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Diet modulates colonic T-cell responses by regulating the expression of a *Bacteroides thetaiotaomicron* antigen

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Abstract

T cell responses to symbionts in the intestine drive tolerance or inflammation depending on the genetic background of the host. These symbionts in the gut sense the available nutrients and adapt their metabolic programs to utilize these nutrients efficiently. Here, we ask whether diet can alter the expression of a bacterial antigen to modulate adaptive immune responses. We generated a CD4⁺ T cell hybridoma, B θ OM, specific for *Bacteroides thetaiotaomicron* (*B. theta*). Adoptively transferred transgenic T cells expressing the B θ OM TCR proliferated in the colon, colon-draining lymph node, and spleen in *B. theta* colonized healthy mice and differentiated into regulatory T (T_{reg}) and effector T (T_{eff}) cells. Depletion of *B. theta*-specific T_{regs} resulted in colitis, showing that a single protein expressed by *B. theta* can drive differentiation of T_{regs} that self-regulate T_{effs} to prevent disease. We found that B θ OM T cells recognized a peptide derived from a single *B. theta* protein, BT4295, whose expression is regulated by nutrients, with glucose being a strong catabolite repressor. Mice fed a high glucose diet had a greatly reduced activation of B θ OM T cells in the colon. These studies establish that the immune response to specific bacterial antigens can be modified by changes in the diet by altering antigen expression in the microbe.

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One Sentence Summary:

Diet alters symbiont-specific immune responses via regulation of the expression of an outer membrane vesicle antigen.

Introduction

Dietary components and metabolites produced by host and microbial enzymes modulate the function of a variety of host immune cells including T cells (1–3). These products can have local effects on the intestinal immune system as well as more distant organs (4). For instance, host enzymes break down starch and various disaccharides in the diet to produce glucose, which is required systemically for maximal effector T (T_{eff}) cell stimulation (5, 6). Microbial metabolites derived from dietary fiber, flavonoids, and amino acids such as tryptophan have immunomodulatory activities (3, 7–10). As examples, short chain fatty acids from fiber fermentation promote the development of intestinal regulatory T (T_{reg}) cells (3), modulate macrophage polarization (11), and suppress innate lymphoid cell development (12). Further, tryptophan catabolites act via the aryl hydrocarbon receptor to induce T cell cytokine production (13); taurine-conjugated bile acids formed from milk-derived dietary fat induces a pro-inflammatory T helper type 1 immune response (14), and lastly, the microbial metabolite desaminotyrosine derived from flavonoids stimulates type I interferons and modulates macrophage activation and cytokine production (15). Recently, ascorbate, a microbial metabolite altered in Crohn's disease, has been shown to modulate T cell activity (16). Other dietary components such as excess salt can change the composition of the microbiome and favor pathogenic T helper 17 (T_H17) responses (17). Conversely, an iron-deficient diet can dampen intestinal inflammation (18). Collectively these studies reveal the dominant effects of dietary components and their immediate or downstream metabolites on the immune system.

$CD4^+$ T cells play a critical role in the response to specific microbial antigens in the intestine (19–23). Symbiotic bacteria that do not damage the host produce tolerogenic T_{regs} responses while pathogens that cause damage elicit T_{effs} responses. In both cases, microbe-specific antigens drive these responses, and these intestinal bacterial are well known to be modulated by diet. However, the effect of diet on T cells that recognize these different groups of symbionts has not been tested. This latter question is of importance due to the effects of diet on the composition and physiology of the microbiome, which has a multitude of effects on the host. It is unclear if specific dietary components have effects at the level of specific bacterial antigens and the T cells that recognize them.

We hypothesize that the $CD4^+$ immune response to specific bacterial antigens can be modified by changes in the diet through effects on antigen expression of the microbe. Progress in this area has been hampered by the lack of a model system in which a $CD4^+$ T cell response can be examined for a specific gut symbiont. To this end, we developed a $CD4^+$ T cell model, termed B θ OM, specific for an outer membrane (OM) antigen from *Bacteroides thetaiotaomicron* (*B.theta*, B θ). *B. theta* is a prototypic gut symbiont that degrades a wide variety of dietary, host, and microbial glycans, and is a representative of a prominent genus found in most human microbiomes (24). In healthy mice gavaged with *B.*

theta, we found that TCR-transgenic B θ OM T cells responded *in vivo* by differentiating into T_{reg} and T_{eff} cells. Deletion of the B θ OM T_{regs} induced colitis by activated B θ OM T cells, revealing that the symbiont-specific CD4⁺ T cells were no longer able to self-regulate to prevent T cell-mediated disease. The *B. theta* antigen recognized by B θ OM T cells was identified to be BT4295, an outer membrane protein contained in one of *B. theta*'s many polysaccharide utilization loci (PUL). We found that we can modify the response of B θ OM T cells to their cognate antigen by altering the salts and glycans available to *B. theta*. Interestingly, glucose was identified as a catabolite repressor of BT4295 expression. Mice fed a high glucose diet had greatly reduced activation of B θ OM T cells, establishing a direct link between dietary regulation of a microbial antigen and CD4⁺ T cell activation. These results show that specific dietary components can alter the T cell driven immune response to dominant symbiotic antigens.

Results

The *B. theta*-specific CD4⁺ T cell response is sensitive to changes in *B. theta* growth media

To determine how dietary components and metabolites can affect the interactions between a symbiont and the host immune system, we developed a bacteria-specific CD4⁺ T cell model. We chose to focus our study on *B. theta*, a model gut symbiont that is known to adapt to changes in the available nutrients, especially by changing expression of carbohydrate utilization gene loci. We immunized C57BL/6J mice with the human *B. theta* strain VPI-5482 (herein referred to as *B. theta*) and produced T cell hybridoma cell lines that responded to *B. theta*. We screened the T cell hybridomas for reactivity against *B. theta* outer membrane vesicles (OMVs), which have been shown to be a source of antigen to the immune system (25). To identify a T cell sensitive to changes in available nutrients, we took advantage of a fortuitous observation that *B. theta* grown in two different formulations of TYG media—classic TYG (TYG) and modified TYG (mTYG) (Table S1)—stimulated T cells differently. We chose one T cell hybridoma clone (herein denoted as *B. theta* outer membrane or “B θ OM”) that showed a robust response to both *B. theta* and OMVs in T cell stimulation assays (Fig. 1, A and B). When we cultured B θ OM T cell hybridomas with bone marrow-derived macrophages (BMDM) along with *B. theta* grown in the different media, B θ OM T cell activation was highest with *B. theta* grown in TYG media (Fig. 1C); no stimulation of these T cells was observed when *B. theta* was grown in mTYG media (Fig. 1C). Thus, B θ OM T cells were sensitive to changes in the nutrients in the media used to grow *B. theta*.

We next created a transgenic mouse line expressing the B θ OM T cell receptor genes on a C57BL/6J-*Rag1*^{-/-}-CD45.1 genetic background (B θ OM *Rag1*^{-/-} mouse strain). The TCR transgenic T cells from this line were I-A^b restricted, expressed V α 1 and V β 12 (Fig. 1D), and were specific for *B. theta* (human or mouse isolates) (Fig. 2A). The peripheral T cells from B θ OM *Rag1*^{-/-} mice were essentially all naive, expressing low levels of CD69, CD25, and CD44 proteins (Fig. 1E); the thymus was also devoid of CD8⁺ T cells (Fig. 1F). We found that B θ OM transgenic mice develop few if any thymic or peripheral T_{regs} compared to non-transgenic C57BL/6J mice (Fig. 1G). Isolated naive T cells from B θ OM *Rag1*^{-/-} mice could be activated when stimulated *in vitro* with BMDM incubated with either *B. theta* or

OMVs (Fig. 2, A and B). Stimulation of the B θ OM TCRtg T cells by *B. theta* was confirmed to be sensitive to nutrients in TYG media (Fig. 2C), enabling the use of B θ OM T cells to study the effect of diet on symbiont-host interactions.

We then evaluated the function of B θ OM T cells *in vivo* by transferring them into antibiotic pre-treated *Rag1*^{-/-} mice. Mice were pre-treated with antibiotics for three weeks to allow colonization with the subsequently gavaged human-isolate of *B. theta*, which we previously showed colonize mice under these conditions (26). Sorted naive (CD44^{lo}CD62L^{hi}) CD25⁻CD4⁺CD45.1⁺ B θ OM T cells (fig. S1A) were transferred into *Rag1*^{-/-} mice that had been previously colonized by *B. theta* for four days (fig. S1B). We identified CD4⁺CD45.1⁺ T cells in the lamina propria, colon- draining lymph node (cdLN) or one lymph node among the mesenteric lymph nodes (mLN) that drains the colon, and spleen 7 days after T cell transfer (Fig. 3, A and B). In these mice, B θ OM T cell localization in the colon lamina propria and cdLNs was dependent on *B. theta* colonization (Fig. 3C). We also found B θ OM T cells in the spleen of *B. theta* colonized *Rag1*^{-/-} mice (Fig. 3C). The B θ OM T cells proliferated in the lamina propria, cdLN, and spleen, revealing that they were exposed to their cognate antigen (Fig. 3, D and E). *B. theta* gavaged B θ OM *Rag1*^{-/-} mice did not have obvious signs of disease such as weight loss (fig. S2A).

B θ OM T cells differentiate into T_{effs} and T_{regs} that self-regulate to prevent colitis

Because *Bacteroides* have been previously shown to be strong drivers of T_{reg} induction (27), we reasoned that the B θ OM T_{regs} would mediate tolerance to *B. theta*. We transferred B θ OM T cells into *Rag1*^{-/-} mice; the transferred cells were pre-sorted for CD4⁺CD44^{lo}CD62L^{hi}CD25⁻ to ensure that there was no transfer of preexisting T_{regs} into recipients (fig. S1A). Characterization of the B θ OM T cells in multiple locations showed a mixture of T_{eff} and FoxP3⁺ T_{reg} cells in the lamina propria and cdLN with a lower percentage of T_{regs} found in the spleen (Fig. 4, A to C). T_{reg} development in the peripheral lymphatics and the colonic tissue was dependent on *B. theta* colonization because few to no T_{regs} were found in PBS gavaged mice (Fig. 4B and fig. S1B). Despite the presence of T_{regs} in both the cdLN and colonic lamina propria, the cdLNs had many more T_{regs} expressing CD25 than the colon, where the majority of T_{regs} expressing FoxP3 lacked CD25 expression (Fig. 4D). Consistent with previous reports with polyclonal T_{regs} exposed to *Bacteroides* in the lamina propria (28, 29), 50% of B θ OM FoxP3⁺ T_{regs} express ROR γ t (fig. S1C). This finding is also consistent with a report showing that in healthy wild-type mice, pathobiont-specific T cells differentiate into ROR γ t-expressing specific iT_{reg} cells in the large intestine (30). Together, these data reveal that the same TCR can differentiate into both T_{eff} and T_{reg} cells (19).

We hypothesized that *B. theta*-specific T_{regs} produced sufficient regulation in the colonic mucosa to prevent *B. theta*-specific CD4⁺ T cells from inducing colitis upon exposure to *B. theta*. To test this hypothesis, we crossed the B θ OM transgenic mouse to FoxP3-DTR-GFP mice, which permits the *in vivo* depletion of T_{regs} upon diphtheria toxin (31) treatment and includes a GFP marker for T_{reg} cell identification (30). We transferred naive, GFP^{lo} B θ OM T cells into *Rag1*^{-/-} mice colonized with *B. theta* that were treated with DT on days 9, 11, and 13 (Fig. 5A). We confirmed depletion of T_{regs} in the cdLNs and spleen (Fig. 5B). We

found that *Rag1*^{-/-} mice that received BθOM-FoxP3-DTR cells and DT developed colitis, with an increase in hyperproliferative crypts, epithelial proliferation, lymphocyte infiltrate, mitotic figures, and crypt height compared to control mice that received BθOM T cells and DT (Fig. 5, C to E). Cells isolated from the mLN of *Rag1*^{-/-} mice transferred with BθOM-FoxP3-DTR T cells and treated with DT to deplete T_{regs} showed an increase in proinflammatory cytokines (IL-17A, IFN γ , IL-6) compared to cells isolated from *Rag1*^{-/-} mice receiving wild type BθOM T cells and treated with DT (Fig. 5F and fig. S3, A and B). Both BθOM-FoxP3-DTR T cells and wild type BθOM T cells isolated from the colon lamina propria and mLN differentiated into T_H1 cells (fig. S4A). BθOM-FoxP3-DTR T cells can also differentiate into T_H17 cells; however, variable levels of T_H17 induction were observed between experiments (fig. S4, B and C). These findings are a direct demonstration that a symbiont-specific CD4⁺ T cell can develop into both T_{eff} and T_{reg} cells and that these T_{regs} can self-regulate.

The antigen recognized by BθOM T cells, BT4295, is expressed in a polysaccharide utilization locus

To elucidate how diet could affect a bacterial antigen expression, we needed to identify the antigen recognized by BθOM T cells. To identify this *B. theta* antigen, we used positive functional fractionation, mass spectrometry, and a loss-of-function screen. Using *B. theta* OMVs as the starting material, we performed a T cell activation assay from 20 fractions of isolated proteins separated based on molecular weight (Fig. 6A and fig. S5A). We found a single fraction of *B. theta* OMV proteins that stimulated BθOM T cells (Fig. 6A). Mass spectrometry analysis of this fraction identified 322 distinct proteins (Fig. 6A). To refine the list of potential antigens, we generated a *B. theta* transposon insertion library (32) and screened individual clones using the *in vitro* T cell activation assay for BθOM T cells (Fig. 6A). In a screen of 2,300 clones, we identified five genes that when knocked out no longer stimulated BθOM T cells (Fig. 6A and fig. S5B). One of the five *B. theta* gene candidates (BT4298) was identified in the mass spectrometry analysis (Fig. 6A). The other 4 hits were all in one additional unlinked locus (BT1220–23) containing genes encoding enzymes in the pentose phosphate pathway.

Expression in *E. coli* of the BT4298 protein identified in both the mass spectrometry and transposon library, unexpectedly, did not stimulate BθOM T cells (Fig. 6C). However, many bacterial genes are organized into co-transcribed operons and this is likely to be true for *B. theta*. For example, the BT4294–4300 polysaccharide utilization locus (PUL) was previously shown to be coordinately activated in response to mucus O-linked glycans (26, 33). We therefore reasoned that the transposon insertion in the BT4298 gene exerts loss of function effects on downstream genes due to polarity (Fig. 6B), including BT4295, which was also identified in our mass spectroscopy analysis (Fig. 6A). Expression of BT4295 in *E. coli* resulted in strong stimulation of BθOM T cells (Fig. 6C), demonstrating the BT4295 was the antigen recognized by BθOM T cells. BT4295 is predicted to be a SusE/SusF lipoprotein that is ultimately trafficked to the outer membrane, including OMVs (fig. S6A) (26). We confirmed that BT4295 was the only antigen recognized by BθOM T cells by generating an in-frame deletion mutant of BT4295 that disrupted its expression (BT 4295), and abolished its ability to stimulate BθOM T cells (Fig. 6D).

To identify the epitope in BT4295 recognized by B θ OM T cells, we expressed amino and carboxyl halves of the protein in *E. coli* (Fig. 6E). We found that the carboxyl half of the protein activated B θ OM T cells (Fig. 6E). We then generated overlapping 20-mer peptides for the entire carboxyl half of BT4295 and tested them for their ability to activate B θ OM T cells. A single peptide (536–555) stimulated B θ OM T cells (Fig. 6F). The antigenic epitope was further defined to be the highly stimulatory 14-mer, (541–554) (EEFNLPPTNGGHAT), which contains a strong predicted I-A^b binding motif (P1 = F543) (fig. S6B). We identified the threonine at the P5 position (T547) to be critical for TCR recognition and generated a point mutation at the P5 position (a threonine to a valine substitution, T547V) that resulted in the complete loss of B θ OM T cell activation (Fig. 6G). Taken together, these findings demonstrate that B θ OM T cells strongly and specifically recognize a single peptide epitope (BT4295_{541–554}) in the BT4295 protein, which is expressed in the *B. theta* outer membrane in response to mucin-type O-glycan (MOG) cues.

Expression of BT4295 is regulated by available nutrients

Having identified BT4295 as the antigen recognized by B θ OM T cells, we determined how specific nutrients altered its expression. Based on the differential ability of *B. theta* grown in TYG versus mTYG media to stimulate B θ OM T cells (Fig. 2C), we asked whether removing specific components (Table S1) from the TYG media or adding them to the mTYG media would alter the stimulatory ability of *B. theta* grown in these modified media. Individually removing vitamin B₁₂, vitamin K₃, histidine, cysteine, FeSO₄, or MgCl₂ from TYG media had no effect on the ability of *B. theta* to stimulate B θ OM T cells (fig. S7, A and B). However, when we removed salts (KH₂PO₄, (NH₂)₄SO₄, and NaCl) from TYG, *B. theta* grown in this altered media no longer stimulated B θ OM T cells (fig. S7, A and B). Because removing salts from the TYG media did reduce *B. theta* growth to some extent, we also tested the addition of these salts to mTYG media that contained significantly lower concentration of salts (KH₂PO₄, K₂HPO₄, and NaCl) (Fig. 7A). Interestingly, adding TYG salts to mTYG media resulted in a significant increase in B θ OM T cell activation (Fig. 7A). The ability of *B. theta* grown in TYG, mTYG, and mTYG with TYG salts to stimulate B θ OM T cells directly correlated with the level of BT4295 protein expression as determined by a quantitative ELISA (Fig. 7B, fig S7C).

Previous transcriptional analysis showed that in the absence of dietary glycans, *B. theta* *in vivo* increases the expression of the BT4294–4300 PUL likely to breakdown endogenous mucin glycans, which is supported by *in vitro* expression of this PUL in response to purified mucin glycans (34, 35). Therefore, we tested whether growing *B. theta* in mTYG with porcine MOG would increase expression of BT4295 and drive B θ OM T cell activation. We found that *B. theta* grown in mTYG supplemented with MOG now strongly activated B θ OM T cells (Fig. 7C) and led to increased BT4295 protein expression (Fig. 7D). Thus, BT4295 expression can be up-regulated by MOG in mTYG media, which alone did not induce expression. Together these findings demonstrate that by changing available nutrients (salts or glycans), the expression of a specific symbiont-derived antigen can be dramatically affected.

Glucose catabolically represses BT4295

The four transposon mutants hits in the pentose phosphate pathway that significantly decreased expression of BT4295 (Fig. 6A) implicated glucose metabolism as another potential regulator of BT4295 expression. To test the involvement of glucose on regulation of BT4295 expression, we eliminated glucose from the TYG and mTYG media (Table S1). *B. theta* grew in both media in the absence of glucose, but at slightly reduced rates. Remarkably, we found that BθOM T cells were now stimulated by *B. theta* grown in mTYG in the absence of glucose (Fig. 8A). Similarly, *B. theta* grown in TYG without glucose also stimulated BθOM T cells, even stronger than in the presence of glucose (Fig. 8A). Thus, glucose appeared to be acting as a repressor of BT4295 expression. Catabolite repression is a well-established regulatory process in bacteria, including *B. theta*, in which other metabolic pathways are repressed in the presence of glucose or other high priority nutrients (36, 37). Using a quantitative ELISA for BT4295 protein, we tested if the increase in stimulatory ability of *B. theta* grown in the absence of glucose was due to increased BT4295 protein expression. Removing glucose from the mTYG media resulted in a 14.5-fold increase in the expression of BT4295 and removing it from TYG media resulted in a 4-fold increase (Fig. 8B). This finding again shows a direct correlation between the level of BT4295 protein expression and the ability to stimulate BθOM T cells, providing proof that glucose is acting as a repressor of BT4295 expression. From these findings we conclude that in the presence of glucose, *B. theta* shuts down the expression of the BT4294–4300 PUL, thereby reducing production of the BT4295 antigen.

Dietary glucose decreases the stimulation of BθOM T cells *in vivo*

We next determined if exogenous glucose affected the ability of BθOM T cells to be stimulated *in vivo* by decreasing BT4295 expression. We added 30% glucose to the drinking water of recipient mice and maintained them on the standard chow throughout the course of the experiment. The addition of 30% glucose to the drinking water had no effect on *B. theta* colonization levels (fig. S8A). Dramatically, the number of BθOM T cells in the colon and cdLN decreased in the recipient mice fed 30% glucose (Fig. 8C). Although there was no difference in T_{regs} (fig. S8B), the number of activated BθOM T cells was also decreased (Fig. 8D). Thus, with a high glucose diet, BT4295 antigen expression is decreased, resulting in weaker stimulation of the BθOM T cells. This finding establishes that diet can affect the expression of a specific symbiont antigen and modulate a CD4⁺ T cell response *in vivo*.

Discussion

We developed a symbiont-specific T cell model to study how diet could affect the interactions between a symbiont and the host immune system. We show that BθOM T cells respond to *B. theta* and OMVs, but not other *Bacteroides* family members. Next, we identified BT4295, a SusE/F homolog, as the BθOM T antigen. Transfer of BθOM T cells into *B. theta* colonized *Rag1*^{-/-} mice showed that antigen-specific T cells differentiate into T_{regs} and T_{eff} cells. Upon depletion of BθOM T_{regs}, the BθOM T_{eff} cells cause colitis. We show that the expression of BT4295 can be altered by glycans, salts, and glucose. A high glucose diet reduced activation of the BθOM T cells, making BT4295 a nutrient-sensitive

antigen able to alter T cell responses to microbes. This study definitively shows that diet can play a role in altering antigen expression thereby affecting immune responses.

TCR transgenic models have been previously developed to study antigen-specific responses to gut microbes. T cells specific for segmented filamentous bacteria (SFB) in the small intestine have revealed how symbiotic microbes contribute to driving organ-specific autoimmunity (23). The CBir1 Tg T cell is widely used to study antigen-specific microbial interactions (21); however, it does not recognize its antigen during homeostasis despite the abundance of microbial antigen in the lumen (38). More recently, *Helicobacter* species-specific transgenic T cells were shown to respond differently during homeostasis and mucosal injury/inflammation (19, 29). In all of these cases, microbial antigens were not shown to cross the epithelial barrier except in the context of inflammation. Therefore, we developed a symbiont-specific T cell that responds to *B. theta* and OMVs, a relevant source of antigen that crosses the colonic epithelium and interacts with the host immune system during homeostasis (25, 39).

Though our study focused on a single T cell and its cognate antigen, this approach is likely relevant because of the concept of immunodominance. Despite a theoretically large number of potential microbial epitopes, which can be recognized by CD4⁺ T cells, the immune system generally focuses on a few immunodominant epitopes. As one example, the CD4⁺ T cell response in mice to SFB focuses on two dominant antigens of this microbe (23). We propose that the TCR we identified in this study may be specific for a dominant *B. theta* antigen.

Our data directly shows the conversion of a naive *B. theta*-specific T cell into T_{regs}. Using the DTR system, we deplete *B. theta*-specific T_{regs} and show that in the absence of these cells, symbiont-specific T cells cause colitis. To determine the mechanism of T_{reg} induction, we identified the antigen driving T cell activation. Previous reports in *B. fragilis* identified capsular polysaccharides on OMVs that induce T_{regs} (40) suggesting that bacterially derived polysaccharides have immunomodulatory effects on the host immune system. Our study extends the types of *Bacteroides* antigens that can participate in T cell development, including induction of T_{regs}.

One potential factor we have not controlled for is a direct effect of glucose on T cells. There is significant literature showing that glucose enhances T cell responses (31, 41, 42). To our knowledge, there are no reported studies showing that increased glucose *in vivo* would decrease T cell responses or homeostatic proliferation. Although we cannot definitively rule out that increased glucose *in vivo* was directly inhibiting B θ OM T cells, the literature supports our conclusion that increased dietary glucose caused a decrease in T cell proliferation due to a direct effect on BT4295 protein expression.

Inflammatory bowel disease (IBD) involves a potentially definable number of chronically activated T cells and microbial antigen specificities. We now show that specific TCR/cognate antigen pairs can be modulated by altering dietary components to affect gene expression of such a key microbial antigen. Future work developing additional TCR/antigen systems from other symbionts, including those that are enriched in IBD subjects, will be

valuable to test if this paradigm established with *B. theta* can be extended to other key microbial antigens. If glucose repression or salt stimulation of dominant microbial antigens is widespread, then such dietary manipulations may become effective for therapy.

Materials and Methods

Study Design

The objective was to generate a *B. theta*-specific T cell system (B θ OM T cells) to identify the interactions between immune system and an antigen expressed on a highly prevalent colonic symbiont and identify the role that diet plays in altering that interaction. We designed and performed experiments in cellular immunology, protein biochemistry, and mass spectrometry. The number of independent experiments is outlined in the figure legends.

Mice

All experimental procedures were performed under approval by Washington University's Animal Studies Committee. Mice were housed in an enhanced specific pathogen-free facility. B θ OM transgenic mice on the *Rag1*^{-/-} background were maintained by breeding to a non-transgenic *Rag1*^{-/-} mouse. B θ OM-FoxP3-DTR mice were generated by breeding B θ OM transgenic mice with FoxP3-DTR mice (30).

Generation of the B θ OM Transgenic mouse

B. theta was grown to confluence and washed with phosphate buffered saline (PBS). C57BL/6J mice were immunized subcutaneously in the rear footpads with *B. theta* mixed with incomplete Freund's adjuvant (Difco) in a 1:1 ratio. One week later, draining popliteal lymph nodes were harvested and stimulated *in vitro* with *B. theta* for 3 days. Stimulated T cells were fused following a standard protocol. Hybridomas were selected for responsiveness to *B. theta* presented by IFN- γ stimulated bone marrow-derived macrophages (BMDM). The B θ OM clone was selected for further analysis and its T cell receptor genes sequenced and cloned into TCR expression vectors (43). TCR α and β constructs were coinjected into C57BL/6J pronuclei in the Washington University Department of Pathology and Immunology's Transgenic Core Facility. Transgenic mice were identified by PCR amplification of the V α 1 and V β 12 transgenes from tail DNA (V α 1 forward primer GTTTCACAGCAGGTGTGAGGAG and reverse primer CAAAACGTACCAGGGCTTACC; V β 12 forward primer CTTCTCTTCTAGGTGATGCTG and reverse primer CCCAGCTCACCGAGAACAGTC)

Antibodies and Reagents

The following reagents were purchased from BD Biosciences: CD62L (MEL-14), CD45.1 (A20); Biolegend: CD4 (GK1.5), CD69 (H1.2F3), CD45.1 (A20), CD44 (IM7), CD25 (PC61), CD45.2 (104), CD25 (PC61), V β 12 (MR11-I), Mouse T_H1/T_H2/T_H17 Cytometric Bead Array Kit; eBiosciences: CD25 (eBio3C7), CD4 (RM4-5), FoxP3 (FJK-16s), IFN γ (XMG1.2), and IL-17A (TC11-18H10.1); Life Technologies: CellTrace CFSE Cell Proliferation Kit, LIVE/DEAD Fixable Blue Dead Cell Stain Kit; Roche: DNaseI from bovine pancreas grade II; Sigma: Collagenase from *Clostridium histolyticum*. Homemade

cocktail antibodies for negative selection of CD4⁺ T cells were purchased from Tombo: anti-mouse Ter-119, CD11c (clone N418), CD11b (M1/70), CD8 α (53–6.7), CD19 (1D3), and CD45R/B220 (RA3–6B2); Biolegend: CD49b (DX5), CD24 (M1/69); Miltenyi Biotec: anti-biotin microbeads.

Media recipes

TYG medium: BD Bacto: 10g/L tryptone, 5g/L yeast extract; Sigma: 4g/L D-glucose, 100mM KH₂PO₄, 8.5mM (NH₂)₄SO₄, 15mM NaCl, 10 μ M Vitamin K₃, 2.63 μ M FeSO₄•7H₂O, 0.1mM MgCl₂, 1.9 μ M hematin, 0.2mM L-histidine, 3.69nM Vitamin B₁₂, 413 μ M L-cysteine; Mallinckrodt: 7.2 μ M CaCl₂•2H₂O.

mTYG medium: BD Bacto: 20g/L tryptone, 10g/L yeast extract; Sigma: 5g/L D-glucose, 8.25mM L-cysteine, 78 μ M MgSO₄•7H₂O, 294 μ M KH₂PO₄, 230 μ M K₂HPO₄, 1.4mM NaCl, 7.9 μ M Hemin (hematin), 4 μ M Resazurin, 24 μ M NaHCO₃; Mallinckrodt: 68 μ M CaCl₂•2H₂O.

Preparation of OMVs

B. theta OMVs were purified with multiple rounds of centrifugation and filtering. (25)

Functional *in vitro* macrophage-T cell assay

BMDM were stimulated with IFN- γ at 2,000 U/ml in I-10 media (IMDM, 10% FBS, glutamine and gentamicin) and plated on a 96-well plate at 1 $\times$ 10⁵ cells per well. The cells were washed with PBS 24 hours later and kept in 100 μ l of fresh I-10 media without IFN- γ for another 24 hours. 5 $\times$ 10⁵ splenocytes or 1 $\times$ 10⁵ isolated B θ OM CD4⁺ T cells were added per well in 50 μ l with 50 μ l of half log dilutions of *Bacteroidaceae* strains and OMV.

Bacteroidetes were grown in a 5mL TYG or mTYG culture at 37°C overnight to mid-log phase. Cultures were washed twice with PBS and resuspended in media prior to adding to the assay. 24 hours later, the supernatant containing the T cells was transferred to a fresh 96-well plate and spun down at 1200rpm. The cells were washed with FACS buffer and stained for CD69 expression.

In vivo experiments

Bacterial stocks: *Bacteroidetes* were grown anaerobically from single isolates in standing culture in TYG at 37°C for 24 hours (33). Each culture was concentrated by centrifugation, mixed with sterile, pre-reduced PBS and glycerol to a final concentration of 20% glycerol, and frozen at –80°C in single-use aliquots.

Gavage: *Rag1*^{–/–} mice were placed on antibiotics at 3–4 weeks of age for 3–4 weeks. Antibiotic treatment consisted of 0.66mg/ml ciprofloxacin, 2.5mg/ml metronidazole (Sigma), and 20mg/ml sugar-sweetened grape Kool-Aid Mix (Kraft Foods) in the drinking water (44). Mice were gavaged with 100 μ l of antibiotic water on the first 2 days and the last 2 days of the 3–4 week duration. For the bulk of the experiments, mice were taken off antibiotic water and given Kool-Aid. For the *in vivo* glucose experiments, mice were taken off antibiotic water and given water or 30% glucose water. Two days later mice were

gavaged with 100µl of *B. theta* strains at a concentration of 1×10^8 CFU/ml. Fecal pellets were obtained on day 0, 4, and 7 to determine colonization.

BθOM T cell transfer: Three days after gavage, *Rag1*^{-/-} mice were injected with BθOM T cells isolated from the peripheral lymph nodes (axillary, brachial, and inguinal), mesenteric lymph nodes, and spleen. Cells were enriched by negative selection using a homemade cocktail of antibodies (see reagents) and sorted for CD4⁺CD44^{lo}CD62L^{hi}CD25⁻ T cells. $1-2 \times 10^5$ cells were injected retroorbitally.

Lamina propria dissociation: Seven days after T cell transfer, mice were sacrificed and leukocytes were isolated from the lamina propria following the Lamina Propria Dissociation Kit protocol published by Miltenyi Biotec.

Peripheral tissue processing: The colon draining lymph node (cdLN) and spleen were removed and processed using frosted microscope slides (Fisher Scientific). Samples were filtered through a 70µm filter.

Diphtheria toxin depletion of BθOM FoxP3⁺ Tregs

Treg depletion: Antibiotic treated *Rag1*^{-/-} mice were gavaged with *B. theta* and injected with enriched and sorted 1×10^5 BθOM-FoxP3-DTR or BθOM T cells. Intraperitoneal injections of diphtheria toxin (10µg/kg) were performed on days 9, 11, and 13 post gavage. Depletion was confirmed by staining for T_{regs} on day 21 post gavage in mLNs and spleen.

Cytokines: On day 21 post gavage, 5×10^4 mLNs and 2×10^6 splenocytes were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 5 hours at 37°C. T_H1/T_H2/T_H17 cytokines were quantified in the supernatant using the BD™ Cytometric Bead Array following the manufacturer's instructions. Supernatants from splenocytes samples were diluted 1:2.

T cell differentiation: On day 24 post gavage, cells isolated from the colon lamina propria and mLN were stimulated with PMA (50ng/ml) and ionomycin (500ng/ml) for 1 hour at 37°C, Brefeldin A was added (5µg/ml), and the cells were stimulated for 4 additional hours at 37°C. T_H1 and T_H17 cells were identified by intracellular staining with IFNγ and IL-17A antibodies.

Tissue Harvest, Fixation, and Preparation for Histology

Ceca and colons were fixed in methacarn for 12–16 hours at 24°C. Samples were washed with 100% methanol for 30 min x 2, followed by 100% ethanol for 20 min x 2 and then stored in 70% ethanol. 5µm sections were stained with hematoxylin and eosin (H+E). Representative images of cecal histology were taken with an Olympus BX51 microscope. Blinded microscopic analysis for mitotic figures using H+E-stained histologic sections was performed at 20X magnification on well-oriented crypts as previously described (44).

Fecal Bacterial DNA extraction and qPCR amplification

Fecal bacterial DNA extraction and qPCR amplification was performed according to a previously published protocol (25, 45).

T cell Western Assay

B. theta OMV antigens were separated using a T cell Western blot assay as described (46). Briefly, 500µg of OMVs were separated on a 10% SDS PAGE gel on both left and right sides of the gel with molecular weight standards on both sides. For the left side, the proteins were transferred to nitrocellulose, each lane cut into 20 strips, dissolved in DMSO, and precipitated with sodium carbonate/sodium bicarbonate. The nitrocellulose particles from each strip was tested for their ability to stimulate B00M T cells using BMDM as APCs. The corresponding position of the active fraction on the right side of the SDS-PAGE gel were further analyzed by mass spectrometry.

Proteomic analysis of OMVs

Proteomic analysis of the corresponding T cell stimulatory SDS-PAGE fraction of OMVs from TYG grown *B. theta* was performed using standard procedures at MS Bioworks, Ann Arbor, MI. Briefly, the gel slices were digested with trypsin and analyzed by nano LC-MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Q Exactive. The data were searched using Mascot against the UniProt *B. theta* reference proteome. Mascot DAT files were parsed into Scaffold for validation, filtering, and to create a non-redundant list per sample, requiring at least two unique peptides per protein.

*B. theta*iotaomicron Transposon Mutagenesis Library and Screen

Transposon mutagenesis of *B. theta* was performed as described previously (32). Briefly, mutagenesis was carried out on an Acapsular *B. theta* strain (CPS) lacking all capsular polysaccharide loci, which was previously characterized (37). Here, we used the pSAM_Bt vector containing *mariner* transposon and an *ermG* cassette. S17 *E. coli* was used to deliver the vector through conjugative transfer into *B. theta*. DNA isolation from selected mutants was performed using the Qiagen DNeasy Blood and Tissue Kit. Two-round PCR was performed to identify the transposon insertion site with the following conditions: round 1; 1 cycle at 95°C (3 min); 5 cycles 95°C (30 sec), 30°C (30 sec), 72°C (45 sec); 32 cycles at 95°C (30 sec), 55°C (30 sec), 72°C (45 sec). The PCR reactions from step 1 were purified using a Qiagen PCR Purification Kit and 100–200ng of product was used as template for round 2; 1 cycle at 95°C (3 min); 35 cycles at 95°C (30 sec), 55°C (30 sec), 72°C (45 sec). Reactions from round 2 were run on a 2% Agarose-TBE gel and bands were extracted using the Qiagen Gel Extraction Kit. These products were then sequenced using the primers previously described (32).

The library was frozen in 96-well plates. The plates were thawed, spun down and the media was removed, washed 1X in 200µl PBS then suspended in 100µl complete media. 10µl of each was screened using the *in vitro* macrophage-T cell assay during the primary screen and hits were retested in duplicate for conformation before sequencing.

Generation of the BT4295 mutant

BT4295 gene deletion and amino acid substitutions within this gene were done using allelic exchange as described previously (47). Briefly, all manipulations were done in a *tdk* strain background of *B. theta* using the pExchange-tdk vector (48) and primers listed in Table S2. All *Bacteroides* strains and mutants were grown in TYG medium or brain-heart infusion agar with 10% horse blood added. Antibiotics were used as needed: gentamicin (200µg/ml), erythromycin (25µg/ml), and 5-fluoro-2'-deoxyuridine (200µg/ml).

Generation of the BT4295 T->V mutant

Construction of the T547V mutation was done using site-directed mutagenesis via overlapping PCR. Forward and reverse primers were synthesized containing the desired mutation and outside primers were constructed to contain the entire *BT4295* gene. Once a verified construct was sequenced as containing the mutation, we followed a similar strategy to construct the deletion mutants (e.g. 4295 or SPdeletion). *E. coli* containing the T547V construct were mated with the BT4295 deletion strain, therefore complementing the *BT4295* gene back, but with a T547V mutation so that it no longer stimulated T cells.

Expression of BT4295 and BT4298 in *E. coli*

To express BT4295 and BT4298 in *E. coli*, we use the Lucigen Expresso™ T7 Cloning and Expression System and followed the manufacturer's protocol. Briefly, we expressed BT4295 and BT4298 in the pETite N-His Kan vector and designed oligonucleotides for cloning full length or partial proteins listed in Table S2.

Sequence confirmed clones of each were transformed into BL21(DE3) *E. coli* and grown overnight at 37°C with shaking. Fresh 2ml cultures were inoculated and grown to an OD₆₀₀ of 0.5, induced with 1mM IPTG and grown for 5 hours at 37°C with shaking, harvested by centrifugation, washed once with PBS, and suspended in 1ml PBS. Samples were heat inactivated for 20 min at 95°C, then stored at 4°C until use.

Production of recombinant BT4295

BT4295 was expressed in Pet-ite expression vector by cloning the sequence distal to the SPII cleavage motif and including a 5' 6 His tag using the oligos CATCATCACCACCATCACTCGCCCGATTACGAAACCGAGTT (forward) and GTGGCGGCCGCTCTATTATATACTGCAGTTAAATGCCTAG (reverse) (49). The construct was verified by sequencing and expressed in the *E. coli* strain BL21(DE3). Bacteria were grown 37°C until mid-log phase growth was reached. The culture was induced with 1mM IPTG and grown overnight at 19°C. Cells were collected by centrifugation, lysed (50mM NaH₂PO₄, 300mM NaCl, 10mM imidazole, 1mg/ml lysozyme (HEL), and protease inhibitors at pH 8.0) for 30 minutes on ice, sonicated, and centrifuged to remove insoluble material. Supernatants were passed over a Qiagen NiNTA column, washed and eluted in 50mM NaH₂PO₄, 300mM NaCl, 250mM imidazole, and protease inhibitors at pH 8.0. Eluted material was buffer exchanged into PBS with an Amicon Ultra 15, 10kDa concentrator to 1–2ml final volume and quantified by A₂₈₀.

Generation of monoclonal antibodies against BT4295

C57BL/6J mice were immunized subcutaneously with 100µg of recombinant protein (rBT4295) emulsified in complete Freund's adjuvant (CFA) and boosted twice with 100µg of rBT4295 in incomplete Freund's adjuvant (IFA) every four weeks, followed by an IV boost of 50µg rBT4295 three days prior to harvest. Splenic B cells were fused with P3Ag8.6.5.3 myeloma cells to create hybridomas. Hybridomas were screened by ELISA against rBT4295, and positives were screened against whole *B. theta* or OMV preparations to confirm specificity. Two clones (ERC-11 and 4E9) were selected for further characterization. They were subcloned by limit dilution, and both antibodies isotypized as IgG2b,κ. The antibodies were purified from culture supernatants on a Protein A-Sepharose column. Purified 4E9 was biotinylated using the Pierce Ez-Link Sulfo-NHS-SS-Biotin reagent following the manufacturers protocol.

Quantitative ELISA for BT4295

BT4295 protein levels in *B. theta* samples were determined using a quantitative ELISA assay. Samples were obtained from equivalent numbers of *B. theta* from OD₆₀₀ measured cultures. Bacteria were lysed in 100mM CHAPS detergent (Sigma) and incubated with agitation for 1 hour at RT. Insoluble material was removed by centrifugation and samples were stored at 4°C. Purified anti-BT4295 antibody, ERC11, was coated on Immunolon 2 ELISA plate overnight at 5µg/ml in pH 9.6 carbonate coating buffer at 4°C. Plates were washed and blocked with buffer (PBS with 0.5% BSA and 0.1% Tween-20) for 1 hour at RT. Plates were washed and samples were added for 2 hours at RT, washed again, and then 5µg/ml of the anti BT4295 antibody biotin-4E9 was added for 1.5 hours at RT. Plates were washed again and 1:5000 dilution of streptavidin HRP (Southern Biotech) was added for 1 hour RT. Plates were washed and developed with ABTS to completion and A₄₀₅ determined. Unknown sample concentrations were quantitated by comparison to a standard curve of rBT4295 performed in the same ELISA using GraphPad Prism software.

Statistical Analysis

Differences between two groups were evaluated using Student's *t* test (or Mann-Whitney test, for non-normally distributed data) and those among more than two groups were evaluated using ANOVA with Tukey's multiple comparison test (or Kruskal–Wallis with Dunn's posttest for non-normally distributed data) using GraphPad Prism. *P* values of less than 0.05 were considered to be significant. Data are summarized as means ± SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Generation and characterization of the BθOM TCR transgenic mouse.

(A-B) IL-2 levels in pg/ml after generated T cell hybrid clones were cultured with BMDMs loaded with (A) *B. theta* (n=2, 1 experiment) or (B) OMVs (n=2, 1 experiment). (C) IL-2 levels in pg/ml after the BθOM T cell hybrid was cultured with BMDMs loaded with *B. theta* grown in TYG or mTYG (n=2, both replicates are shown). (D) Representative flow cytometry plot with Vβ12 staining on blood leukocytes of C57BL/6J mice (left) or BθOM transgenic mice (middle) (n=3, 3 experiments). Representative TCRα1 PCR on DNA isolated from tails of C57BL/6J mice and BθOM transgenic mice (right) (x=3, 3 experiments). (E) Representative histograms of CD69, CD25 and CD44 expression (left) and quantification of the percentage of CD69, CD25 and CD44 cells among all CD4 cells (right) isolated from the mLNs and spleen of C57BL/6J mice (red) or BθOM transgenic mice (blue) (x=5, 3 experiments). (F) Representative flow cytometry plots of CD4 and CD8 staining of thymic cells isolated from C57BL/6J mice or BθOM transgenic mice (x=5, 3

experiments) and quantification of the percentage of CD8 T cells among the thymic leukocyte population. **(G)** The percentage of T_{regs} in the thymus (n = 6, n=3 experiments), colon-draining lymph node (cdLN) (n = 10, n=6 experiments), spleen (n = 10, n=6 experiments), and colon (n=4, n=4 experiments) of C57BL/6J mice (black) or B6.129SvEv transgenic mice (gray). Student's *t* test: **(E)** **P*<0.1, ***P*<0.01 **(F)** ****P*=0.0004 **(G)** *****P*<0.0001, ****P*=0.0001.

Fig. 2. *B. theta* activates B0OM T cells in a nutrient-dependent manner.

(A-B) The percentage of CD69 expressing B0OM T cells after a 24-hour culture with BMDM loaded with (A) *Bacteroidaceae* family (human: *B. thetaiotaomicron* (n=4, 4 experiments); mouse: *B. fragilis*, *B. vulgaris*, *P. goldsteinii*, *E. coli*, *B. sartorii* (n=3, 3 experiments), or (B) human *B. theta* OMVs (75µg/ml: n=7, 6 experiments; 37.5µg/ml: n=6, 6 experiments; 18.75µg/ml: n=5, 4 experiments; 10µg/ml: n=8, 6 experiments; 1µg/ml: n=3, 3 experiments; 0.1µg/ml: n=4, 4 experiments; 0.01µg/ml: n=3, 3 experiments). Flow cytometry plots are gated on CD4⁺ CD45.1⁺ leukocytes. (C) the percentage of CD69 expressing B0OM hybridoma T cells after a 24-hour culture with BMDM loaded with human *B. theta* grown in TYG (n=13, 5 experiments) or mTYG media (n=5, 5 experiments). One-way ANOVA analysis: (A) *** $P < 0.001$, **** $P < 0.0001$. Means with asterisks are significantly different by Tukey's multiple comparisons test. Student's *t* test: (C) **** $P < 0.0001$, *** $P = 0.0001$, ** $P < 0.01$.

Fig. 3. BθOM T cells proliferate in the colon in *B. theta* colonized mice.

(A) Schematic of adoptive transfer of BθOM T cells into *Rag1*^{-/-} mice gavaged with PBS or *B. theta*. (B) Representative flow cytometry plots of CD45.1⁺CD4⁺ BθOM T cells in the colon of *B. theta* gavaged mice compared to PBS gavaged mice. (C) Number of BθOM T cells among live leukocytes that are CD45.2⁺CD45.1⁺CD4⁺ in PBS or *B. theta* gavaged mice in the colon (n 6, 5 experiments), cdLN (n 5, 3 experiments), and spleen (n 6, 4 experiments). (D) Representative histograms of adoptively transferred CFSE-labeled BθOM T cells in the colon (n 3, 3 experiments), cdLN (n 3, 3 experiments), and spleen (n 3, 3 experiments) of *B. theta* gavaged mice compared to PBS gavaged mice. (E) Quantification of the percentage of proliferated CFSE low CD45.2⁺CD45.1⁺CD4⁺ T cells in the colon (n 3, 3 experiments), cdLN (n 3, 3 experiments) and spleen (n 3, 3 experiments). Mann-Whitney test for non-normally distributed data: (C) *****P* < 0.0001, ****P* = 0.0006. Student's *t* test: (E) *****P* < 0.0001, ****P* = 0.0005, **P* = 0.0160

Fig. 4. BθOM T cells in the colon differentiate into T_{regs}.

(A) Flow cytometry plots of CD45.1⁺CD4⁺ BθOM T cells in the colon, cdLN, and spleen of PBS or *B. theta* gavaged *Rag1*^{-/-} mice transferred with naive CD25⁻ BθOM T cells. (B) The number of CD4⁺ CD45.1⁺FoxP3⁺ BθOM T_{regs} cells in the colon (n = 6, 5 experiments), cdLN (n = 5, 3 experiments), and spleen (n = 6, 4 experiments) of PBS or *B. theta* gavaged *Rag1*^{-/-} mice after CD25⁻ BθOM T cell transfer. (C) The percentage of FoxP3⁺ T_{regs} in the colon (n=27, 9 experiments), cdLNs (n=25, 7 experiments), and spleen (n=20, 7 experiments) of *Rag1*^{-/-} mice that received naive CD25⁻ BθOM T cells and were gavaged with *B. theta*. (D) The percentage of CD25^{high} versus CD25^{low} CD4⁺FoxP3⁺ T_{regs} in the colon (n=27, 9 experiments) and cdLNs (n=25, 7 experiments) of *Rag1*^{-/-} mice gavaged with *B. theta* and injected with naive BθOM T cells. Mann-Whitney test for non-normally distributed data: (B)*****P*<0.0001, ***P*=0.004. Kruskal-Wallis with Dunn's posttest, for

non-normally distributed data: **(C)** **** $P<0.0001$. Two-way ANOVA analysis: **(D)** **** $P<0.0001$, * $P=0.0161$.

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Fig. 5. Depletion of BθOM Tregs drives BθOM CD4⁺ T_{eff} to cause colitis.

(A) Schematic of adoptive transfer of BθOM or BθOM-FoxP3-DTR T cells into *Rag1*^{-/-} mice gavaged with PBS or *B. theta* and treated with diphtheria toxin (31) to deplete BθOM T_{regs}. (B) Percentage of BθOM T_{regs} after depletion in the mLN (n = 12, 5 experiments) or spleen (n = 14, 5 experiments). (C-E) (C) Histology, (D) quantification of the number of mitotic figures/100 crypts, and (E) average crypt height in cecal sections from *Rag1*^{-/-} mice given BθOM T cells and DT (n=6, 3 experiments) compared to those given BθOM-FoxP3-DTR T cells and DT (n=10, 3 experiments). Bars: 120 μm. (F) Cytometric bead array used to quantify IFN-γ (n = 10, 3 experiments), IL-17A (n = 10, 3 experiments), and IL-6 (n = 10, 3 experiments) after cells isolated from the mLN were stimulated with PMA for 5 hours. Student's *t* test: (B) ***P=0.0002, **P=0.0055, (D) **P=0.0029, (E) ****P<0.0001, (F) *P=0.0205, **P=0.098.

Fig. 6. B00M T cells specifically recognize the BT4295(541-554) epitope.

(A) Two parallel methods, T cell Western with Proteomics (left) and Transposon Mutagenesis (TM) (20) Screen (right), used to identify the antigen that stimulates B00M T cells. (B) Schematic of the polysaccharide utilization locus (PUL80) affected by BT4298 disruption by TM. The arrow represents the direction of transcription. (C-G) The percentage of CD69 expressing B00M T cells after culture with BMDM loaded with (C) *E. coli* expressing the full length BT4295 (n=3, 3 experiments for each dilution) or three consecutive segments of BT4298 (BT4298A, B, C) (n=3, 3 experiments for each dilution), (D) *B. theta* (n=4, 4 experiments) or 4295 (n=4, 4 experiments), or (E) *E. coli* expressing two consecutive segments of BT4295 (BT4295A and B) (n=3, 3 experiments for each dilution). (F) Synthetic 20-amino acid peptides overlapping by 12 amino acids. The asterisks represent the P5 position. (G) *B. theta* (n=4, 4 experiments, same data as Fig. 2E) or 4295 (n=3, 3 experiments). One-way ANOVA analysis: (C) ** $P < 0.01$, *** $P < 0.001$,

**** $P<0.0001$. Means with asterisks are significantly different by Tukey's multiple comparisons test. Student's t test: **(D)** *** $P<0.001$, **** $P<0.0001$, **(E)** * $P<0.1$, ** $P<0.01$, *** $P<0.001$, **(G)** *** $P<0.001$, **** $P<0.0001$.

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Fig. 7. Salt and glycan regulate BT4295 expression and alter BθOM T cell activation.

(A) The percentage of CD69 expressing BθOM T cells after a 24-hour culture with BMDM loaded with *B. theta* grown in mTYG (n=4, 4 experiments), TYG (n=2, 2 experiments), and mTYG supplemented with TYG salts (n=4, 4 experiments). (B) The concentration in μg/mL of BT4295 protein expressed in *B. theta* grown in TYG, mTYG, and mTYG supplemented with TYG salts (n=4, 4 experiments) as determined by a quantitative ELISA. (C) The percentage of CD69 expressing BθOM T cells after a 24-hour culture with BMDM loaded with *B. theta* grown in mTYG, TYG, mTYG supplemented with MOG, and TYG supplemented with MOG (n=2, 2 experiments). (D) The concentration in μg/mL of BT4295 protein expressed in *B. theta* grown in mTYG, TYG, mTYG supplemented with MOG, and TYG supplemented with MOG (n=3, 3 experiments) as determined by a quantitative ELISA. One-way ANOVA analysis: (A) * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, (B) ** $P = 0.0093$, (D) **** $P < 0.0001$, ** $P = 0.0065$. Means with asterisks are significantly different by Tukey's multiple comparisons test.

Fig. 8. Dietary glucose represses BT4295 expression, decreasing the activation of BθOM T cells *in vivo*.

(A) Representative plot of the percentage of CD69 expressing BθOM T cells after culture with BMDM loaded with *B. theta* grown in TYG and mTYG media with or without glucose (n=6, 3 experiments). (B) The concentration in μg/mL of BT4295 protein expressed in *B. theta* grown in TYG and mTYG media with or without glucose (n=6, 3 experiments). The percent difference in the number of (C) CD4⁺CD45.1⁺ BθOM T cells or (D) CD4⁺CD45.1⁺CD44⁺CD62L⁻ activated BθOM T cells in the colon (n=26, x=3 experiments) and cdLN (n=16, 2 experiments) of *B. theta* colonized mice given water or 30% glucose water and adoptively transferred with 200,000 CD4-enriched BθOM T cells. (C-D) The percent difference was calculated from the mean of each experiment. ANOVA-multiple comparison analysis: (A) **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, (B) *****P*<0.0001, **P*=0.0190. Means with asterisks are significantly different by Tukey's multiple comparisons test. Mann-Whitney test for non-normally distributed data: (C) ****P*=0.0002, ***P*=0.0052, (D) ****P*=0.0002, **P*=0.0115.