Bacteroides thetaiotaomicron Ameliorates Colon Inflammation in Preclinical Models of Crohn's Disease

Margaret Delday,[*,†] *Imke Mulder , PhD,*[*] *Elizabeth T. Logan,*[*,†] *and George Grant, PhD*[*,†]

Background: Alterations in the gut microbiota are strongly associated with the development of inflammatory bowel disease (IBD), particularly with Crohn's disease, which is characterized by reduced abundance of commensal anaerobic bacteria including members of the *Bacteroides* genus. Our aim was to investigate the protective effects of *Bacteroides thetaiotaomicron*, an abundant member of this genus, in different rodent models of IBD.

Methods: We assessed the effect of *B. thetaiotaomicron* administration on primary readouts of colitis (weight loss, histopathology, and immune parameters) in dextran sodium sulphate (DSS) and interleukin-10 knockout (IL10KO) models of IBD. Efficacy of a freeze-dried bacterial formulation and a purified recombinant protein of *B. thetaiotaomicron* was also investigated.

Results: *B. thetaiotaomicron* showed protective effects in both DSS and IL10KO rodent models, as demonstrated by significant amelioration of weight loss, colon shortening, histopathological damage and immune activation. This efficacy was not exclusive to actively growing bacterial preparations but was retained by freeze-dried cells of *B. thetaiotaomicron*. A pirin-like protein (PLP) of *B. thetaiotaomicron*, identified by microarray analysis during coculture of the bacterial strain with Caco-2 cells, reduced pro-inflammatory NF-κB signalling in these intestinal epithelial cells. Recombinant PLP partially recapitulated the effect of the whole strain in a rat DSS model.

Conclusions: *B. thetaiotaomicron* displays strong efficacy in preclinical models of IBD and protects against weight loss, histopathological changes in the colon and inflammatory markers. These data indicate that the live strain or its products may be a novel alternative to current treatment options for Crohn's disease.

Key Words: *Bacteroides thetaiotaomicron*, Crohn's disease, dextran sodium sulphate, interleukin-10 knockout, inflammatory bowel disease

INTRODUCTION

Crohn's disease (CD) is a chronic, relapsing-remitting, autoimmune inflammatory bowel disease (IBD) that can affect any part of the gastrointestinal tract, unlike the other common form of IBD, ulcerative colitis (UC), which mostly affects the colon. Crohn's disease is characterized by discontinuously affected areas of transmural granulomatous inflammation and/or fistula.[1] Its clinical presentations include bloody diarrhea, abdominal cramps and pain, with patients often requiring long-term medical therapy, periodic hospitalization and even surgical intervention.[2] Linear growth deficiency and delayed puberty are detected in up to 85% of patients diagnosed with CD in childhood.[3] In addition, extra-intestinal complications may develop in systemic organs such as the joints, skin and eyes.[4, 5] Adult-onset IBD, which presently accounts for 60%–65% of all cases, first presents between 18 to 40 years of age, while elderly-onset IBD (10%–15% of cases) appears around 50 to 70 years of age.[6–9] Pediatric-onset IBD first occurs in childhood, and approximately 20%–30% of all CD cases are diagnosed before the age of 20.[7, 9]

Aminosalicylates and corticosteroids are often the first-line therapy for mild to moderate IBD, whereas biologics such as tumor necrosis factor (TNF)-alpha inhibitors (eg, infliximab, adalimumab and golimumab) are generally reserved for patients with fistulising CD or disease that is nonresponsive to other therapies. However, there has been an exponential increase in the number of patients exposed to higher doses of anti-TNFs earlier in their disease course.[10] There are safety concerns regarding the long-term use of potent immunosuppressive agents due to the development of severe side effects,[2, 11–13] and this presents an ongoing, unmet clinical need for alternative treatment options.

The exact causes of IBD are still unknown, but the disease is thought to develop as the result of abnormal intestinal immunity and altered gut microbiota related to environmental

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factors such as diet and infection in genetically susceptible individuals. While the gut microbiota is now considered an essential factor in the IBD inflammatory cascade, it is yet to be resolved whether it is an initiating trigger or secondary to the development of IBD. Crohn's disease is characterized by decreased microbiota diversity in the gut, an altered and unstable microbiota composition, increased numbers of bacteria attaching to the intestinal epithelium and altered relative abundances of health-associated taxa and pathobionts.[14–17] In particular, reduced relative abundance of commensal anaerobic bacterial species of the genera *Bacteroides*, *Faecalibacterium*, *Eubacterium* and *Lactobacillus* and increased abundance of pathobionts, including *Fusobacterium* and *Escherichia*, has been observed[16–18]

Bacteroides thetaiotaomicron is a prevalent species within the *Bacteroides* genus of the human gut microbiota. It has previously been shown that *B. thetaiotaomicron* has anti-inflammatory properties, can increase mucosal barrier function and can limit pathogen invasion.[19–21] On the basis of this information, coupled with data from our in-house *in vitro* screening platform, we decided to assess the activity of *B. thetaiotaomicron* type strain DSM 2079 (BT) in well-characterized rodent models of colitis. We here demonstrate that BT is efficacious in reducing the primary disease readouts in dextran sulphate sodium (DSS) mouse and rat models of colitis and in interleukin-10 knockout (IL10KO) mice. Treatment with BT could, thus, potentially reverse inflammatory responses and ultimately reduce symptoms of CD.

MATERIALS AND METHODS

B. thetaiotaomicron and Pirin-Like Protein (PLP) Treatment

Bacteroides thetaiotaomicron type strain DSM 2079 (BT) was cultured in anaerobic Wilkin's Chalgren Medium (Oxoid, UK) overnight at 37°C before dosing. Freeze-dried BT was fermenter-grown, freeze-dried, milled and sieved; mice were subsequently dosed with 250 mg of freeze-dried BT per day. Pirin-like protein BT_0187 (PLP; 861-YHH-02P; 29.2 kDa) was produced at GTP Technology (Labege, France) and encapsulated in size 9 capsules at Encap (Edinburgh, UK), with each capsule containing 4 nmol PLP.

Colitis Models

Specific-pathogen free (SPF) female C57BL/6 mice (6 weeks old) were obtained from Harlan (UK) and housed in pairs in flexifilm isolators. After an acclimatization period of 7–10 days, the mice were dosed orally daily with a viable overnight culture (1×10^{10} CFU/kg BW) of BT for 14 days. Control animals were dosed with culture medium alone. From day 8 onward, the mice were given 3% dextran sodium sulphate (DSS, 36-50kDa, MP Biomedicals UK) in their drinking water for 6 days until the end of the study. Untreated controls remained on culture medium and had free access to sterile water. Experiments were performed as 2 independent cohorts.

SPF male Hooded Lister (9–10 weeks old) rats were dosed orally daily with BT (3×10^{10} CFU/kg BW) for 14 days, and 16 controls were given culture medium alone. On days 8–14, the BT-treated rats and 8 controls were given DSS in sterile water for 7 days (4% DSS for 5 days and 2% DSS for 2 days). Nontreated controls had free access to sterile water.

SPF male Hooded Lister rats were dosed orally with PLP capsules (12 nmol/rat/d) or placebo for 7 days. Rats were also given DSS in sterile distilled water (4% DSS for 5 days and 2% DSS for 2 days) during this time. Nontreated control rats had free access to sterile water.

SPF C57BL/6 homozygous IL10KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). They were housed and bred over 3 generations within flexifilm isolators. Weanling mice (20 days old) were orally dosed for 13 weeks (2–3 times a week) with a viable culture (1×10^{10} CFU/kg BW) of BT. Experiments were performed as 2 independent cohorts.

Animals on all studies were checked twice daily for general health condition and in particular for blood in the feces. Water consumption, food intake and body weight were monitored daily. Animal sacrifice, tissue sampling and processing of all studies were carried out using similar procedures. At termination, ascending colon tissues were collected and fixed in neutral buffered formalin (NBF; Sigma-Aldrich, St. Louis, MO, USA) for histology or stored in RNAlater (Qiagen, Manchester, Lancs, UK) for molecular analysis.

The University of Aberdeen is licensed under the UK Animals (Scientific Procedures) Act 1986. The local Animal Welfare and Ethical Review Board (AWERB), the animal welfare unit, and the appropriate governmental inspectorate monitor and review all animal studies. The management and experimental procedures used were approved by AWERB and conducted under the auspices of PPL 60/4360 and 60/3690.

Histopathology Analysis of Ascending Colons

Immediately post dissection, colon length was measured. Ascending colon samples were dissected at the exact same gut location between experiments and further divided into 3 pieces for the different analyses carried out. This was paramount for intestinal sampling due to the heterogeneity along its length.[22] Samples for histology were fixed in 10% NBF and processed to 8100-resin (TAAB Laboratories, Aldermaston, Berks, UK) according to manufacturer's instructions. Multiple ascending colon pieces were orientated for transverse sectioning per tissue/block. Four-micron sections were cut (Reichert Jung microtome) and stained with hematoxylin and eosin (H&E) (Sigma-Aldrich). Two slides were prepared for each sample, and sections were taken at least 400 µm apart to increase the area of tissue examined. An Axioskop 50 upright microscope (Zeiss, Cambridge, Cambs, UK) equipped with x20/0.50

Plan Neofluar objective and Qimaging QICAM Fast cooled 12-bit camera controlled by Image Pro Plus software (Media Cybernetics, Wokingham, UK) was used to image the complete transverse cross section of at least 2 colon pieces. This resulted in a mean number of digitized images of 70 per ascending colon, which represented 2 complete transverse cross sections. The number of fields of view per tissue varied due to the thickening of the mucosa, seen particularly in the IL10KO groups. Only complete transverse sections of ascending colons were imaged and used for histopathological scoring. This was found to be particularly important in the DSS experiments as the highest grade of pathology was usually adjacent to the mesenteric attachment.

Two experienced, blinded individuals used a pathology scoring method based on Berg et al[23] to allocate a single score per field of view. The scores ranged from 0 to 4, where 0 had no pathology, the fields of view displayed shallow crypts with no or few infiltrating inflammatory cells, an intact epithelium was observed and goblet cells appeared full of mucin. Fields scoring 1 displayed some epithelial hyperplasia resulting in a slight increase in the depth of the crypts, and there was slight depletion of mucin from the goblet cells, but the epithelium remains intact. Fields scoring a 2 had crypts which seemed deeper with distinct evidence of epithelial hyperplasia and depletion of mucin from goblet cells, but infiltrating inflammatory cells were evident and could be multifocal in nature, though the infiltrates were not seen in the submucosa. Grade 3 fields of view displayed lesions involving a larger area of the mucosa or were more frequent than that seen in grade 2. The lesions did not involve the submucosa. The luminal epithelial cells could exhibit small erosions. The lesions were not transmural. Grade 4 pathology was characterized by the crypt epithelium seeming to be eroded, luminal epithelial cells seeming irregular—sometimes with complete loss. Transmural infiltrate was often associated with complete loss of epithelial cells into the lumen. Areas of almost complete mucin depletion could be seen. The mean percentage of fields of view at a given score was calculated, and a comparison was made between treatment groups.

Microarray Analysis

Caco-2 cells were seeded into 35-mm dishes in 10% FBS/4 mM L-glutamine/1 × antibiotic antimycotic/DMEM media (fetal bovine serum [FBS]), 200 mM L-glutamine, antibiotic antimycotic solution 100 × stabilized, Dulbecco's Modified Eagle's Medium—high glucose (DMEM) (all from Sigma-Aldrich) and incubated at 5% CO_2 at 37°C for 6 days. Cells were washed twice with Hank's Balanced Salt Solution (HBSS) (Sigma-Aldrich) and then synchronized in serum and antibiotic free media (DTS) for 24 hours (DMEM , 200 mM L-glutamine, 500 µl 20 µgml^{-1} sodium selenite, and 500 µl 500 µgml^{-1} apo-Transferrin, Sigma-Aldrich). Cells were treated with flagellin of *Salmonella enteritidis* (10 ng/well), phorbol-12-myristate-13-acetate (PMA; 150 ng/well) or interleukin-1 (IL-1; 10 ng/well). *Bacteroides thetaiotaomicron* type strain DSM 2079 was added at 10^9 CFU/mL and incubated for 2 hours. Bacterial culture was then removed, and RNA was prepared using Nucleospin II RNA Extraction Kit and reverse transcribed to cDNA with the SuperScript II Reverse Transcriptase (ThermoFisher, Hemel Hemstead, Hertfordshire, England) following manufacturer's instructions. Samples were processed using the One-Color DNA Labeling kit, Hybridization kit, Sample Tracking Control kit and Wash Buffer kit (Roche NimbleGen Inc., Madison, WI, USA) and hybridized to a *Bacteroides thetaiotaomicron* VPI-5482-specific microarray (Roche NimbleGen Inc.) according to the manufacturer's instructions. The files were then analyzed using NimbleScan software (Madison, WI, USA).

Mouse ascending colon tissues were lyzed in Trizol (Invitrogen, Carlsbad, CA, USA). RNA was isolated using standard chloroform/isopropanol steps. Total RNA was further purified with the RNeasy Kit (Qiagen), including an RNase-free DNase I (Qiagen) digestion step. Total RNA was processed into biotin-labeled cRNA using the One-Cycle Target Labeling Kit (Affymetrix, Santa Clara, CA, USA) or biotin-labeled aRNA using the 3' IVT Express Kit (Affymetrix). Hybridization to the GeneChip NuGO Mouse Array (Affymetrix) on a GeneChip Fluidics Station 450 (Affymetrix) was performed at the Institute of Medical Sciences Microarray Core Facility (University of Aberdeen, Aberdeen, UK). Chips were scanned with an Affymetrix GeneChip Scanner 3000. Image quality analysis was performed using the Gene Chip Operating Software (GCOS) (Affymetrix). Further data analysis was performed using R (www.rproject.org) and Bioconductor (www.bioconductor.org). The moderated *F* test provided by the Bioconductor package limma was used to test for differential expression. Differences were considered significant with $P < 0.05$ using the Benjamini and Hochberg false discovery method. Microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (accession number GSE118526; www.ncbi.nlm.nih.gov/geo).

Quantitative Reverse Transcriptase Polymerase Chain Reaction

mRNA was extracted from the ascending colon using TRIzol (Invitrogen) followed by the RNeasy kit and Oligotex mRNA mini kit (Qiagen) according to the manufacturer's instructions; 2 µg of total eukaryotic RNA, isolated from the ascending colon, was reverse-transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers. This was followed by quantitative polymerase chain reaction (qPCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems), with the QuantiFast SYBR Green PCR Kit (Qiagen) and QuantiTect Primer Assays (Qiagen), for hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), interleukin-6 (*Il6*), interleukin-1β (*Il1b*), tumor necrosis factor α (*Tnfα*), C-X-C motif chemokine 10 (*Cxcl10*), chemokine (C-C motif) ligand 3

(*Ccl3*) and regenerating islet-derived protein 3-gamma (*Reg3g*). Polymerase chain reaction cycling conditions were as follows: 1 cycle at 95°C for 5 minutes, followed by 40 cycles at 95°C for 10 seconds and at 60°C for 30 seconds, ending with a dissociation step. All samples were run in triplicate. Hypoxanthine-guanine phosphoribosyltransferase was used for normalization purposes. All data were analysed on a logarithmic scale with base 2 by one-way ANOVA with a significance cutoff of $P < 0.05$. Differences were back-transformed to calculate fold changes.

Luciferase Assay

Caco-2 cells were transfected by adding DMEM plus 4 mM of L-glutamine media, including appropriate quantities of PLP in expression vector, pLuc-GL3\NF-κB, Renilla luciferase construct, and Fugene 6 transfection reagent (Promega, Southampton, Hants, UK) to appropriate wells; cells were then incubated for 48 hours at 5% CO2 at 37°C. Cells were synchronised 24 hours before stimulation in serum-free media. Subsequently, cells were treated with flagellin of *Salmonella enteritidis* (10 ng/well), phorbol-12-myristate-13-acetate (PMA; 150 ng/well) or interleukin-1 (IL-1; 10 ng/well) for 9 hours (5% CO2 at 37°C). The media was removed and the 24 well plates containing the cells were frozen at −80°C. The plates were warmed to room temperature for 30 minutes, 150 µl of Dulbecco's Phosphate Buffered Saline (PBS; Sigma-Aldrich) was added to each well and cells were scraped off and 100 µl transferred to a 1.5-mL microfuge tube. Luciferase activities in the 100 µl samples were determined using the Dual-Glo luciferase assay system (Promega, UK) on an Envision 2102 Plate Reader.

Statistical Analysis

Analysis of data was conducted using GraphPad Prism 7. One-way ANOVA was applied to datasets comparing more than 2 treatment conditions at a single timepoint followed by the Tukey multiple comparison test. Two-way ANOVA was applied to datasets comparing 2 or more treatment conditions where data was also collected over multiple timepoints. Two-way ANOVA was followed by the Sidak multiple comparison test when comparing a single condition to another condition or the Tukey multiple comparison test when comparing multiple conditions to multiple other conditions. Unpaired Student *t* test was used to analyse means from 2 conditions only at a single timepoint. All data were plotted as mean ± standard deviation, except quantitative PCR, which was plotted as mean ± standard error of the mean.

RESULTS

B. thetaiotaomicron Is Protective in Chemically-Induced Acute DSS-Colitis in Mice

Dextran sodium sulphate (DSS) causes acute colitis with similarities to IBD in C57Bl/6 mice.[24] This well-established preclinical model was used to test if administration of *B. thetaiotaomicron* (BT) could prevent or limit this severe, chemically induced colitis in mice. C57Bl/6 mice were dosed daily with BT for 8 days, while control mice were dosed with culture medium. The BT-dosed mice and control mice were then treated with 3% DSS for 6 days. Untreated control mice were dosed with culture medium without DSS treatment.

Mice given DSS alone displayed notable weight loss ($P < 0.001$ versus control), mainly over days 4–6 of DSS exposure (Fig. 1A). This weight loss was significantly reduced in mice dosed with BT ($P < 0.01$ versus DSS at day 4 and $P < 0.0001$ at day 5 and 6; Fig. 1B). Food and water intake was measured daily, and mean total intakes were not significantly different between groups (data not shown). A grading assessment scheme based on Berg et al[23] (Fig. S1) was used to assess colon histopathology with grades ranging from 0 (no pathology) to 4 (highest pathology). DSS-induced histopathological damage was generally moderate-severe, with 27.1% of the fields of view showing high scores (grades 2–4) and only 27.6% fields of view with no pathology (Fig. 1C). All pathology grades were significantly different when comparing the control group with the DSS-treated group (grade 0, $P < 0.0001$; grade 1, $P = 0.0218$; grade 2, $P = 0.0013$; grade 3, $P = 0.0229$; grade 4, $P = 0.0010$). Most mice dosed with BT had only mild-moderate disruption of the colon. The majority of fields of view had no (grade 0) or low (grade 1) pathology, with only 9.3% of the fields of view having higher pathology (grades 2–4). Histopathological scores were significantly reduced (grade 0, $P = 0.0031$; grade 4, $P = 0.0006$) in DSS-treated mice dosed with BT compared with DSS alone. In the control group, 92.2% of fields of view had grade 0 (no pathology), with 7.8% at grade 1 (low-grade pathology). Cellular infiltration, mucin depletion and epithelial erosion were all less evident in mice dosed with BT (Fig. 1D). This was reflected in the significant downregulation of expression of inflammation-related genes such as *Il6*, *IL1b*, *Tnfα* and *Cxcl10* compared with DSS alone (Fig. 1E).

B. thetaiotaomicron Is Protective in an IL-10KO Mouse Model of Colitis

Interleukin-10 knockout mice spontaneously develop intestinal inflammation and transmural pancolitis between 2 and 4 months of age. Disease progression has many features of CD, so IL10KO mice are widely used as a model for this disease.[24–26] The aims of this study were to establish if BT could ameliorate the moderate spontaneous colitis that develops in the IL10KO mouse and to investigate the effects of bacterial dosing on gut integrity and immune parameters. Interleukin-10 knockout mice were dosed with BT 3 times a week from weaning at 3 weeks old, for a total period of 13–14 weeks. Control IL10KO mice were dosed with culture medium alone.

Bacteroides thetaiotaomicron treatment significantly improved weight gain, both over time ($P < 0.05$ at days 97–100 interval; Fig. 2A) and assessed as total weight change at day 93

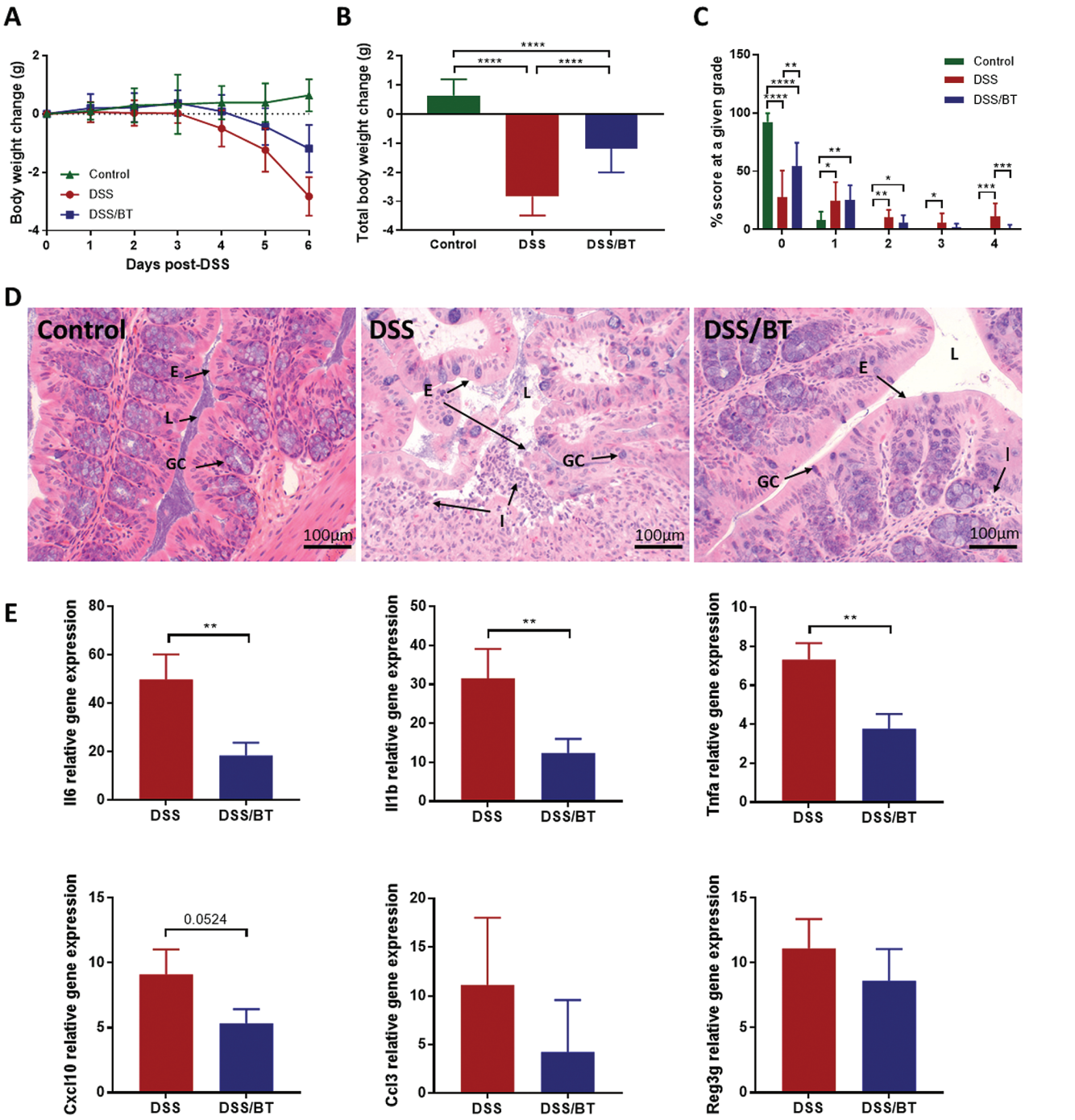

FIGURE 1. *B. thetaiotaomicron* is protective in C57Bl/6 mice with acute DSS-colitis. A and B, Weight change of DSS-treated mice dosed with *B. thetaiotaomicron* (BT) or culture media for 15 days. Mice given DSS alone displayed major weight loss, mainly over days 4–6 of DSS exposure, and this weight loss was significantly reduced in mice dosed with BT. C, Histopathology scores showed BT treatment of DSS-induced colitis significantly increased the percentage fields of view with 0 score (no pathology) and significantly decreased the percentage fields of view at score 4 (highest pathology). D, Representative images of H&E stained sections of ascending colons from indicated groups; L = lumen; GC = goblet cell; E = epithelium; I = infiltrate. E, The relative gene expression of the inflammatory mediators *Il6*, *IL1b*, *Tnfa* and *Cxcl10* was significantly reduced with BT treatment.

(P = 0.0050), which was the final time-point before staggered termination (Fig. 2B). Interleukin-10 knockout mice displayed moderate-severe pathology with 57.83% of the fields of view having the highest grades (3 and 4) of pathology (Fig. 2C). BT-dosed IL10KO mice had a mild-moderate pathology in which most fields of view (68%) had low levels (grades 0 and 1)

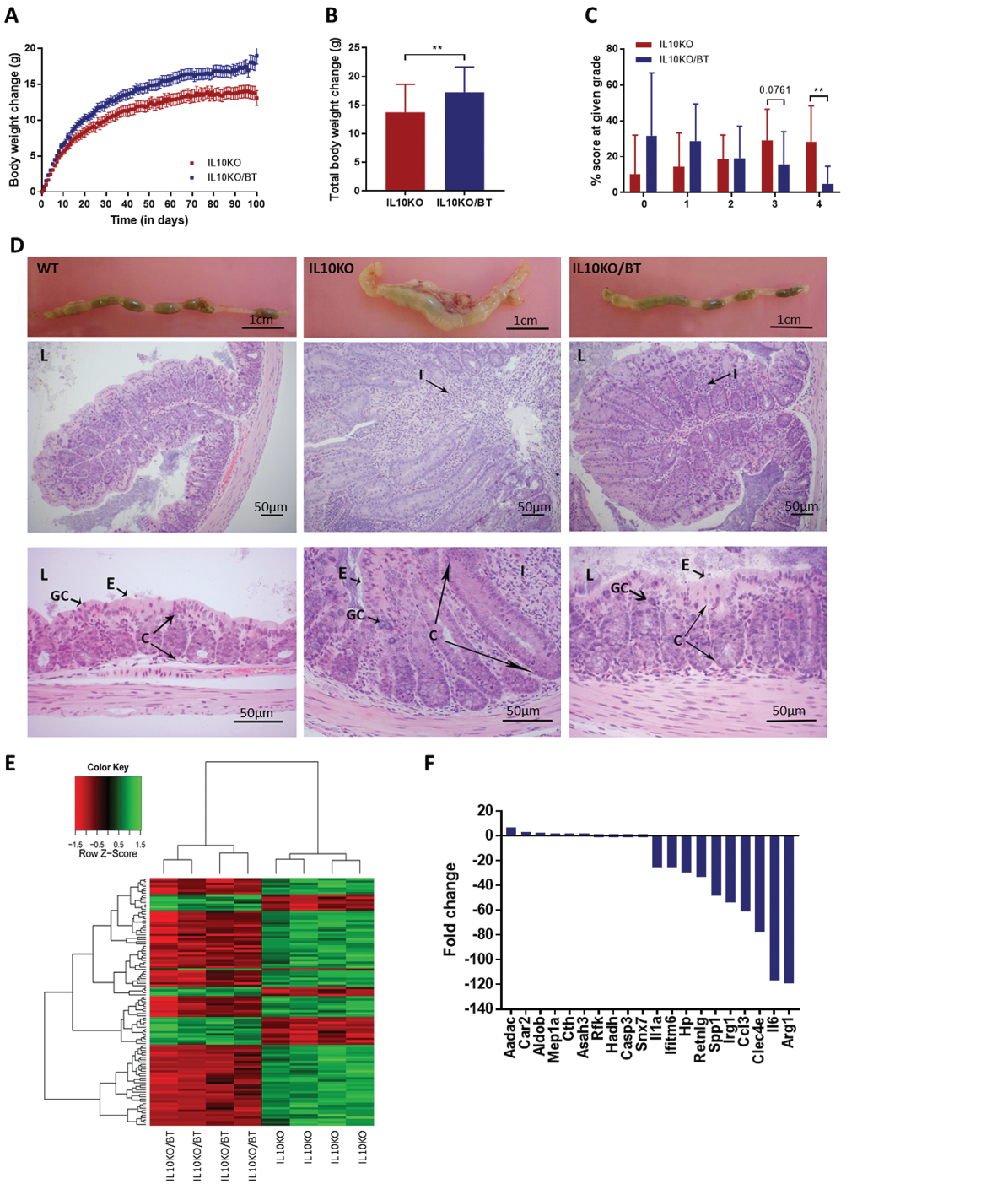

FIGURE 2. *B. thetaiotaomicron* is protective in an IL10KO mouse model of colitis. A, Body weight monitoring of IL10KO mice dosed 3 times weekly with BT or culture media for 13–14 weeks from weaning showed that BT treatment significantly improved weight gain, both over time and (B) assessed as total weight change at day 93. C, BT treatment of IL10KO mice resulted in an increase in the percentage fields of view with no pathology and a reduction in the fields of view scoring the highest grade of pathology. D, Macroscopic images (top panel) demonstrate the increase in ascending colon diameter from IL10KO mice compared with the WT and IL10KO/BT groups. Representative images of H&E stained ascending colon sections (middle panel) illustrate the degree of crypt hyperplasia and infiltrating inflammatory cells which occurred in these mice relative to the WT and IL10KO/BT; I = infiltrating cells; L = lumen. Representative images from WT, IL10KO, and IL10KO/BT (bottom panel) illustrate the types of images used for pathology scoring; L = lumen; E = epithelium; GC = goblet cells; C = crypt; I = infiltrate. E and F, Transcriptomics of ascending colon of BT-treated IL-10KO mice shows differential gene expression of 107 transcripts between IL10KO- and IL10KO/BT-treated mice. BT treatment particularly reduced the expression of genes related to the inflammatory response.

of pathology. There was a significant difference at grade 4 (P = 0.0025), with grade 3 trending towards significance (P = 0.0761). Macroscopically, colons varied from being very swollen and distended—indicative of extensive inflammation, immune cell infiltration and disruption to the tissue—through to the narrow form of wildtype controls. An overall visual assessment of the colons indicated that most IL10KO mice had severe pathological changes, whereas most BT-dosed IL10KO mice exhibited only moderate disruption (Fig. 2D). Microscopic examination illustrated the degree of crypt hyperplasia in IL10KO mice relative to IL10KO/BT and wildtype mice (Fig. 2D). The increased thickness of the mucosa was primarily due to crypt hyperplasia, and this, along with an increase in infiltrating cells, was responsible for the overall increased colon diameter.

Comparative transcriptome analysis of ascending colon tissue of IL10KO and IL10KO/BT-treated mice showed differential expression of a wide range of genes associated with inflammatory responses between the 2 treatment groups (Fig. 2E), with 107 gene expression levels significantly different. Expression of pro-inflammatory genes (such as *Arg1*, *Il6*, *Ccl3*, *Spp1* and *Il1a*) and genes involved in pathogen recognition (*Clec4e*, *Irg1*) was particularly lower in IL10KO/BT mice (Fig. 2F).

Lyophilized *B. thetaiotaomicron* Maintains Its Protective Activity in Murine DSS-Colitis

Next, the activity of BT in a freeze-dried formulation (BTFD) was assessed for its ability to ameliorate colitis as the preferred target formulation for clinical use. C57Bl/6 mice were dosed daily with BTFD for 8 days. Another group of mice were dosed with a growing culture of BT, and control mice were dosed daily with culture medium. All animals were then given 3% DSS for 6 days. Untreated control mice were dosed with culture medium without DSS treatment. Administration of BT or BTFD limited weight loss caused by DSS in C57Bl/6 mice (Fig. 3A and B). There was a major decline in body weight in mice dosed with DSS, but the losses were significantly reduced when the mice were also dosed with liquid culture of BT (P < 0.001 versus DSS) or freeze-dried BT (P < 0.0001 versus DSS). Weight loss was significantly reduced by BT from day 4 of DSS treatment onward, whereas BTFD showed significant effects from day 5 of DSS exposure onward. Colon lengths in mice treated with DSS were reduced compared with control mice (P < 0.001; Fig. 3C) but were less affected by DSS if mice were also given BT (P < 0.001 versus DSS) or BTFD (P < 0.001 versus DSS). DSS-induced colon inflammation was reduced in mice dosed with BT and BTFD (Fig. 3D and E). Ascending colon pathology in DSS-treated mice was generally moderate-severe, with 34.8% of the fields of view showing high histopathology scores (grades 2–4) and only 38% fields of view with no pathology (Fig. 3D and E). All grades of pathology showed significant differences when comparing the control with DSS-treated mice (grade 0, P < 0.0001; grade 1, P = 0.0004; grade 2, P = 0.0001; grade 3, P = 0.0029; grade 4, P = 0.02). In contrast, in BT- and BTFD-dosed mice most fields of view (94.9% for both treatments) scored 0–1. This was comparable to control mice, in which 98.5% of fields of view had no pathology, and the remainder scored grade 1. When comparing DSS/BT and DSS/BTFD mice with the DSS group, a significant improvement was observed in all grades of histopathology scores apart from grade 1 (grade 0, P = 0.0001 for BT and P = 0.0001 for BTFD; grade 2, P = 0.0025 and P = 0.0007; grade 3, P = 0.0029 and P = 0.0081; grade 4, P = 0.0215 and P = 0.0479).

B. thetaiotaomicron Is Partially Protective Against DSS in Hooded Lister Rats

Adult rats are commonly used in studies of DSS colitis.[27] The aim of this experiment was to assess if prophylactic treatment with BT could limit the severity of acute colitis in a different animal model. Hooded Lister rats were dosed with BT for 7 days. A group of control rats was dosed with culture medium daily. The BT-dosed rats and controls were then also given DSS for 7 days (4% DSS for 5 days and 2% DSS for 2 days).

While weight gain was reduced by dosing with DSS, daily administration of BT had no significant effect on preventing this weight loss (Fig. 4A and B). However, while colon length was significantly reduced (P < 0.001) in rats administered DSS alone (Fig. 4C), this reduction was not evident when animals were treated with BT. Indeed, colon lengths in these rats were not significantly different to control animals.

Dextran sodium sulphate caused mild histopathological disruption in the ascending colon of rats, with 34% of fields of view having major (grade 2 or 3) pathology, 40% with grade 1 pathology, and 26% with no pathology (Fig. 4D). *Bacteroides thetaiotaomicron* significantly reduced the severity of this DSS-colitis. Rats administered BT had reduced cellular infiltration, over 50% of fields of view had no pathology (P < 0.05 versus DSS alone), and only 13% had grade 2 or 3 pathology (P < 0.05 DSS/BT versus DSS).

A Pirin-Like Protein of *B. thetaiotaomicron* Is Protective in Rats With DSS-Colitis

To identify potential bacterial protein(s) involved in the anti-inflammatory effect of BT in colitis, BT was incubated with Caco-2 human intestinal epithelial cells for 2 hours, and bacterial gene expression was assessed on a *Bacteroides thetaiotaomicron* VPI-5482 specific microarray. Forty-three BT genes were upregulated by 5-fold or more, and of these, 20 genes encoded hypothetical proteins (Table S1). These included the pirin-like protein BT_0187 (gene ID 1075517; gene symbol BT_0187; accession no. NC_004663.1). A second hypothetical pirin-related protein, BT_1576, was also elevated, albeit to a lesser extent, after exposure of BT to epithelial cells. As

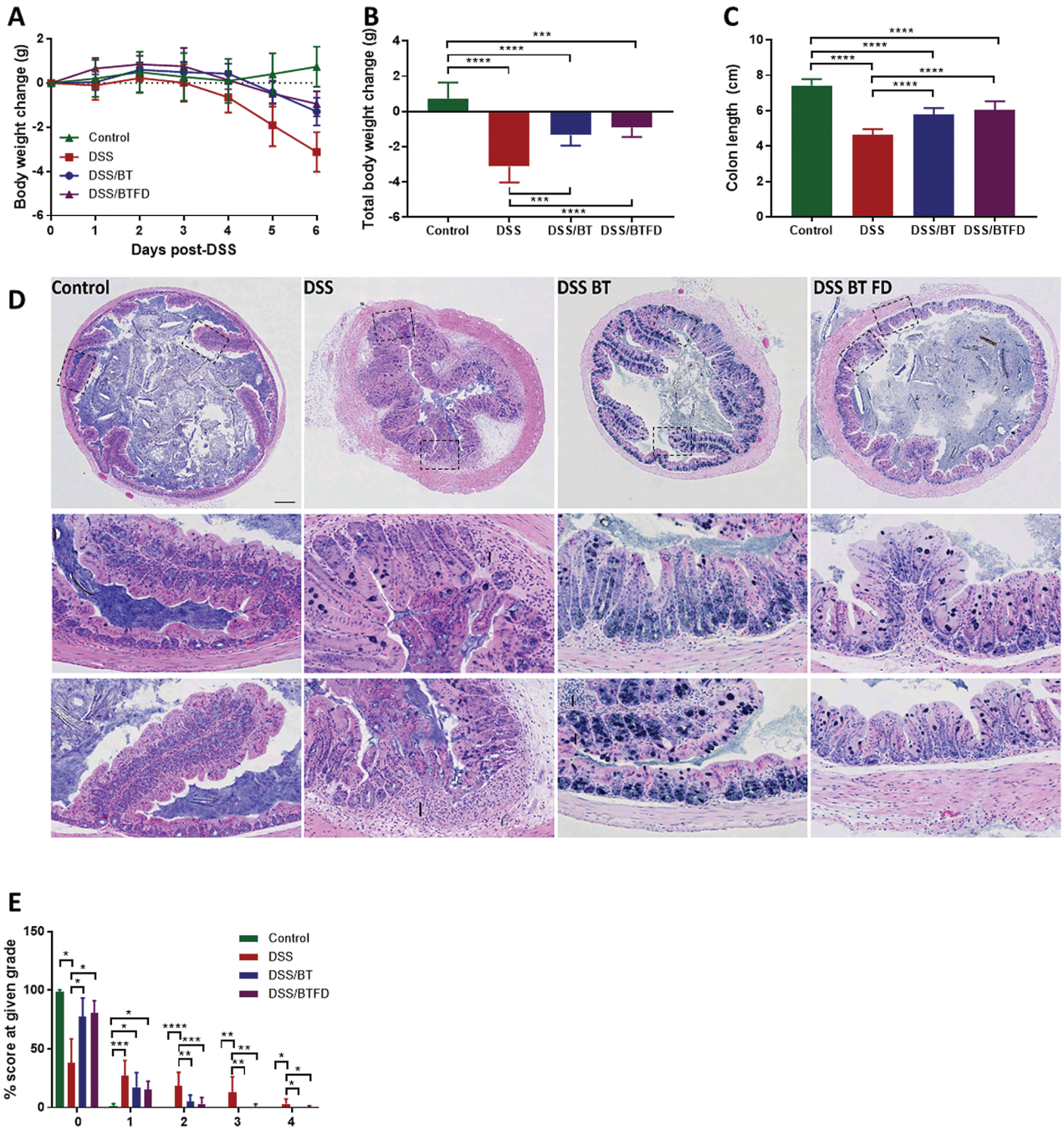

FIGURE 3. Lyophilized *B. thetaiotaomicron* maintains its protective activity in murine DSS-colitis. A, Weight change of DSS-treated mice dosed with *B. thetaiotaomicron* (approximately 1×10^{10} CFU /kg BW) or culture media for 8 days. Both BT and BTFD treatment reduced weight loss caused by DSS, and (B) this effect was maintained until time of termination. C, DSS treatment significantly reduced colon length; this was partially ameliorated in the BT treated group and in the BTFD group. D, DSS-induced histopathology was reduced in mice dosed with BT and BTFD. Top panel shows representative scans of H&E stained complete transverse cross sections of ascending colons. The images below show areas selected from the scans and digitally zoomed, illustrating the degree of pathology observed between groups. E, Histopathology scores show that mice treated with DSS exhibited a high degree of pathology which was significantly reduced with BT and BTFD treatment

mammalian pirin is an iron-binding nuclear protein and an important cotranscription factor that promotes activation of NF-κB-dependent genes[28] and BT was previously shown to affect NF-κB signalling,[21] we postulated that pirin-like protein BT_0187 (PLP) could influence this inflammatory pathway in host cells. Pirin-like protein BT_0187 was transfected into

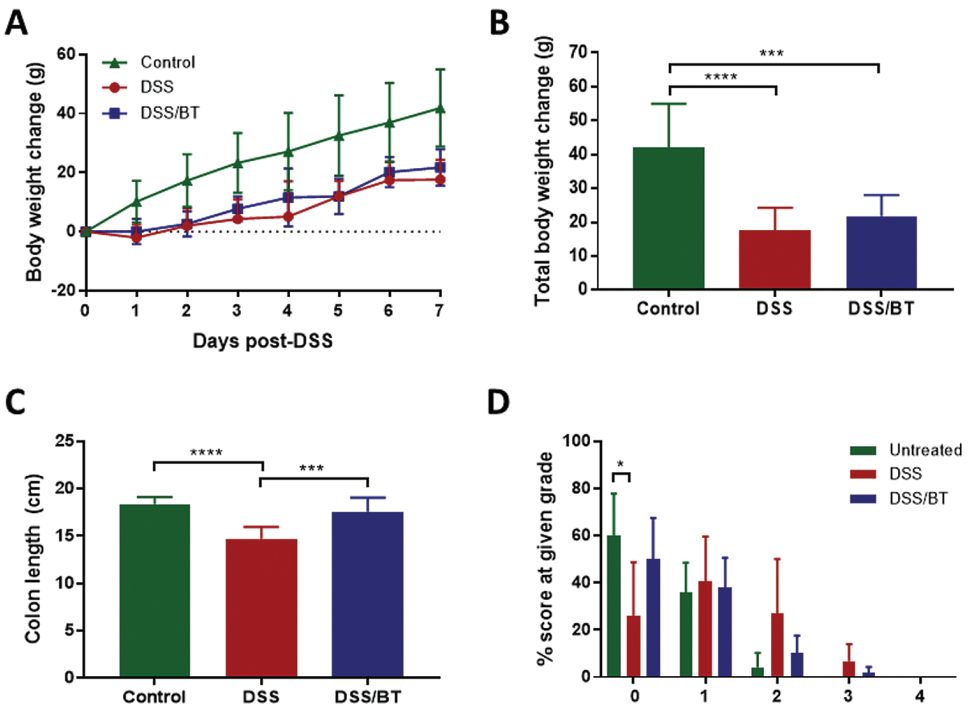

FIGURE 4. *B. thetaiotaomicron* is partially protective against DSS-colitis in Hooded Lister rats. A, Weight gain was significantly reduced in animals given DSS. This loss of condition was not prevented in rats pretreated with BT compared with DSS alone. B, Total body weight change of DSS/BT-treated rats was not significantly different from DSS-treated rats; however, (C) the reduction in colon length was statistically improved with BT treatment, and (D) there was a statistical improvement in the percentage of fields of view with no pathology when compared with DSS.

Caco-2 cells to investigate if it could affect NF-κB-dependent pro-inflammatory responses. Interestingly, epithelial cells transfected with PLP had lower basal levels of NF-κB-responsive luciferase activity (Fig. 5A). Furthermore, PLP greatly reduced the NF-κB-dependent responsiveness of the cells to flagellin, phorbol-12–myristate-13 acetate (PMA), and IL-1 (Fig. 5A).

Recombinant PLP (6His-Tev-Yhhw) was produced in *Lactococcus lactis* and encapsulated into size 9 capsules. Its ability to modulate DSS-induced colitis was evaluated in Hooded Lister rats given DSS in water for 7 days (as described previously). The rats were orally dosed daily with capsules of PLP (12 nmole/rat/d) or placebo during this same time period.

Rats given DSS alone lost weight over the experimental period (Fig. 5B and C). In contrast, animals treated with PLP gained weight slowly, similar to control rats. Colon length was significantly reduced due to intake of DSS ($P < 0.05$ compared with control and $P < 0.01$ compared with PLP; Fig. 5D). However, this reduction in colon length was prevented when rats were treated with PLP. Dextran sodium sulphate caused moderate disruption of the colonic mucosa of Hooded Lister rats (Fig. 4 and 5). As in other models of colitis, there was interanimal and local-site variation in the nature, extent, and severity of the disruption caused in the colon. Histopathology scores after PLP treatment were not statistically different from DSS-treated mice, although the increase in the percentage of fields showing 0 scores showed the same trend as in the previous study (Fig. 5E). Thus, PLP was able to partially recapitulate the protective effect of whole BT in a DSS model of colitis.

DISCUSSION

The genus *Bacteroides* is recognized as a dominant and biologically important group of commensal bacteria in the microbiota of the human gastrointestinal tract.[16] *Bacteroides* spp. have been attributed with symbiotic traits related to immunoregulatory, metabolic and homeostatic functions. As one of the most dominant *Bacteroides* species within the human gut,[29, 30] *B. thetaiotaomicron* has been the subject of a number of studies regarding its beneficial effects on the host.[19, 20, 31–33] Importantly in the context of CD, *B. thetaiotaomicron* maintains immune homeostasis in the gut.[20, 21, 34, 35] In germ-free mice, this species can recapitulate the effects of the entire conventional microbiota on immune system maturation, in particular related to regulatory T cell pathways.[35] *Bacteroides thetaiotaomicron* also exerts immunomodulating actions on the host through altering the activity of the transcription factor NF-κB and preventing the downstream induction of inflammatory factors such as IL-8, TNF-α, and IL-1β.[21] These functional effects on the host are particularly relevant for IBD because disease

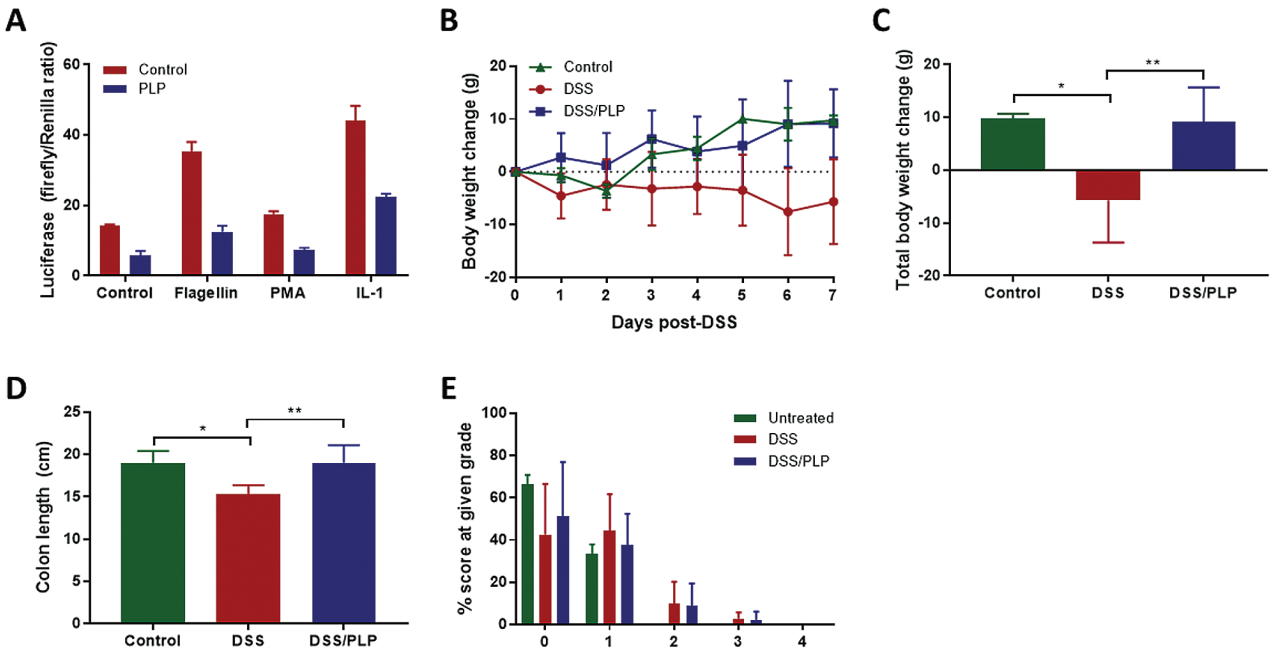

FIGURE 5. A hypothetical protein produced by *B. thetaiotaomicron* is protective in rats with DSS-colitis. A, Luciferase ratios obtained for Caco-2 cells transfected with pTarget-PLP and incubated with flagellin of *Salmonella enteritidis,* phorbol-12–myristate-13 acetate (PMA) or IL-1 show a reduction in NF-κB-dependent responsiveness by PLP treatment. B, Body weight change across the duration of the trial showed that DSS-treated animals had the greatest growth reduction, whereas PLP treatment improved weight gain to almost similar levels as control animals. C, The total body weight change at termination of study was statistically improved in the PLP group over DSS alone. D, Colon length was significantly reduced due to intake of DSS, but this reduction in colon length was prevented when rats were also treated with PLP. E, Histopathology scores were not statistically different from DSS-treated mice, although the increase in the percentage of fields showing 0 scores showed the same trend as in the previous study.

is associated with increased inflammatory cytokine levels and NF-κB levels in the intestinal lamina propria.[36]

Here, we demonstrate that *B. thetaiotaomicron* has protective effects on the primary efficacy readouts of colitis including weight loss, colon length, histopathological scoring and inflammatory mediators. The striking effects of *B. thetaiotaomicron* treatment on mucosal histopathology are of specific interest as therapeutic strategies in the IBD clinical field are moving towards a treat-to-target approach, with endoscopic mucosal healing the most predictive factor for sustained remission.[2, 37]

Bacteroides thetaiotaomicron showed efficacy in 2 different preclinical IBD models with relevance for CD: the DSS model and the IL10KO model. Although no single animal model of IBD can fully recapitulate the complexity of human disease, the selected models display characteristics similar to that of human CD.[24, 38] The DSS colitis model is considered a suitable model for investigating pathogenesis, therapeutic options and the dysplasia-adenocarcinoma sequence of IBD.[39] Colon damage in this model has similar pathophysiological features to human disease, with extensive ulceration of the epithelial layer, fibrotic thickening of the mucosa, loss of crypts and a dense cellular infiltrate that includes neutrophils and inflammatory macrophages.[24, 40] The C57BL/6 mouse strain in particular shows high susceptibility to DSS-induced colitis compared with other strains.[41] Treatment with *B. thetaiotaomicron* had striking ameliorating effects on disease pathology in these animals. The response to *B. thetaiotaomicron* treatment in DSS-treated rats was not as significant as the results obtained in mice, with significant amelioration of colon shortening and histopathological damage but no reduction in weight loss. This lack of effect on body weight may be attributed to the less severe colitis induced in this species, and the response to BT might have been more pronounced with a higher DSS concentration. Additionally, disease course, severity and location of pathological changes of DSS-induced intestinal inflammation vary between animal species and strains.[42, 43] This variability is likely due to a combination of different factors including genetic background, husbandry conditions and the gut microbiome composition. How these factors affect the species- and strain-specific response to DSS administration warrants further investigation.

The IL10KO B6.129P2-*Il10*^{tm1Cgn}/J mutant mouse strain spontaneously develops a chronic form of enterocolitis associated with altered lymphocyte and myeloid profiles, altered responses to inflammatory or autoimmune stimuli and increased prevalence of colorectal adenocarcinoma. Similar to the human disease, the IL10KO model of CD is responsive to anti-TNF therapy and thus is a relevant model for evaluating novel therapeutic approaches.[38] As *B. thetaiotaomicron* effectively ameliorated colitis in IL10KO mice, it may be a targeted therapy for patient populations associated with loss of function

mutations in IL-10 or the IL-10 receptor, such as infant-onset (under 2 years old) and very-early-onset (2–6 years old) IBD.[44] Disorders in these populations are particularly complex—even in first presentation at clinic—and are poorly responsive to standard therapies.[45, 46]

Overall, *B. thetaiotaomicron* treatment reduced the 3 main histopathology categories assessed in the current studies, namely crypt hyperplasia, epithelial integrity and infiltrating inflammatory cells.[47] Although not scored as an individual pathology, colonic goblet cell mucin depletion also appeared to be reduced in *B. thetaiotaomicron*–treated animals. These findings correlate well with histopathological findings in human IBD, which include inflammatory cell infiltration of eosinophils, neutrophils, monocytes and mast cells into the gut mucosa, goblet cell depletion, crypt abscesses and distortion of mucosal glands.[48] Prevention of goblet cell depletion by *B. thetaiotaomicron* is of specific interest as an article by Wrzosek et al[20] has previously shown that *B. thetaiotaomicron* enhances goblet cell differentiation in the colon of gnotobiotic rat and affects the specific composition of mucin O-glycans.

Animals treated with *B. thetaiotaomicron* showed strong downregulation of proinflammatory genes including *Arg1*, *Il6*, *Ccl3*, *Spp1*, *Il1a*, and genes involved in pathogen recognition, such as *Clec4e* and *Irg1*. In particular, *Arg1* was identified as a potential biomarker of treatment response. Arginase I is an endogenous antagonist to nitric oxide (NO) synthase.[40] Nitric oxide regulates many processes including blood flow, vascular permeability, mucosal defence and immune regulation. Nitric oxide production is decreased in chronically inflamed gut blood vessels in CD and UC, whereas arginase is upregulated.[49] Reconstitution of NO metabolism by inhibition of arginase activity has potential therapeutic effects in IBD.[40] Increased expression of the transcripts for *Il6*, *Ccl3*, *Spp1*, *Il1a*, *Clec4e* and *Irg1* was also observed. All of these genes are increased in human IBD and play roles in the inflammatory cascade through their effects on different innate immune populations including macrophages and dendritic cells.[50] For instance, *Spp1* is the murine ortholog of Osteopontin (OPN), a Th1 inflammatory mediator which is increased in the lamina propria of CD and UC patients and is directly related to the severity of inflammation.[51, 52]

Protective effects of some other *Bacteroides* species in animal models of colitis have been demonstrated in previous studies. *Bacteroides vulgatus*[53] and *Bacteroides ovatus*[54] have shown beneficial effects in DSS models. *Bacteroides fragilis* has shown efficacy in different experimental colitis models,[55, 56] and a protective role has been identified for polysaccharide A[55] and outer membrane vesicles[57] produced by this bacterial species. We present preliminary evidence of a pirin-like protein that ameliorated DSS-colitis when given orally to rats. While more work is needed to understand the exact mechanisms by which this protein conveys protection, our working hypothesis is that it modulates nuclear shuttling of NF-κB Rel A in stimulated cells in a manner similar to the whole bacterium. Further investigations should also show whether this pirin-like protein is solely responsible for the beneficial effects of *B. thetaiotaomicron* or whether additional signalling molecules or mechanisms are involved.

CONCLUSION

This study shows that treatment with *B. thetaiotaomicron* has protective effects in DSS and IL10KO models of colitis in both mice and rats. The strain we used was able to protect against weight loss, histopathological changes of the colon and inflammatory parameters. This efficacy was not exclusive to actively growing bacterial preparations but was maintained in a freeze-dried formulation. These encouraging data also indicate that a pirin-like protein produced by *B. thetaiotaomicron* was able to at least partially recapitulate the effect of the whole strain. Further work is needed to precisely understand the specific mechanisms by which *B. thetaiotaomicron* and its products interact with the host to exert its therapeutic effects.

SUPPLEMENTARY DATA

Supplementary data is available at *Inflammatory Bowel Diseases* online.

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