Experimental colonization with *Blastocystis* ST4 is associated with protective immune responses and modulation of gut microbiome in a DSS-induced colitis mouse model

Lei Deng1,2 · Lukasz Wojciech3 · Chin Wen Png1 · Eileen Yiling Koh1 · Thet Tun Aung3 · Dorinda Yan Qin Kioh4 · Eric Chun Yong Chan4 · Benoit Malleret3,5 · Yongliang Zhang1 · Guangneng Peng2 · Nicholas Robert John Gascoigne3 · Kevin Shyong Wei Tan1

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**Abstract**

**Background** *Blastocystis* is a common gut protistan parasite in humans and animals worldwide, but its interrelationship with the host gut microbiota and mucosal immune responses remains poorly understood. Different murine models of *Blastocystis* colonization were used to examine the effect of a common *Blastocystis* subtype (ST4) on host gut microbial community and adaptive immune system.

**Results** *Blastocystis* ST4-colonized normal healthy mice and *Rag1*-/- mice asymptotically and was able to alter the microbial community composition, mainly leading to increases in the proportion of *Clostridia* vadinBB60 group and *Lachnospiraceae* NK4A136 group, respectively. *Blastocystis* ST4 colonization promoted T helper 2 (Th2) response defined by interleukin (IL)-5 and IL-13 cytokine production, and T regulatory (Treg) induction from colonic lamina propria in normal healthy mice. Additionally, we observed that *Blastocystis* ST4 colonization can maintain the stability of bacterial community composition and induce Th2 and Treg immune responses to promote faster recovery from experimentally induced colitis. Furthermore, fecal microbiota transplantation of *Blastocystis* ST4-altered gut microbiome to colitis mice reduced the severity of colitis, which was associated with increased production of short-chain fat acids (SCFAs) and anti-inflammatory cytokine IL-10.

**Conclusions** The data confirm our hypothesis that *Blastocystis* ST4 is a beneficial commensal, and the beneficial effects of *Blastocystis* ST4 colonization is mediated through modulating of the host gut bacterial composition, SCFAs production, and Th2 and Treg responses in different murine colonization models.

**Keywords** *Blastocystis* · Gut microbiota · Th2 · Treg · Colitis

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**Abbreviations**

IBD  Inflammatory bowel disease  
IBS  Irritable bowel syndrome  
SSU  Small subunit  
Th2  T helper 2  
Treg  T regulatory  
FGFP  Flemish Gut Flora Project  
AGP  American Gut Project  
IMDM  Iscove’s modified Dulbecco’s medium  
PBS  Phosphate-buffered saline  
DSS  Dextran sulfate sodium  
DAI  Disease activity index  
H&E  Hematoxylin and eosin  
SI  Small intestine  
SEM  Scanning electron microscopy  
QIIME  Quantitative Insights into Microbial Ecology  
RPMI  Roswell Park Memorial Institute  
FCS  Fetal calf serum  
FBS  Fetal bovine serum  
IL  Interleukin  
IFN-γ  Interferon gamma  
TNF-α  Tumor necrosis factor alpha  
LP  Lamina propria  
SCFAs  Short-chain fatty acids  
CDI  *Clostridium difficile* Infection

Guangneng Peng  
pgn.sicau@163.com

Kevin Shyong Wei Tan  
mictank@nus.edu.sg

Extended author information available on the last page of the article
Background

*Blastocystis*, classified under the stramenopile phylum, is the most common unicellular intestinal parasite found in humans and various animals, with an estimate of more than 1 billion people colonized worldwide [1]. The clinical significance of *Blastocystis* remains controversial, although it has been widely studied for more than 100 years [2]. *Blastocystis* has been associated with inflammatory bowel disease (IBD) and irritable bowel syndrome (IBS) [3, 4]. Some microbiome studies have reported that *Blastocystis* colonization decreases the abundance of beneficial bacterial *Bifidobacterium* in humans and mouse models [5, 6]. However, most *Blastocystis*-gut microbiota association studies have revealed that *Blastocystis*-colonized individuals have a higher gut bacterial diversity and lower levels of *Bacteroides* compared to *Blastocystis*-free individuals, suggesting that *Blastocystis* may be a beneficial commensal rather than a pathogen [7, 8]. These discrepancies may be influenced by the complex nature of *Blastocystis* wherein several genetically distinct subtypes exist [1]. Different subtypes exhibit extensive differences in genome size, effects on gut microbiota, and immune responses [8–10]. However, there is still no consensus on the existence of pathogenic and non-pathogenic subtypes to date, although some in vitro *Blastocystis*–host studies reveal subtype-associated pathological outcomes [5, 11].

Based on analyses of the small subunit (SSU) rRNA gene, 25 subtypes have been identified in humans and a wide range of animals [12]. Among them, ST1–4 are the most reported subtypes in humans, accounting for around 90% of human infected cases [13]. The prevalence of ST4 appears to be more geographically variable, as it is mainly reported in Europe and rarely found in South America, Africa and Asia [14, 15]. Interactions among host, gut microbiota, and *Blastocystis* can drive the development of the immune system in colonized individuals and play a role in maintaining intestinal homeostasis. Indeed, it has been reported in vitro and in mouse models that *Blastocystis* is involved in the host’s innate and adaptive immunity in regulating the function and differentiation of the immune cell repertoire of the gut [16, 17]. T helper 2 (Th2) cells and T regulatory (Treg) cells are well known for their crucial roles in fighting extracellular parasite infection and suppressing intestinal inflammation [18]. However, it is unclear whether these immune cells are involved in the process of *Blastocystis* colonization.

Although several studies have investigated the association between *Blastocystis* and the gut microbial composition, only limited studies have analyzed this association at the subtype level. *Blastocystis* ST4 was originally isolated from a Wistar rat [19] and is the most prevalent subtype observed in the Flemish Gut Flora Project (FGFP) [8], TwinsUK [20] and American Gut Project (AGP) [21]. In the current study, we explored the interactions between *Blastocystis* ST4, gut microbiota, and host immunity in different mice models. Furthermore, we evaluated the impact of *Blastocystis* ST4 colonization on experimentally induced colitis. Our findings showed that *Blastocystis* ST4 colonization not only alters the gut microbial composition, but also enhances the accumulation of Th2 and Treg cells in the colonic mucosa. Additionally, *Blastocystis* ST4 colonization prevents loss of microbiota diversity and contributes towards attenuation of disease in a murine model of experimental colitis. These results revealed a previously unrecognized mutualistic relationship between *Blastocystis* ST4, gut microbiota and host immunity.

Methods

Culture of *Blastocystis*

The axenized *Blastocystis* isolate WR1 belonging to ST4 was used in this study. ST4-WR1 was originally isolated from a healthy Wistar rat during an animal survey at National University of Singapore [19]. ST4 is a common zoonotic subtype frequently detected in humans and a wide range of animals, including nonhuman primates, bovines, goats, dogs, rodents, and birds [22]. *Blastocystis* was maintained in 10 ml of pre-reduced Iscove’s modified Dulbecco’s medium (IMDM) (Gibco) supplemented with heat-inactivated 10% horse serum (Gibco). Cultures were incubated under anaerobic conditions in an Anaerojar (Oxoid) with gas pack (Oxoid) at 37 °C and subcultured every 3–4 days. *Blastocystis* cell counts were assessed manually using hemocytometer (Kova International).

Mice and treatments

The animal experiments were performed according to the Singapore National Advisory Committee for Laboratory Animal Research guidelines. All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee of National University of Singapore. C57BL/6 and *Rag1*<sup>−/−</sup> (*Rag1<sup>tm1Mom</sup>) mice of 8–12 weeks of age were bred and maintained in the animal facilities of the National University of Singapore (NUS). Littermates of the same sex and age were randomly assigned to the different experimental groups. Mice were colonized with *Blastocystis* ST4 via oral gavage with 5 × 10<sup>7</sup> live *Blastocystis* cells suspended in sterile phosphate-buffered saline (PBS) three times per week before euthanization at day 3.
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post last gavage. The control mice were orally gavaged with equal amounts of PBS at the same time. The mice used in all the experiments were age and sex matched.

For dextran sulfate sodium (DSS)-induced colitis with ST4 colonization model: C57BL/6 mice were administered 2% DSS (molecular mass = 36,000–50,000 Da; MP Biomedicals) w/v in drinking water for 7 days. Mice were then orally gavaged with $5 \times 10^7$ live Blastocystis cells three times per week for two consecutive weeks. In all experiments, mice were monitored daily for changes in body weight, stool consistency and presence of fecal blood. Disease activity index (DAI) was used to assess the severity of colitis as previously described [23]. In brief, daily calculation of DAI for each mouse was based on weight loss, occult blood, and stool consistency/diarrhea. Each parameter was scored on a scale of 1–4, with a maximum DAI score of 12. Score 0: no weight loss, normal stool, no blood; score 1: 1–3% weight loss, softer stool; score 2: 3–6% weight loss, loose stool, blood visible in stool; score 3: 6–9% weight loss, diarrhea, blood visible in stool; score 4: > 9% weight loss, diarrhea, gross bleeding.

Scanning electron microscopy

Mouse cecum and colon tissues were processed by opening the gut longitudinally without disturbing the intestinal contents with the help of binocular dissecting microscope. The opened tissues were pinned down to a silicone mat in four corners and fixed in 2.5% glutaraldehyde at 4 °C overnight as previously described [24]. The overnight fixed samples were washed two times (20 min each) with PBS and kept at 4 °C until further processing. Afterward, they were processed by post-fixing in 1% osmium tetroxide for 1 h, followed by dehydration with increasing concentrations of ethanol and critical-point dried. The dried samples were coated with 25 nm of gold and imaged on a field emission JSM-6701F Scanning Electron Microscopy (SEM) at a voltage of 10 kV.

DNA extraction and real-time quantitative PCR

DNA of the mouse fecal microbiota was isolated by using QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturers. Real-time quantitative PCR (qPCR) to estimate the number of Blastocystis cells in feces was performed in accordance with a previously published protocol [25]. Briefly, 2 μl of the extracted DNA was added to a mixture of 5 μl SsoAdvanced Universal SYBR Green Supermix and 0.5 μM forward primer BL18SSPF1 (5′- AGT AGT CATACGCTCGTCTCAA -3′) and 0.5 μM reverse primer BL18SR2PP (5′- TCTTGTTACCGGTGTACTGC -3′) for the SSU rRNA gene of Blastocystis. The qPCR was performed on an ABI 7500 real-time PCR system instrument (Life Technologies) at 95 °C for 5 min followed by 45 cycles of 95 °C for 15 s, 68 °C for 10 s, and 72 °C for 15 s, and completed with melting curve analysis. Each sample was quantified in triplicate. A standard curve was produced with a dilution series (10⁰, 10¹, 10², 10³, 10⁴, 10⁵, 10⁶ cells/ml) of Blastocystis ST4. The Ct values of each sample were compared with that of the standard curve and the number of Blastocystis cells was calculated.

All mice were examined for the presence of Blastocystis by SEM and qPCR amplification of the Blastocystis SSU rRNA gene. Mice were considered to be successfully colonized after Blastocystis was observed in the gut lumen by SEM, and SSU rRNA gene was successfully amplified from the stool samples.

Fecal microbiota transplantation

Fecal microbiota transplantation (FMT) was performed through oral gavage of feces preparations from donor mice as previously described [26]. In brief, microbiota for FMT was obtained from control (PBS-gavaged) Rag1−/− mice or ST4-colonized Rag1−/− mice administered with Blastocystis ST4. Feces were collected, diluted with PBS (50 mg/ml), and then administered to recipients by oral gavage (10 mg/mice) three times a week.

Histology

For histological studies, the small intestine (SI), cecum and colon tissues were fixed in 4% neutral buffered formalin before processing and embedding in paraffin based on standard protocols. 4.5 μm sections were prepared and stained with hematoxylin and eosin (H&E). Histology scoring was performed in a blinded fashion, whereby changes in intestinal crypt architecture, level of tissue damage, goblet cell loss, and inflammatory cell infiltrates were scored as previously described [27].

16S rRNA gene sequencing

The V3–V4 region of the 16S rRNA gene was amplified using the 341-F (CCTAYGGRBGASCAG) and 806-R (GGACTACNNGGTATCTAAAT) primers. Gene amplification was carried out using Phusion High-Fidelity PCR Master Mix (New England Biolabs). Single amplifications were performed in 50 μl reactions with 50 ng of template DNA. Cycling protocol consisted of 94 °C for 4 min, followed by 30 cycles of 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 30 s, with a final extension of 72 °C for 5 min. The size of the amplicons was determined using 2% agarose gel electrophoresis. Samples with size between 400 and 450 bp were extracted and purified from agarose gel using Qiagen Gel Extraction kit (Qiagen, Germany). Sequencing libraries
were generated from the amplicons using NEBNext Ultra DNA Library Pre® Kit for Illumina, following manufacturer’s recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Qualified library was sequenced on an Illumina Novaseq platform (Illumina, San Diego CA, USA) and 250 bp paired-end reads were generated.

**Bioinformatic and statistical analysis**

Paired-end reads were assigned to samples based on their unique barcodes and truncated by cutting off the barcode and primer sequences. Paired-end clean reads were merged using FLASH version 1.2.7, which was designed to merge paired-end reads when the reads overlap the read generated from the opposite end of the same DNA fragment [28], and the splicing sequences were called tags. The tags were compared with the reference database using UCHIME algorithm to detect chimera sequences [29], and these chimera sequences were subsequently removed, resulting in effective tags. Next, effective tags were trimmed to 200 bp to remove the low-quality portion of the sequences (mean quality score < 20) using the DADA2 plugin for Quantitative Insights into Microbial Ecology (QIIME2 version 2021.02) [30, 31]. Taxonomic assignment was performed using the BLAST fitted classifier trained on the SILVA 138 reference database with the feature-classifier plugin for QIIME2 [32] based on 100% similarity. Biodiversity index analysis was calculated using QIIME2 and displayed with R software (Version R-4.0.3). Alpha diversity analysis was done using the metrics Shannon, Simpson, and Richness index. Pairwise comparisons of microbial communities in different groups were carried out using permutational multivariate analysis of variance (PERMANOVA, Bray–Curtis distance) in the q2-diversity-plugin in QIIME. Principal coordinate analysis (PCoA) and heatmap analysis were performed using R package.

**Isolation of lamina propria cells**

To analyze intestinal lymphocytes, the intestines were longitudinally opened and washed with ice-cold PBS to remove luminal contents. The tissues were cut into 1 cm pieces and incubated in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) containing 1 mM EDTA (Sigma-Aldrich) and 1 mM DTT (Sigma-Aldrich) at room temperature for 20 min under slow rotation and spun down to remove the supernatant. The remaining pieces were incubated in RPMI containing 25% HEPES, 10% fetal calf serum (FCS), 1 mM EDTA, and 1 mM DTT at 37 °C for 1 h under slow rotation and then washed by PBS to remove epithelial cells and intraepithelial lymphocytes. Tissue pieces were digested with 0.3 mg/ml collagenase D (Sigma-Aldrich), 0.4 mg/ml dispase (Gibco) and 40 μg/ml DNase I (Roche) at 37 °C for 30 min under slow rotation. The digested tissue pieces and supernatants were filtered by 70 μm cell strainer and glass wool separately. After centrifugation, pellets containing the lamina propria (LP) lymphocytes were harvested.

**Flow cytometric analysis**

Lymphocytes were stimulated for 6 h with a cell stimulation cocktail of PMA (50 ng/ml), ionomycin (750 ng/ml) and 0.7 μl/ml GolgiStop (monensin, BD Biosciences). Live/dead stain was used to evaluate the viability of the cells. For surface staining, stimulated cells were stained with anti-CD4 (APC/FITC; Biolegend). Fixation and permeabilization buffers from Biolegend were used for intracellular cytokine staining. Fixed and permeabilized cells were stained with fluorochrome-conjugated anti-mouse antibodies against interleukin (IL)-4 (BV421; Biolegend), IL-5 (PE; Biolegend), IL-13 (PE; Biolegend), IL-10 (BV421; Biolegend), IL-17 (PerCP-Cy5.5; eBioscience, CA, USA), interferon gamma (IFN-γ) (BV711; Biolegend), and tumor necrosis factor (TNF-α) (APC; eBioscience) at 4 °C for 10 h. Flow cytometric analysis was performed on Fortessa X-20 (BD biosciences) and the data were analyzed using FlowJo_V10 software. The gating strategies are shown in Supplementary Fig. 4.

**LC/MS/MS assay**

Liquid chromatography/tandem mass spectrometry (LC/MS/ MS) was carried out for analysis of short-chain fatty acids (SCFAs) in derivatized stool extracts as previously described [33]. In brief, 500 μl of ice-cold extraction solvent containing 10 μM of d5-benzoic acid as internal standard (IS) was added to 250 mg of stool sample and subjected to vortex mixing for 5 min at ambient temperature. The suspension was then centrifuged at 18,000g for 10 min at 4 °C. The supernatant was carefully removed and centrifuged again at 18,000g for 10 min at 4 °C. An aliquot of 100 μl was subsequently derivatized using a final concentration of 10 mM aniline and 5 mM EDC for 2 h at 4 °C. The derivatization reaction was quenched using a final concentration of 18 mM succinic acid and 4.6 mM 2-mercaptoethanol for 2 h at 4 °C. All samples were stored at 4 °C until analysis on the same day. Analysis was performed using an Agilent 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA) interfaced with an AB Sciex QTRAP 5500 hybrid linear ion-trap quadrupole mass spectrometer equipped with a TurboIonSpray source (Applied Biosystems, Foster City, CA). Details of the LC/MS/MS and calibration methods were similar as previously described [33].
**Statistical analysis**

Statistical analysis was performed using R-4.0.3 software and GraphPad Prism 8 software (GraphPad Software, CA, USA). Two independent replicates were performed for each experiment. The unpaired two-tailed Student’s *t* test was used to evaluate differences between two groups. Two-way ANOVA and one-way ANOVA analysis with Tukey multiple comparisons test was used for comparison of more than two groups. Graphs show mean ± SEM. * * * * p < 0.05, ** * * * p < 0.01, *** * * * * p < 0.001.

**Results**

**Blastocystis ST4 colonization exerts no harmful effects on normal healthy mice**

*Blastocystis* ST4 is commonly found in humans and a variety of animals worldwide, predominantly in rats [34]. Although some in vitro studies have shown that ST4 infection can increase epithelial permeability and release pro-inflammatory cytokines [16, 35], the effects of ST4 in vivo are less well understood. To determine the effects of *Blastocystis* ST4 colonization on host intestinal bacterial communities and immune responses, we established a mouse model of oral *Blastocystis* colonization. Specifically, C57BL/6 mice were orally inoculated with 5 × 10⁷ cells of *Blastocystis* three times per week, and the mice were euthanized 3 days after the final gavage with *Blastocystis* (Fig. 1a). Interestingly, we observed that *Blastocystis* ST4 colonization did not cause any abnormalities, characterized by no significant difference in weight change and DAI between ST4-colonized and non-colonized control mice (Fig. 1b). The number of *Blastocystis* ST4 cells was quantitated by qPCR analysis (Fig. 1c), and scanning electron microscopic analysis of the contents of the cecum and colon from ST4-colonized mice revealed the presence of *Blastocystis*, which colonizes the intestinal lumen and closely adheres to intestinal microbes (Fig. 1d). Additionally, we examined the histopathology of SI, cecum and colon, and scored these based on the degree of inflammation and tissue damage. The mice colonized with ST4 showed intact mucosal epithelium without any ulcerated lesions or an abnormal level of inflammatory cell infiltration (Fig. 1e, f; Supplementary Fig. 1), which is consistent with previous findings in rats experimentally colonized with *Blastocystis* ST4 [17, 36]. Overall, these findings indicate that *Blastocystis* ST4 colonization did not cause abnormal phenotypic changes or any gut histopathology within the duration of the experiment.

**Blastocystis ST4 colonization alters the bacterial community composition in normal healthy mice**

*Blastocystis*-colonized individuals showed distinct difference in bacterial community structure when compared to non-colonized individuals [37], suggesting *Blastocystis* has the ability to modulate intestinal microbiota. To investigate the effects of *Blastocystis* ST4 colonization on gut bacterial communities, fecal samples of control and ST4-colonized mice were collected at day 0 and day 7, and bacterial compositions were investigated using 16S rRNA gene sequencing. Rarefaction analysis was used to estimate the total number of observed features that could be identified from the samples; this showed that a credible number of reads (average 101, 487) had been measured in each group (Supplementary Fig. 2). No significant differences in bacterial diversity and richness were detected in ST4-colonized and control mice over time, as measured by Shannon, Simpson and Richness indices (Fig. 2a). However, we observed significant difference in bacterial community composition in the ST4-colonized group between day 0 and day 7, measured by beta diversity of Bray–Curtis dissimilarity (PERMANOVA *p* = 0.026; Fig. 2b; Supplementary Table 1). The heatmap showed that differences in the relative abundances of various taxa between control and ST4-colonized mice (Fig. 2c). Specifically, we observed higher levels of unclassified *Clostridia* vadinBB60 group, *Tizardella*, and *Peptococcaceae* uncultured, and lower levels of *Odoribacter*, and *Lachnospiraceae* ASF356 at day 7 post-*Blastocystis* ST4 colonization (Fig. 2d, Supplementary Fig. 3). In contrast, the significantly reduced bacterial taxa in ST4-free mice were *Lachnospiraceae* NK4A136 group, *Odoribacter*, *Lachnospiraceae* uncultured, *Blautia* and *Oscillibacter*, while *Alloprevotella*, *Bacteroides*, and *Paraprevotella* were the most significantly increased (Fig. 2d, Supplementary Fig. 3). Overall, these data indicated that *Blastocystis* ST4 colonization alters the bacterial community compositions in normal healthy mice.

**Blastocystis ST4 colonization induces the accumulation of Th2 and Treg cells**

*Blastocystis* has been reported to involve the host adaptive immune responses. However, these effects are mainly based on in vitro studies [38]. To comprehensively investigate the effect of *Blastocystis* ST4 colonization on adaptive immune responses in a murine model, we examined the polarization status of T cell subsets in the colonic LP from control and ST4-colonized mice (Supplementary Fig. 4). The CD4 T cell population from the *Blastocystis*-associated group was enriched with IL-5-producing cells, reflecting a Th2 differentiated phenotype [39] (Fig. 3a, b). Furthermore, a substantial increase of the Th2 compartment (as defined by the
IL-13 expression) was observed in the ST4-colonized group (average 6% and 22% of IL-13-positive cells within the CD4 subset in the control and ST4 group, respectively) (Fig. 3a, b). Notably, together with an increase of the colonic Th2 compartment, the ST4-colonized group displayed a substantial increase in IL-10-producing CD4+ Treg cells (Fig. 3c). In contrast, the Th1 compartment (defined by the IFN-γ and TNF-α cytokines) and the Th17 subset (expressing IL-17) were comparable in ST4-colonized mice and control mice (Fig. 3c, d). These data suggest that colonic Th2 and Treg responses are induced by *Blastocystis* ST4 colonization.

**Blastocystis** ST4 colonizes asymptomatically in Rag1−/− mice

Th2 and Treg cells play a crucial role in the host’s resistance to parasite infection and in limiting intestinal inflammation [40]. *Blastocystis* ST4 could asymptomatically colonize normal healthy mice, possibly because it activates the host Th2 and Treg cells that promote mucus production, and increases gut motility to maintain intestinal homeostasis [41]. To investigate the role of adaptive immunity in *Blastocystis* ST4 colonization, we carried out experimental infections in immunodeficient Rag1−/− mice that lack all
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Fig. 2 *Blastocystis* ST4 colonization alters the murine fecal bacterial community compositions. a Alpha diversity was measured by Shannon, Simpson, and Richness indexes in the fecal samples of control and ST4-colonized mice (n = 4 mice per group). b PCoA of fecal gut microbiota in control and ST4-clonized mice at day 0 and day 7. c Heatmap of ST4 colonization-associated taxonomic markers at day 7. d Bacterial genera (relative abundance in the top 35) showing significant differences in their relative abundance between control and ST4-colonized mice.
mature lymphocytes. *Rag1−/−* mice were orally inoculated with *Blastocystis* ST4, with extension of the inoculation duration, from 7 to 14 days, to further investigate the effect of *Blastocystis* ST4 colonization in mice (Fig. 4a). Microscopic analysis showed the presence of *Blastocystis* in both ST41week and ST42weeks colonized mice (Fig. 4b). Histological examination of the colon tissues revealed no difference in histological scores, and showed intact structure and no inflammatory cell filtrate among the three groups (Fig. 4c, d). Similarly, there was no significant difference in colon length between groups (Fig. 4d). The number of *Blastocystis* cells was determined by qPCR amplification of SSU rRNA gene, and the mice from ST42weeks colonized group showed higher proportion of *Blastocystis* than ST41week colonized.
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Fig. 4  *Blastocystis* ST4 colonizes asymptomatically in *Rag1* −/− mice.  

a Experimental design.  

b Scanning electron microscopy (SEM) of colonic and cecum tissues from control and ST4-clonized mice. *Blastocystis* are indicated with red asterisk (*). Scale bar = 1 μm.  

c Representative micrographs of H&E-stained colon sections from ST4-colonized and control mice at day 14. Scale bar = 100 μm.  

d Colon length and colonic histological scores at endpoint. *Blastocystis* ST4 cells per milligram of stool in ST4-clonized mice.

Impact of *Blastocystis* ST4 colonization on the bacterial microbiome of *Rag1* −/− mice

Host adaptive immune responses can regulate the gut microbial compositions [42]. It is unclear whether the changes of intestinal microbiome are caused by the ST4 itself or the interaction of the ST4 and host adaptive immune system. Thus, we further explored the impact of *Blastocystis* ST4 colonization on gut microbial composition in *Rag1* −/− mice. Rarefaction curves showed that most of the reads were obtained, thus allowing us to undertake further analysis (Supplementary Fig. 5). There was no significant difference in Shannon index among control, ST41week, and ST42weeks groups during the experimental period, whereas ST41week mice revealed decreased Simpson index and ST42weeks mice.
mice increased Richness index at day 14 post-colonization (Fig. 5a). Bacterial community composition changed significantly over time for both ST4-colonized groups (PERMANOVA \( p < 0.01 \); Fig. 5b; Supplementary Table 1). The relative abundance of various taxa among different groups at day 0 and day 14 are presented as a heatmap (Fig. 5c). Of these bacterial taxa, six genera \textit{Prevotellaceae} UCG-001, unclassified \textit{Clostridia} vadinBB60 group, unclassified \textit{Rhodospirillales}, \textit{Muribaculum}, \textit{Anaeroplasma}, and \textit{Escherichia–Shigella} were significantly decreased in the fecal microbiota of both ST4\textsuperscript{week} and ST4\textsuperscript{2weeks} groups following \textit{Blastocystis} ST4 colonization (Fig. 5d; Supplementary Fig. 6). In contrast, two genera \textit{Lachnospiraceae} NK4A136 group and \textit{Oscillospiraceae} UCG-003 were significantly enriched in ST4-colonized groups (Fig. 5d; Supplementary Fig. 6). These data collectively suggest that \textit{Blastocystis} ST4 can modulate the gut microbiota in \textit{Rag I}\textsuperscript{−/−} mice, even without the participation of B and T immune cells.

**Blastocystis ST4 colonization promotes recovery from DSS-induced colitis**

\textit{Blastocystis} ST7 infection caused colonic tissue damage and ulceration in DSS-induced colitis mice [5], while long-term colonization with \textit{Blastocystis} ST3 promotes a faster recovery from colitis in rats [43], suggesting different subtypes exert differential effects on the host. To further explore whether \textit{Blastocystis} ST4 colonization can modulate the severity of disease in an experiment model of DSS-induced colitis, DSS-treated mice were orally inoculated with \textit{Blastocystis} ST4 three times per week for two consecutive weeks (Fig. 6a, b). Mice with \textit{Blastocystis} ST4 colonization showed faster recovery after DSS-induced colitis, as compared to that in \textit{Blastocystis}-free mice, as reflected by weight changes and DAI (Fig. 6c). Histological examination of the colon revealed reduced mucosal ulceration and damage, and lower histological scores in ST4-clonized mice (Fig. 6d). Similarly, there was significant difference in colon lengths between ST4-colonized mice and control mice (Fig. 6d). These data indicate that \textit{Blastocystis} ST4 colonization promotes recovery of mice from DSS-induced colitis.

**Gut microbiota changes after Blastocystis ST4 colonization in DSS-induced colitis mice**

Accumulating evidence indicates that the gut microbiome plays a pivotal role in host health and immune homeostasis [44]. The changes in gut microbial composition induced by helminth infection have the ability to reduce intestinal inflammation in an experimental mouse model [45]. To investigate if the protective effect of \textit{Blastocystis} ST4 on DSS-induced colitis mice is related to alterations in the gut bacterial community, we compared the variations in the microbial communities in ST4-colonized and control mice over time. Reduced alpha diversity (Shannon and Richness indexes) was observed in control mice, whereas \textit{Blastocystis} ST4 colonization maintained a stable alpha diversity over time (Supplementary Fig. 7; Fig. 7a). Both ST4-colonized and control group showed pronounced differences in bacterial composition during the experiment period (PERMANOVA \( p < 0.05 \); Fig. 7b; Supplementary Table 1). The relative abundance of various taxa are shown as a heatmap among groups (Fig. 7c). In particular, the genera \textit{Lachnospiraceae} NK4A136 group, unclassified \textit{Clostridia} vadinBB60 group, \textit{Alistipes}, \textit{Oribacter}, \textit{Oscillobacter}, \textit{Cloidextribacter}, unclassified \textit{Oscillospiraceae}, unclassified \textit{Ruminococcaceae}, \textit{Roseburia}, and uncultured \textit{Mitochondria} were significantly reduced in control mice over experimental time, whereas the genera \textit{Bacteroides}, \textit{Escherichia–Shigella}, \textit{Lachnoclostridium}, and \textit{Paraprevotella} were progressively expanded in control mice over time (Fig. 7d; Supplementary Fig. 8). Conversely, colonization with \textit{Blastocystis} ST4 appears to maintain a relatively stable microbiota communities compared to \textit{Blastocystis}-free mice after DSS administration. Higher proportions of unclassified \textit{Muribaculaceae} and lower abundance of \textit{Oscillibacter}, \textit{Cloidextribacter}, unclassified \textit{Oscillospiraceae} and unclassified \textit{Ruminococcaceae} were observed in ST4-colonized mice over time (Fig. 7d; Supplementary Fig. 8). Overall, these results suggest that the protective effect of \textit{Blastocystis} ST4 on DSS-induced colitis mice was associated with a stable bacterial diversity and microbiota communities.

**Protective effects of Blastocystis ST4 in DSS-induced colitis mice may be mediated by activation of Th2 and Treg cells responses**

Helminth-mediated Th2 or Treg cell responses have been exploited to ameliorate experimental colitis in a mouse model [46, 47]. We asked if \textit{Blastocystis} ST4 also regulates these immune responses to confer protection from DSS-induced colitis. Colonic tissues from DSS-induced colitis mice with \textit{Blastocystis} ST4 colonization demonstrated increased expression of Th2 (IL-4, IL-5, and IL-13) cytokines relative to control mice (Fig. 8a, b).
The immunophenotype of ST4-colonized DSS-treated mice was consistent with the previous observations on normal mice (Fig. 3a, b). Importantly, the size of the anti-inflammatory cytokine IL-10-producing Treg cell subset in colonic LP was also elevated in ST4-colonized mice (Fig. 8c, d). Similar to what we observed in normal healthy mice, the numbers of Th1-associated cytokine expressing cells (IFN-γ+ and TNF-α+), as well as Th17 cells (IL-17) were similar in ST4-colonized and control mice (Fig. 8c, d). Collectively, the composition of the Blastocystis-associated CD4 compartments in colonic LP from the DSS-induced colitis model indicated this organism’s direct interaction with the host’s adaptive immune system. Furthermore, Blastocystis-driven changes in the gut microbiome with the accompanying shift within immune compartments are plausible key factors that account for attenuation in the severity of DSS-mediated colitis.

**Transfer of fecal microbiota from ST4-colonized Rag1−/− mice reduces inflammation in experiment-induced colitis**

Next, to determine the effect of Blastocystis ST4-altered microbial communities that was independent of adaptive immunity-mediated microbiota changes on experiment-induced colitis, we performed FMT from ST4-colonized Rag1−/− mice into DSS-treated mice (Fig. 9a). DSS+FMT + ST4− colonized mice showed faster recovery and better health status, as measured by weight changes and DAI (Fig. 9c), compared to DSS+FMT + control. In addition, lower levels of intestinal inflammation and higher colon length were detected in DSS+FMT + ST4− colonized mice, although did not reach significance difference (Fig. 9e). We further evaluated the bacterial communities in experiment-induced mice upon different FMT conditions. Rarefaction curves showed
that the depth of sequencing was sufficient for analysis (Supplementary Fig. 9). DSS FMT + ST4–colonized mice showed a stable bacterial α-diversity when compared to DSS FMT + Control mice, which showed significant decreases, as indicated by the Shannon, Simpson and Richness indices (Fig. 10a). The gut microbial communities changed significantly over time for DSS FMT + ST4–colonized and DSS FMT + Control groups (PERMANOVA p < 0.05; Fig. 10b; Supplementary Table 1). The differences in the relative abundances of various taxa were observed in heatmap format (Fig. 10c). We observed higher levels of commensals and SCFA-producing taxa, Akkermansia, unclassified Clostridia vadinBB60 group, unclassified Rhodospirillales, and Clostridia UCG.014 in DSS FMT + ST4–colonized mice (Fig. 10d; Supplementary Fig. 10). DSS FMT + Control mice showed the enrichment of the genera Lachnospiraceae NK4A136 group, unclassified Clostridia vadinBB60 group, Prevotellaceae UCG.001, Bacteroides, Blautia, and Clostridia UCG.014 (Fig. 10d; Supplementary Fig. 10). Altogether, these findings suggest that transfer of ST4-altered gut microbiome are beneficial to the recovery of experimental colitis.

**FMT influences SCFAs and Treg cells IL-10 production in DSS-induced colitis recipients**

The host microbiome plays an important role in regulating physiology through microbiota-derived metabolites, especially SCFAs, during host–microbiome interactions [48]. To gain mechanistic insight into the faster recovery from intestinal inflammation in DSS FMT + ST4–colonized mice, we firstly quantitated eight SCFAs (acetic, propionic, butyric, isoobutyric, valeric acid, isovaleric, 2-methylbutyric, and caproic acid) of feces by LC/MS/MS. The levels of five SCFAs in fecal samples of DSS FMT + ST4–colonized–2 weeks mice exhibited significantly higher concentration changes than DSS FMT + Control mice (Fig. 11a). SCFAs are critical to the immune system and can serve as substrates for host energy metabolism [49]. We also detected increased SCFAs in Rag1−/− mice after Blastocystis ST4 colonization for one or two weeks (Supplementary Fig. 11). Besides, it has been determined that microbiota-derived SCFAs have the ability to modulate Treg cell function and can alleviate the colonic inflammation [50, 51]. We then assessed whether re-colonization of DSS treated mice with microbiota from ST4-colonized mice via FMT influences colonic immune cells. Interestingly, we observed an increased number of CD4+ cells expressing IL-10 and decreased number of CD4+ cells expressing TNF-α in DSS FMT + ST4–colonized mice (Fig. 11b, c). Taken together, these data indicate that transfer of ST4-altered gut microbiota in DSS-induced colitis recipients increases SCFAs production and induces accumulation of IL-10-producing Treg cells.

**Discussion**

Although the pathogenicity of Blastocystis is controversial, accumulating evidence shows that it is often present in asymptomatic individuals and is associated with healthy gut microbiota [52]. To understand the causal role of Blastocystis ST4 on the host gut microbiota and mucosal immune responses, we established murine models of oral colonization to investigate the effects of Blastocystis colonization on gut microbiota, and adaptive immune responses. Our results suggest that Blastocystis ST4 colonization in normal healthy and Rag1−/− mice did not cause any pathological lesions or inflammatory cell infiltration in colonic mucosa. Furthermore, Blastocystis ST4 colonization and transfer of ST4 colonization-altered gut microbiota to experimentally induced colitis mice promoted faster recovery from experimental caused colitis.

Microbial alpha diversity is considered an important marker for gut health, and high bacterial diversity implies stability and resilience of the gut ecosystem [53]. We monitored changes in the alpha diversity over the course of colonization with Blastocystis ST4 in several mouse models. Interestingly, alpha diversity (measured by Richness index) was significantly increased in Rag1−/− mice colonization with Blastocystis ST4 at day 14 compared with day 0 (Fig. 5a). Similarly, although it’s a different model of infection, a previous study showed infection with ST4 cysts increase in bacterial richness in rats [36]. The higher bacterial richness was also observed in individuals with Blastocystis colonization in the majority of microbiome studies [54, 55]. On the other hand, colonization with ST4 appears to maintain a stable fecal bacterial alpha diversity in normal healthy and DSS-induced colitis mice, while a significant decrease in alpha diversity was detected in control mice after DSS administration (Figs. 2a, 7a, and 10a). Loss of alpha diversity has been implicated in IBD patients [56, 57], and it is also a sign of dysbiosis in many other human diseases [58]. Thus, the decreased alpha diversity in control mice but not ST4-colonized mice may explain the differences in recovery from colitis.

Blastocystis and gut microbiota co-inhabit the host intestinal tract and are capable of interacting with each other. We observed that ST4 colonization significantly changed the bacterial community compositions, but there were some differences in specific taxa, depending on the mouse model utilized. For example, ST4 colonization mainly increased the abundance of Clostridia vadinBB60 group, belonging to the Clostridia class, in normal healthy mice, which was positively correlated with the Treg cell counts [59]. Lachnospiraceae NK4A136 group, one of the known short-chain fatty acid (SCFA)-producing bacteria that degrade complex polysaccharides [60], was the most enriched bacteria in...
Experimental colonization with Blastocystis ST4 is associated with protective immune responses. Although we did not dissociate the effect of Blastocystis ST4 in a study to demonstrate that FMT from a donor colonized with ST4 improves the intestinal inflammation in normal healthy mice and DSS-induced colitis mice. It has been determined that Th2 cells are important sources of type 2 cytokines (IL-4, IL-5, and IL-13) and are also important effector cells during the inflammatory process. Recent data showed an expansion of IL-13- and IL-4-producing CD4+ T cells in Nod2−/− mice contributes to ameliorating the intestinal injury response [62]. Another interesting study in primates demonstrated that the experimental administration of Trichuris trichiura can ameliorate colitis by both induction of the colonic CD4+ T cells producing IL-4 and modulation of microbial populations [71].

In addition, we also found that Blastocystis ST4 colonization increases the number of IL-10-producing Treg in the colonic mucosa of DSS-induced mice. The cytokine IL-10 produced by Treg cells is required for containment of inflammatory responses in mucosal tissues [72]. Both humans and mice deficient in IL-10 or IL-10 receptor (IL-10R) are prone to develop severe intestinal inflammation [73–75], highlighting the importance of IL-10 in preventing this disease process. On the other hand, the gut microbe-derived SCFAs can enhance the expression of Foxp3 and IL-10-expressing colonic Treg cells by inhibition of histone deacetylase (HDAC) or in a GPR43-dependent manner [50, 76, 77]. Although the three most abundant SCFAs, acetate, propionate, and butyrate, did not change significantly after FMT and ST4 colonization, there was elevation in the other five SCFAs, isobutyric acid, valeric acid, isovaleric acid, 2-methylbutyric acid, and caproic acid, which have been reported to induce the accumulation of anti-inflammatory cytokine IL-10 [78–80]. However, it is unclear whether the increased production of IL-10 is directly caused by Blastocystis ST4 or indirectly through regulating the gut microbiota-derived SCFAs. Future studies should focus on understanding the mechanistic connection between Blastocystis ST4 colonization, IL-10 signaling, and bacterial-derived SCFAs using relevant animal models.

**Conclusions**

We demonstrated that Blastocystis ST4 colonization is able to alter the gut bacterial community composition in an adaptive immunity-independent fashion, evidenced through the use of Rag1−/− mice. In models with intact immune systems, Blastocystis ST4 induces the expression of Th2 cytokines. Notably, alterations in gut microbiota...
composition mediated by *Blastocystis* ST4 colonization is associated with amelioration of colonic inflammation, likely through immunomodulatory effects of SCFAs, Th2 and Treg effectors. These findings represent an important contribution toward the elucidation of the complex interplay between *Blastocystis* ST4, gut microbiota, and host adaptive immune responses.

**Fig. 8** *Blastocystis* ST4 colonization activates Th2 and Treg cells responses in colonic mucosa of DSS-induced colitis mice. **a** Colored contour plots show staining for IL-4, IL-5, and IL-13 within CD4⁺ cells. **b** Bar charts show the percentage of IL-4, IL-5, and IL-13 expressing CD4⁺ T cells. **c** Colored contour plots show staining for IL-10, IL-17, TNF-α, and IFN-γ within CD4⁺ cells. **d** Bar charts show the percentage of IL-10, IL-17, TNF-α, and IFN-γ expressing CD4⁺ T cells. Statistical significance is indicated by *p* < 0.05, **p** < 0.01, and ***p*** < 0.001, unpaired Student’s *t* test.
Fig. 9 Transfer of ST4-altered microbiome to colitis mice reduces colonic inflammation. a Experimental design. b Blastocystis ST4 cells per milligram of stool in DSSFMT + ST4−clonized mice. c Weight changes and DAI between DSSFMT + Control mice and DSSFMT + ST4−clonized mice. d Scanning electron microscopy (SEM) of colonic and cecum tissues from DSSFMT + Control mice and DSSFMT + ST4−clonized mice, Blastocystis are indicated with red asterisk (*). Scale bar = 1 μm. e Representative micrographs of H&E-stained colon sections from DSSFMT + Control mice and DSSFMT + ST4−clonized mice at day 17. Scale bar = 100 μm. Colon length and colonic histological scores at end point.
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Fig. 10 Gut microbiota analysis upon different FMT conditions treatment in DSS-induced colitis mice. a Alpha diversity was measured by Shannon, Simpson, and Richness indexes (n = 4 in control group and n = 5 in ST4-colonized group). b PCoA of fecal gut microbiota in control and ST4-clonized mice at day 0 and day 17. c Heatmap of taxonomic markers among different groups. d Bacterial genera showing significant differences in their relative abundance among groups.

Fig. 11 FMT influences SCFAs and Treg cells IL-10 production in DSS-induced colitis recipients. a Fold change of each SCFA relative to levels at day 0 from DSS\textsuperscript{FMT} mice (recipients). b Colored contour plots show staining for IL-10 within CD4\textsuperscript{+} cells, and bar chart shows the percentage of IL-10 expressing CD4\textsuperscript{+} T cells. c Colored contour plots show staining for TNF-α within CD4\textsuperscript{+} cells, and bar chart shows the percentage of TNF-α expressing CD4\textsuperscript{+} T cells. *p<0.05, **p<0.01, ***p<0.001, Two-way ANOVA (a) and one-way ANOVA (b, c) analysis with Tukey multiple comparison test.
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Author contributions KSWT conceived and designed the study. CWP and LD performed animal experiments. CWP and YZ performed histological analyses. EYK and LD performed 16S rRNA gene sequencing and analyzed the data. LW and LD performed immunological experiments and analyses. TTA and BM performed scanning electron microscopy experiments. DYQK and ECYC performed LC/MS/MS assays. YZ, GP, and NRJG provided scientific insights and critically reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials The datasets generated and analyzed in the current study are available in the Sequence Read Archive (SRA) database at NCBI under BioProject ID PRJNA669121 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA669121).

Declarations

Ethics approval and consent to participate All animal handling and procedures were performed in accordance with the Institutional Animal Care and Use Committee of National University of Singapore (protocol no. R19-1259).

Consent for publication Not applicable.

Conflicts of interest The authors declare no competing interests.

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Authors and Affiliations

Lei Deng1,2 · Lukasz Wojciech3 · Chin Wen Png1 · Eileen Yiling Koh1 · Thet Tun Aung3 · Dorinda Yan Qin Kioh4 · Eric Chun Yong Chan4 · Benoit Malleret3,5 · Yongliang Zhang1 · Guangneng Peng2 · Nicholas Robert John Gascoigne3 · Kevin Shyong Wei Tan1

Lei Deng
e0501856@u.nus.edu.sg
Lukasz Wojciech
micluka@nus.edu.sg
Chin Wen Png
micpcw@nus.edu.sg
Eileen Yiling Koh
mickohy@nus.edu.sg
Thet Tun Aung
mictta@nus.edu.sg
Dorinda Yan Qin Kioh
dorinda.kioh@nus.edu.sg
Eric Chun Yong Chan
eric.chan@nus.edu.sg
Benoit Malleret
micmbjb@nus.edu.sg
Yongliang Zhang
miczy@nus.edu.sg
Nicholas Robert John Gascoigne
micnrjg@nus.edu.sg

1 Laboratory of Molecular and Cellular Parasitology, Healthy Longevity Translational Research Programme and Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University of Singapore, 5 Science Drive 2, Singapore 117545, Singapore
2 The Key Laboratory of Animal Disease and Human Health of Sichuan Province, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu 611130, Sichuan, China
3 Department of Microbiology and Immunology, Immunology Translational Research Programme, Yong Loo Lin School of Medicine, Immunology Programme, Life Sciences Institute, National University of Singapore, Singapore 117597, Singapore
4 Department of Pharmacy, Faculty of Science, National University of Singapore, 5 Science Drive 2, Singapore 117545, Singapore
5 Singapore Immunology Network (SIgN), A*STAR, 8A Biomedical Grove, Immunos Building, Singapore 138648, Singapore