetaiotaomicron, including CPS1, weakly activated B00M T cell blasts, suggesting that antistimulatory CPSs on B. thetaiotaomicron do not inhibit the Ag presentation pathway (Fig. 2A, Supplemental Fig. 1A). Using B00M T cell hybridomas, which only require TCR stimulation to be activated, we next investigated if B. thetaiotaomicron expressing antistimulatory CPSs block Ag processing. B00M 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
expressed on the APC surface. B0OM T cell hybridomas were also strongly activated by \textit{B. thetaiotaomicron} expressing prostimulatory CPSs but weakly activated by \textit{B. thetaiotaomicron} with antistimulatory CPSs (Fig. 2B, Supplemental Fig. 1B), demonstrating that antistimulatory CPSs on \textit{B. thetaiotaomicron} do not block the Ag processing pathway. To determine if antistimulatory single CPS–expressing \textit{B. thetaiotaomicron} strains inhibit the uptake of dominant Ags by APCs, we cultured labeled BMDMs with \textit{B. thetaiotaomicron} strains that expressed a single CPS and GFP and performed live-cell imaging. \textit{B. thetaiotaomicron} strains with prostimulatory CPSs, such as CPS8 and Acap, were highly phagocytosed, whereas \textit{B. thetaiotaomicron} strains with antistimulatory CPSs, such as CPS1, were poorly phagocytosed (Fig. 2C, 2D, Supplemental Fig. 1C, 1D), revealing that \textit{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 \textit{B. thetaiotaomicron} strains can also act in trans. Mixing the prostimulatory CPS8-expressing \textit{B. thetaiotaomicron} strain with either the antistimulatory CPS1-expressing \textit{B. thetaiotaomicron} strain or purified CPS1 capsule failed to decrease B0OM T cell stimulation (Fig. 2E, 2F), suggesting that CPS1 cannot inhibit CPS8 on \textit{B. thetaiotaomicron} in trans. Live-cell imaging of BMDM treated with unlabeled CPS1-expressing \textit{B. thetaiotaomicron} as well as \textit{B. thetaiotaomicron} that expressed CPS8 and GFP revealed that CPS8-expressing \textit{B. thetaiotaomicron} was still readily uptaken by BMDM in the presence of CPS1-expressing \textit{B. thetaiotaomicron} (data not shown). To rule out the possibility that CPS8-expressing \textit{B. thetaiotaomicron} could be enhancing the uptake and B0OM T cell stimulation of CPS1-expressing \textit{B. thetaiotaomicron}, we incubated BMDMs with unlabeled CPS8-expressing \textit{B. thetaiotaomicron} and \textit{B. thetaiotaomicron} that expressed CPS1 and GFP and found no increase in uptake of CPS1-expressing \textit{B. thetaiotaomicron} (data not shown). These findings definitively demonstrate that the anti-stimulatory CPS1 and prostimulatory CPS8 do not function in trans to alter \textit{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.

\textit{Fc} receptor–dependent Ab optimization can rescue immune access to dominant Ag on \textit{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 \textit{B. thetaiotaomicron} and is IgG2a, \kappa (Supplemental Fig. 2A). Because A176 binds to all single CPS–expressing \textit{B. thetaiotaomicron}, A176 recognizes an Ag that is present in all \textit{B. thetaiotaomicron} strains and is not unique to the CPS1 capsule. Adding A176 to our B0OM T cell stimulation assay allowed CPS1-expressing \textit{B. thetaiotaomicron} to now strongly activate B0OM T cells (Fig. 3A, Supplemental Fig. 2B). Live-cell imaging demonstrated that CPS1-expressing \textit{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 \textit{B. thetaiotaomicron} in BMDMs (Fig. 3B, 3C). The addition of A176 had no effect on B0OM T cell activation by CPS1-expressing \textit{B. thetaiotaomicron} when splenic CD11c+ DCs were used as the APCs, suggesting that A176 likely increased CPS1 uptake through a receptor present on BMDM but not splenic CD11c+ 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 FcRγ- BMDM, which lack the Fc receptor common \gamma-chain and do not express any of the activating mouse FcγRs (FcγRI, FcγRII, and FcγRIII). A176 no longer increased CPS1-expressing \textit{B. thetaiotaomicron}’s B0OM T cell stimulation in the presence of FcRγ- BMDM, demonstrating that A176 increases CPS1 uptake and B0OM T cell stimulation by Ab opsonization through

FIGURE 1. Most \textit{B. thetaiotaomicron} (\textit{B. theta}) CPSs are antistimulatory and prevent B0OM T cell recognition of a dominant Ag in \textit{B. thetaiotaomicron}. (A) Percentage of CD69-expressing B0OM T cells after a 24 h culture with CD11c+ splenic DCs loaded with \textit{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).
Antistimulatory CPSs block uptake of a dominant Ag in *B. thetaiotaomicron* (*B. theta*), partly because of capsule thickness. (A) Percentage of CD69-expressing B9OM 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 B9OM T cell hybridomas after a 24-h culture with BMDM loaded with CPS1, CPS8, or Acap (n = 6, three experiments). (C) Percentage of CD69-expressing B9OM T cells after a 24 h culture with BMDM loaded with CPS1, CPS8, or a 1:1 or 5:1 ratio (n = 6, three experiments). ***p = 0.0207. (F) Percentage of CD69-expressing B9OM 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 live-cell images and (H) quantification of uptaken GFP-labeled CPS1, CPS8, or Acap per BMDM after a 4 h culture (n = 150, three experiments). Scale bar, 5 μm. (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 μm. Data represent mean ± SEM. One-way ANOVA analysis. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
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−/− 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 (ΔPSA OMVs), induce CD4+CD25+Foxp3+ Tregs and prevent experimental colitis (28). A limitation of this study is that only ΔPSA OMVs that lack PSA but still express other CPSs were tested. Because our B00M 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 B00M T cells, whereas OMVs with prostimulatory CPSs, such as CPS8 and Acap, strongly activated B00M 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.
(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 B0OM T cell stimulation assay with OMVs containing antistimulatory CPS1 but saw no increase in B0OM 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 B0OM T cell transfer model (18). Rag1 Δ/Δ 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 B0OM 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 B0OM 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 B0OM T cells, whereas prostimulatory CPS8-expressing *B. thetaiotaomicron* strongly activate B0OM T cells in vivo. Although there were no differences in the percentage of naïve CD44+CD62L+ or activated CD44+CD62L+ B0OM T cells (Supplemental Fig. 4C, 4D), CPSs on *B. thetaiotaomicron* also directed the differentiation of Ag-specific T cells as B0OM T cells in the colon differentiated into more IFN-γ+IL-17A+ TH17 and IFN-γ+IL-17A+ TH17 B0OM T cells, whereas CPS1-expressing *B. thetaiotaomicron* induced more Foxp3+B0OM Tregs in the colon, although these trends did not reach statistical significance.

**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 Δ/Δ 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 Δ/Δ BMDM and (C) C57BL/6 BMDC after a 24-h culture with CPS1, CPS8, or Acap (n = 4–6, three experiments). Data represent mean ± SEM. One-way ANOVA analysis (A and B). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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The asterisks above the red/blue triangles are comparing CPS1 versus MyD88
CPS8 OMVs (experiments). These results confirmed that readily phagocytosed
substantial differences in the time course of bacterial clearance in the peritoneal fluid and found
is poorly uptaken by immune cells (Fig. 7A). We then performed a
likely because antistimulatory CPS1-expressing
exhibited enhanced bacterial clearance from the peritoneal fluid 4 h later, and measured the
B. thetaiotaomicron bacteremia (30, 31), we next examined the functional roles of
Because B. thetaiotaomicron has been reported to cause sepsis and
strains expressing antistimulatory CPSs sup-
B. thetaiotaomicron better than an Acap that lacked all
were prostimulatory and activated B. thetaiotaomicron
specific bacterial Ags can be modulated by CPSs on bacteria. Our findings reveal
set of single CPS–expressing
B. thetaiotaomicron did not study how CPSs on
influence the
handful of CPSs, such as the zwitterionic PSA produced by
but current studies have only investigated the immune effects of a
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). 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.

**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 B0OM T cells just as well or better than an Acap that lacked all *B. thetaiotaomicron* CPSs. *B. thetaiotaomicron* strains expressing antistimulatory CPSs suppressed B0OM 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%
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 BöOM 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...
be amenable to APC uptake. This may also explain the inability of A176 to upregulate B00M 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 pathogenic 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 OMVs, such as PSA on B. thetaiotaomicron, can also regulate 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.

Acknowledgments
We thank D. Kraemalmeyer for specific pathogen-free animal care and breeding, M. White and S. Rucknagel for germ-free mouse care and breeding, D. Stewart and T. Tolley for histology, M. Shi for assistance with the confocal microscope. Quick-freeze deep-etch experiments and imaging were performed in part through the use of the Washington University Center for Cellular Imaging.

Disclosures
The authors have no financial conflicts of interest.

References

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 × 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 ± SEM. *p < 0.05, ****p < 0.001, ****p < 0.0001, Student t test.


Supplemental Information

Supplementary Fig. 1. Anti-stimulatory CPSs block uptake of dominant antigen and have thicker capsules.

(A) Percentage of CD69 expressing B0OM T blasts after a 24 hour culture with BMDM loaded with CPS1-8, WT, or Acap (n = 6, three experiments).

(B) IL-2 levels in picogram per milliliter produced by B0OM T cell hybridomas after a 24 hour culture with BMDM loaded with CPS1-8, WT, or Acap (n = 6, three experiments).

(C and D) (C) Representative live cell images and (D) Quantification of uptaken GFP-labeled CPS1-8, WT, or Acap per BMDM after a 4 hour culture (n = 150, three experiments). Scale bar = 5 µm.

(E and F) (E) Representative images and (F) Quantification of capsule thickness of CPS1-8, WT, and Acap stained with India ink (n = 200, three experiments). Scale bar = 2 µm.

(G) Quick-freeze, deep-etch scanning electron micrographs of WT B. theta. The yellow line indicates the width of the capsule. Scale bar = 0.5 µm.

Data represent mean ± SEM.
Supplementary Fig. 2. A176 antibody has no effect on CPS1 activation of the innate immune system.

(A) The amount of A176 that bound to CPS1-8, WT, and Acap was quantified via ELISA.
(B) Titration curve of A176 shown by the percentage of CD69 expressing B0OM T cells after a 24 hour culture with BMDM loaded with CPS1 at a 1:31 dilution.
(C) Percentage of iNOS expressing C57BL/6 or MyD88^{-/-} BMDM after a 24 hour culture with CPS1 only or CPS1 and A176 (n = 2-4, two to three experiments).
(E) IL-6 and TNF levels in picogram per milliliter produced by C57BL/6 or MyD88^{-/-} BMDM after a 24 hour culture with CPS1 only or CPS1 and A176 (n = 2-4, two to three experiments).

Data represent mean ± SEM. One-way ANOVA analysis: (C-D) *p < 0.05, **p < 0.01, ***p < 0.001.
Supplementary Fig. 3. A complete set of CPSs activate the innate immune system in a MyD88-dependent manner.

(A) Percentage of PD-L1 and CD86 expressing BMDM after a 24 hour culture with single CPS-expressing B. theta (n = 4, two experiments).

(B) Percentage of iNOS expressing C57BL/6 or MyD88−/− BMDM after a 24 hour culture with single CPS-expressing B. theta (n = 4, three experiments).

(C and D) IL-6 and TNF levels in picogram per milliliter produced by (C) C57BL/6 or MyD88−/− BMDM and (D) C57BL/6 BMDC after a 24 hour culture with B. theta CPS1-8, WT, or Acap (n = 4-6, three experiments).

Data represent mean ± SEM. Student’s t test: (B and C) *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.000.
Supplementary Fig. 4. Anti-stimulatory and pro-stimulatory CPSs colonize mice equally and may alter T cell differentiation.

(A) Schematic of adoptive transfer of B0OM T cells into Rag1−/− mice gavaged with CPS1 or CPS8. (B) Colonization levels of B. theta (total DNA of B. theta/gram of fecal matter on day 8 or 9 in CPS1 or CPS8-gavaged Rag1−/− mice transferred with B0OM T cells (n = 24 mice, three experiments). (C-G) Percentage of live CD4+ CD45.1+ (C) CD44- CD62L+, (D) CD44+ CD62L-, (E) FoxP3+, (F) IFNγ+ IL-17A-, and (G) IFNγ- IL-17A+ B0OM T cells in CPS1 or CPS8-gavaged Rag1−/− mice transferred with B0OM T cells in the colon, cdLN, and spleen (n = 24 mice, three experiments). (H-M) Percentage of live CD3+ CD4+ (H) CD44- CD62L+, (I) CD44+ CD62L-, (J) FoxP3+, (K) IFNγ+ IL-17A-, (L) IFNγ- IL-17A+ and (M) IFNγ+ IL-17A+ T cells in the colon, cdLN, and spleen of germ-free C57BL/6 mice gavaged with CPS1 or CPS8 (n = 20 mice, two experiments). Data represent mean ± SEM.