Colitogenic *Bacteroides thetaiotaomicron* Antigens Access Host Immune Cells in a Sulfatase-Dependent Manner via Outer Membrane Vesicles

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**SUMMARY**

Microbes interact with the host immune system via several potential mechanisms. One essential step for each mechanism is the method by which intestinal microbes or their antigens access specific host immune cells. Using genetically susceptible mice (*dnKO*) that develop spontaneous, fulminant colitis, we investigated the mechanism of intestinal microbial access under conditions that stimulate colonic inflammation. *B. theta* antigens localized to host immune cells through outer membrane vesicles (OMVs) that harbor bacterial sulfatase activity. We deleted the anaerobic sulfatase maturing enzyme (anSME) from *B. theta*, which is required for post-translational activation of all *B. theta* sulfatase enzymes. This bacterial mutant strain did not stimulate colitis in *dnKO* mice. Lastly, access of *B. theta* OMVs to host immune cells was sulfatase dependent. These data demonstrate that bacterial OMVs and associated enzymes promote inflammatory immune stimulation in genetically susceptible hosts.

**INTRODUCTION**

Host-microbial interactions play a vital role in systemic physiology and pathophysiology; however, such contact, especially in the colon, is greatly limited by host barriers. The colonic epithelium provides important key barriers against a dense (\(10^{11} - 10^{12}\) bacteria/ml) and diverse population of bacteria (Human Microbiome Project Consortium, 2012a, 2012b). Epithelial stem cells at the base of the crypts of Lieberkühn drive rapid epithelial cell turnover of differentiated cell lineages that include absorptive colonocytes and goblet cells, which in turn form two major barriers with the host (Shen et al., 2012), though the mechanisms by which they traverse host barriers are unclear.

*Bacteroidaceae* is a prominent family of intestinal symbiotic organisms. The extent to which these diverse organisms influence host physiology and disease models is unclear beyond a few examples (Bloom et al., 2011; Housseau and Sears, 2010; Shen et al., 2012), and precise mechanisms are still elusive. One member of this family, *Bacteroides thetaiotaomicron* (*B. theta*), is fully sequenced, genetically manipulable, and has a well-defined role in complex carbohydrate metabolism, including interactions with host mucin glycans (Benjdia et al., 2011; Martens et al., 2008; Sonnenburg et al., 2005). These characteristics make *B. theta* an appropriate experimental model to elucidate both beneficial and detrimental host interactions.
among members of this family. Specifically, *B. theta* is well suited to interact with at least one of the barriers, the host mucin glycans, because of polysaccharide utilization loci (PULs) in *B. theta*’s genome that encode several mucin-degrading enzymes such as glycosidase hydrolases (GHs) and sulfatases (Benjdia et al., 2011; Martens et al., 2008). Here, we used *B. theta* (strains dnlkv9 and VPI-5482, thereby referred to in the text as *B. theta*) in an experimental mouse model of intestinal inflammation to decipher the mechanism by which *B. theta* traverses host barriers and contacts the host. We have developed a highly reproducible system, dnKO mice (*CD4-dnTgfβ2; IL10rb−/−*), in which exposure to *B. theta* is sufficient to trigger disease (Bloom et al., 2011; Kang et al., 2008). In this study, we demonstrate that *B. theta* requires sulfatases to cause colitis in dnKO mice, and that *B. theta* OMVs gain access to host immune cells in a sulfatase-dependent manner.

**RESULTS**

**Extracellular Antigens from WT *B. theta* Localize to the Host Peri-Cryptal Mesenchyme in dnKO Mice**

Since *B. theta* is sufficient to trigger colitis in dnKO mice, we first determined *B. theta*’s localization with a panel of monoclonal antibodies. Multiple clones were reactive and specific to *B. theta* by ELISA (Figures 1A and 1B), but the two most promising candidates were selected by in situ luminal staining of intestinal tissue sections from WT mice: 3H2 and 6E9. We found that 3H2 labeled the periphery of bacterial cells in colonic sections of dnKO and control (*IL10rb+/−*/*C0/C0*) mice that were pretreated with antibiotics followed by gavage with WT *B. theta* stained with 3H2 conjugated to Alexa 647 (D and F) and 6E9 conjugated to Alexa 594 antibodies, both in red (E and G). DAPI indicates nuclei in blue. Bars, 3 μm (D and E). Bars, 50 μm (F and G). See also Figure S1.

We found that 3H2 labeled the periphery of bacterial cells in colonic sections of dnKO and control (*IL10rb−/−*) mice that were pretreated with antibiotics followed by gavage with WT *B. theta* (Figures 1C, 1D, and S1A) (Bloom et al., 2011). Based on this staining pattern, we surmised that the target of 3H2 was a highly expressed surface antigen such as one of the eight capsules for *B. theta* (Martens et al., 2009). We found that 3H2 recognized capsule 3 of *B. theta* (Figures S1B and S1C), and thereby stains a subset of whole *B. theta*. However, despite the robust signal in the colonic lumens (Figures 1C and 1D), we did not detect any staining in the mucosa or in the lumen of crypts (Figure 1F; Figure S1D for additional controls). In contrast, 6E9 labeled abundant small particles in the lumen that were not directly associated with *Bacteroides* DNA (Figures 1C, 1E, and S1A), providing us different antibodies to identify whole bacteria versus bacterial particles. Interestingly, 6E9-positive particles were present in mesenchymal cells around the crypt base of dnKO mice where inflammation is typically
initiated in this model (Figure 1G; Figure S1E for additional controls) (Bloom et al., 2011; Kang et al., 2008).

**Host-Penetrant *B. theta* Antigen Localizes to OMVs with Sulfatase Activity**

With immunogold electron microscopy, we found that 6E9 recognized numerous 10- to 80-nm vesicles that were not directly associated with *B. theta* (Figure 2A; Figure S2A for the isotype control). The size and shape of these vesicles were consistent with expelled OMVs that are classically produced by the physiological process of pinching off a portion of the outer membrane of Gram-negative bacteria (Kulp and Kuehn, 2010). Immunogold labeling of cryo-sectioned fecal pellets from *dnKO* mouse gavaged with WT *B. theta* revealed that both *B. theta* and its OMVs had 6E9-positive membranes (Figure 2B).

Because we demonstrated that *B. theta* produced OMVs in vivo, we next wanted to determine the molecular mechanism by which the OMVs accessed the host. OMVs have multiple properties that would facilitate passage through host barriers including small size (Elhenawy et al., 2014), enzymes such as sulfatases and glycoside hydrolases (GHs) (Elhenawy et al., 2014) that may assist in glycan breakdown, and the ability to be endocytosed by host cells (Irving et al., 2014). Desulfation is necessary for the degradation of sulfated mucin glycans, which are abundant in the colon. Of the nearly 5,000 genes present in the *B. theta* genome, 28 of these genes encode sulfatases (Benjdia et al., 2011). Thus, we hypothesized that the sulfatase activity of *B. theta* may be important for the development of colitis in *dnKO* mice.

Fortuitously, a single gene in *B. theta*, the anaerobic sulfatase maturing enzyme (anSME) gene, is required for post-translational activation of all 28 of *B. theta*'s sulfatase genes (Berteau et al., 2006). An engineered mutant strain of *B. theta* (ΔanSME strain) abrogates sulfatase activity (Benjdia et al., 2011). The ΔanSME strain produced 6E9-positive OMVs that are similar to WT *B. theta* OMVs in appearance (Figure 2C; Figure S2B for isotype control and concentration (Figures S1D and S2C) (Chutkan et al., 2013). 6E9 also recognized a single 19-kD antigen in OMVs from both strains (Figure 2D; see Figure S2D for additional controls). Immunoprecipitation followed by mass spectrometry analyses showed this protein was homologous to a predicted membrane protein, BT3901a (Figure S2E), that is widely conserved in *Bacteroides* and highly expressed by *B. theta* both in vivo and in vitro (Sonnenburg et al., 2005). BT3901a was confirmed as the 6E9 antigen on an immunoblot comparing WT *B. theta* and a BT3901a expressing *E. coli* (Figure S2F). As expected, the ΔanSME strain of *B. theta* showed reduced growth using a sole energy source of gastric mucin O-glycans (Figure S2G) that contained sulfated glycan structures (Table S1). Also, WT *B. theta* OMVs, but not ΔanSME OMVs, contained detectable sulfatase activity when comparing equivalent amounts of protein (Figure 2E; Figure S2H for bacterial comparisons).

In addition to this in vitro validation of *B. theta* anSME function, we next determined the feasibility of testing its function in vivo. First, we examined the populations of *Lactobacillus, Enterococcaceae*, and *Lachnospiraceae/Ruminococcaceae* via 16 s qPCR (Bloom et al., 2011) in six different groups: both *dnKO* and *IL10rb*+/− mice gavaged with either PBS, WT *B. theta*, or the ΔanSME strain of *B. theta*. No significant differences were noted in levels of any of these families between any groups (Figures S2I–S2L). Second, *B. theta* sulfatase genes are found within PULs that also encode GHs and other enzymes.
involved in glycan degradation (Benjdia et al., 2011; Martens et al., 2008). Because previous transcriptional studies of B. theta were isolated to the mouse cecum and revealed low expression of many sulfatase-containing PULs (Sonnenburg et al., 2005), we confirmed that multiple B. theta sulfatase-containing PULs were highly expressed in the distal colon (Figures S3A–S3K). Also, because germ-free mice produce varying amounts of sulfated mucin glycans in different regions of the colon (Holmén Larsson et al., 2013), we measured the thickness of the inner sulfated mucus layer, which is Alcian blue positive at pH = 1 (Lev and Spicer, 1964), in the distal colons of dnKO and control IL10rb+/− mice (Figures S3L–S3R). All groups showed similar thickness regardless of genotype or gavage inoculum (Figures S3L–S3R).

The B. theta anSME Gene Is Necessary for Causing Colitis in dnKO Mice
To determine if the anSME gene was required for B. theta’s colitogenic potential, we pre-treated dnKO and littermate controls (IL10rb+/−) with antibiotics for 3 weeks beginning at weaning as previously described (Kang et al., 2008). We gavaged the mice with ΔanSME mutant and WT B. theta strains 2 days after the cessation of antibiotics and performed analysis 3 weeks thereafter. Compared with WT B. theta, the ΔanSME strain was unable to elicit disease in dnKO mice as determined by histologic analysis of the colons (Figure 3A; Figure S3S for additional controls) and quantification of crypt loss and epithelial hyperproliferation (Figures 3B and 3C). As a control, when the ΔanSME B. theta mutant was genetically complemented (ΔanSME::anSME), it

Figure 3. The B. theta anSME Gene Is Necessary and Sufficient for Causing Colitis in dnKO Mice
(A) H&E-stained rectal sections from dnKO and littermate controls (IL10rb+/−) 3 weeks after gavage with B. theta strains or PBS. dnKO mice were gavaged with PBS (A1), WT B. theta (A3), ΔanSME (A4), and ΔanSME::anSME (A5), and a littermate control (IL10rb+/−) was gavaged with WT B. theta (A2). For each low-power image (100x) shown per group, a high-power image (400x) is included (boxed region adjacent to the low-power image). Bars, 200 μm for 100x images. Bars, 30 μm for 400x images.
(B and C) Graphs of average (B) crypts per 400x field (0.55 mm) and (C) M-phase cells per 100 crypts are shown for different groups of gavaged dnKO mice ± SEM. 
(D) Graph of colonization at day 4 of dnKO mice and littermate control by B. theta strains via qPCR ± SEM. One-way ANOVA analysis: (B) F = 15.70, p < 0.0001, n ≥ 7 per group; (C) F = 21.79, p < 0.0001, n ≥ 7 per group; (D) F = 116.4, p < 0.0001, n ≥ 9 per group. Means with different letters are significantly different by Tukey’s multiple comparisons test. See also Figure S3.
stimulated colitis similarly to the WT parent strain (Figures 3A–3C). All B. theta strains colonized control and dnKO mice to similar levels demonstrating that the observed colitogenic differences between WT B. theta and the ΔanSME strains were not due to quantitative differences in overall colonization (Figure 3D; Figures S3T, S3U, and S1D for additional controls). These data support a role for bacterial sulfatases in B. theta’s colitogenic potential.

**OMVs Gain Access to Host Immune Cells in B. theta-Colonized dnKO Mice**

We next tested if B. theta’s OMVs accessed host immune cells in a sulfatase-dependent manner. Immunogold localization in dnKO mice gavaged with WT B. theta showed 6E9-positive vesicles in mesenchymal cells that were morphologically consistent with macrophages (Figures 4A and S4A–S4D). Then, to determine if OMVs required anSME to activate macrophages in the absence of the mucus barrier, we incubated bone marrow macrophages from dnKO mice with OMVs from either WT or ΔanSME B. theta strains in vitro and found that OMVs from both strains were internalized by these cells (Figures 4B, 4C, and S4E). As expected, macrophages from dnKO mice were more potently activated by an OMV stimulus when compared to macrophages from WT mice. However, OMVs from either bacterial strain were equally able to elicit TNFα production from either macrophages (Figure 4D). To determine the production of inflammatory modulators by colonic macrophages exposed in vivo to B. theta, we extracted colonic macrophages after gavage and then analyzed gene expression. mRNA for Ptg2 (a.k.a. Cox-2), TNFα, and IL1-β were all significantly enriched in dnKO mice gavaged with WT B. theta as compared to PBS and ΔanSME B. theta groups (Figures S4F–S4H). Lastly, we performed double-label immunofluorescence using 6E9 and F4/80 on dnKO mice gavaged with WT B. theta. In these mice, we readily identified macrophages that were 6E9 positive (Figures 4E and 4F). In contrast, in dnKOs gavaged with ΔanSME B. theta, we rarely observed macrophages with 6E9 staining (Figures 4E and 4F). Additionally, we performed double-label immunofluorescence using 6E9 and CD11c on colonic sections of dnKOs gavaged with WT B. theta and did not see any co-localization, suggesting these cells are not dendritic cells. Together, these data show that only WT B. theta OMVs can efficiently access mucosal macrophages in vivo and suggest that B. theta accesses and activates the host immune system in a sulfatase-dependent manner.

**DISCUSSION**

In this study, we showed that antigen from B. theta, a symbiotic bacterium, localizes to the mucosa of a host that is genetically susceptible to the development of colitis. We also determined that B. theta’s anSME gene, which activates all of its sulfatases, is necessary and sufficient for B. theta’s colitogenic behavior in dnKO mice. Furthermore, we found that B. theta OMVs localized to host immune cells in vivo in a sulfatase-dependent manner. Taken together, these results suggest that B. theta OMVs can access the host’s immune cells in a sulfatase-dependent fashion.

OMVs are pinched off fragments of the outer membrane and periplasm of Gram-negative bacteria such as B. theta that are 20–250 nm in diameter (Kulp and Kuehn, 2010). Most studies of OMVs to date have been performed using E. coli and Pseudomonas, which are relatively poor producers of OMVs as evidenced by the fact that OMVs make up < 1% of the outer membrane material in bacterial cultures (Bauman and Kuehn, 2006; Gankema et al., 1980). In contrast, B. theta is very efficient in producing OMVs, making it an ideal model organism for further studies (Elhenawy et al., 2014). OMVs have three general features that highlight B. theta’s mechanism of access. First, OMVs enable bacterial products to spread to locations that are inaccessible to bacteria (Kulp and Kuehn, 2010). Second, OMVs can be readily engulfed by host cells (Irving et al., 2014). Third, OMVs contain a variety of functional bacterial products including protein toxins, adhesins, and enzymes (Elhenawy et al., 2014; Ellis and Kuehn, 2010). Specifically, B. theta produces a variety of enzymes that degrade host-derived carbohydrates (Elhenawy et al., 2014; Martens et al., 2008, 2009), and some of these enzymes have recently been shown to be contained in OMVs produced by this species (Elhenawy et al., 2014). These properties of OMVs likely contribute to the ability of B. theta OMVs to access the host’s immune cells.

OMVs are an obvious candidate for mediating microbial-host interactions of certain symbiotic families. The challenge is how to visualize the localization of parent bacteria and their specific OMVs with respect to the host. For the parent microbe, a number of tools exist for denoting localization including 16S DNA probes (Swidsinski et al., 2005) and antibodies (Round et al., 2011). However, there are few reagents to determine OMV localization. The monoclonal antibodies described in this study, 3H2 and 6E9, are efficient and specific to Bacteroides species and their OMVs, respectively. We anticipate that these reagents will be valuable tools in further studies of B. theta-host interactions.

OMVs can be taken up by a variety of host cells (Irving et al., 2014). In our study, we showed that F4/80-positive, CD11c-negative immune cells, consistent with macrophages, engulfed B. theta’s OMVs. The gastrointestinal tract contains a high number of macrophages with a large proportion in the colon (Hume et al., 1984; Lee et al., 1985). Intestinal macrophages typically reside in the lamina propria adjacent to the base of crypts (Hume et al., 1984). Interestingly, WT macrophages have been proposed to lack pro-inflammatory responses when primed by bacteria or their products (Bain et al., 2013; Smythies et al., 2005). In contrast, it has been suggested that macrophages in IL10RII-deficient mice fail to respond to tolerogenic signals and become pro-inflammatory and potentially drive colitis (Shouval et al., 2014). Future studies are necessary to better understand how resident macrophages are involved in inflammatory diseases.

It is possible that B. theta sulfatases may be involved in disease in genetically susceptible human hosts. Normally, gastrointestinal mucus acts as a protective barrier, lubricant, and bacterial habitat (Johansson et al., 2008). Mucin glycans are protected from enzymatic breakdown by sulfate, sialic acid, and ester modifications (Mian et al., 1979; Podolsky and Iselbacher, 1983; Tsai et al., 1992). Clinical studies of patients with inflammatory bowel disease suggest that alterations in sulfatase activity may be important in this disease. For example, human fecal samples from IBD patients have elevated sulfatase...
Figure 4. OMVs Gain Access to Host Macrophages in B. theta-Colonized dnKO Mice

(A) TEM image of 6E9-positive vesicle located within a cell consistent with a macrophage in the colonic mesenchyme of a WT B. theta-gavaged dnKO mouse labeled with 6E9 mAb/goat anti-mouse IgG antibody conjugated to 18 nm colloidal gold. Bar, 100 nm.

(B) Co-localization of macrophages (CFSE, green) derived from dnKO mice cultured with OMVs from WT B. theta (Dil Vybrant dye, red). Bar, 50 μm. Bar, 5 μm (inset).

(C) Graph of the percent of CFSE+ macrophages that co-localized with OMVs ± SEM. Unstimulated macrophages from IL10rb+/- and dnKO mice were used as a control.

(D) Concentration of TNF-α (pg/ml) in the macrophage supernatant from IL10rb+/- or dnKO mice cultured with OMVs from WT or ΔanSME B. theta ± SEM.

(E) Staining of colonic mucosa from dnKOs gavaged with WT or ΔanSME B. theta with F4/80 (green) and 6E9 (red) antibodies. White dashed lines, outlined crypts. Bar, 20 μm. Bar, 2.5 μm (inset).

(F) Graph of percentage of double-positive F4/80+ and 6E9 + cells per crypt-associated mesenchyme in dnKO gavaged with PBS or B. theta strains ± SEM. One-way ANOVA analysis: (C) F = 10.65, p < 0.0001, n = 4 per group; (D) F = 96.11, p < 0.0001, n = 4 per group; (F) F = 5.86, p = 0.01, n ≥ 6 per group. Means with different letters are significantly different by Tukey’s multiple comparisons test. See also Figure S4.
activity (Corfield et al., 1992). Also, colonic mucin in ulcerative colitis patients was shown to be degraded much more quickly than normal mucin (Corfield et al., 1996). Thus, the findings of our study emphasize how B. theta sulfatases may play a role in inflammatory diseases and have the potential as targets for drug research.

In conclusion, this work has opened up several avenues of research into microbial access to the host immune system. First, we have shown OMVs to be a mode by which bacteria access the mucosa of a genetically susceptible host. Second, we have introduced antibodies that can identify Bacteroides in situ. Third, we have given credence to the idea that macrophages may be critical in the development of inflammatory diseases in a genetically susceptible host. Lastly, we have revealed a new potential microbial drug target for inflammatory intestinal diseases, (i.e., anSMa), which is present in most anaerobic gut bacteria.

EXPERIMENTAL PROCEDURES

For more details, please see Supplemental Experimental Procedures.

Mice

All experimental procedures were performed under approval by Washington University’s Animal Studies Committee. Il10r2−/− and dnKO mice were housed in an enhanced specific pathogen-free facility. Antibiotic treatment with ciprofloxacin and metronidazole began at weaning.

Anti-B. theta Monoclonal Antibody Generation

CS7BL/6 mice were immunized with killed B. theta (BTS482) and boosted, and then splenic B cells were fused with P3Ag8.6.5.3 myeloma cells to create hybridomas (Kearney et al., 1979). The monoclonal antibodies were fluorescently labeled with Alexa dyes.

Tissue Harvest, Fixation, and Preparation for Histology

Mouse tissues were fixed in methacarn, rinsed in methanol and ethanol, and then stained with H&E and Alcian blue (pH 1.0).

Immunofluorescence Analysis

The 3H2 mAbs were directly conjugated to Alexa 647, and the 6E9 mAbs to Alexa 594. The M.O.M. Kit was the blocking agent for 3H2 staining, and the SNIPER for 6E9.

B. theta ELISA

B. theta were grown to log phase, then fixed in methacarn. Immulon 2 plates were coated with poly L-lysine. Plates were coated with dilutions of fixed bacteria O/N at 4 °C. Plates were washed, blocked, and incubated. The ELISA was developed with biotin-goat anti-mouse IgG followed by streptavidin peroxidase and 1-Step Ultra TMB-ELISA.

Preparation of OMVs

B. theta OMVs were purified with multiple rounds of centrifugation and filtering (Chutkan et al., 2013).

Immunogold Labeling

Samples were allowed to absorb onto formvar/carbon-coated copper grids, washed in dH2O, and stained with 1% aqueous uranyl acetate. Samples were viewed on a JEOL 1200EX transmission electron equipped with an 8 megapixel digital camera (Advanced Microscopy Techniques).

Immunoblots for B. theta Antigen

Bacteria or OMVs were resuspended in PBS and sonicated. Samples were loaded on gels for SDS-PAGE and western blotting using the Amersham ECL Prime Western Blotting Detection Reagent following the manufacturer’s instructions. Primary antibody 6E9 was used at a 1:5,000 dilution, and HRP goat anti-mouse IgG was used at 1:3,000.

Sulfatase Assay

A sulfatase assay was performed (Benjdia et al., 2011). Briefly, sulfatase activity was measured at 25 °C in 600-µl mixture of 10 mM of the substrate p-nitrophenyl sulfate. One unit of activity was defined as the release of 1 µmol of product per minute per mg of protein.

Development of Bacterial B. theta Mutants

The B. theta strain VPI-5482/ATCC 29148 was manipulated for generation of all mutants. Genetic deletion of the B. theta anSMa gene was performed using allelic exchange (Benjdia et al., 2011; Cameron et al., 2014).

Colitis Experiments

dnKO mice and littermate controls (IL10−/−) were given antibiotics for 3 weeks after weaning and then taken off antibiotics for 2 days prior to gavage and sacrificed at 21 days (Bloom et al., 2011).

In Vitro Macrophage Assays

Bone marrow cells were isolated from dnKO or IL10−/− littermate controls and cultured in M-CSF (Hume et al., 1985). Macrophages were co-cultured with purified OMVs from either WT B. theta or ΔanSMa. Supernatants were collected and assayed for the concentration of TNF-α by ELISA.

Statistical Analysis

Statistical analysis was performed using Prism v3.02 and v5.01 (GraphPad Software). Significance was determined with one-way ANOVA and defined as p < 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2015.04.002.

AUTHOR CONTRIBUTIONS


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