Title

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TGR5 protects against colitis in mice but vertical sleeve gastrectomy increases colitis severity

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Abstract

Background and Aims: Bariatric surgery, such as vertical sleeve gastrectomy (VSG), is the most effective long-term treatment for obesity. However, there are conflicting reports on the effect of bariatric surgery on inflammatory bowel disease (IBD). Bariatric surgery increases bile acid concentrations, which can decrease inflammation by signaling through the bile acid receptor, TGR5. TGR5 signaling protects against chemically-induced colitis in mice. VSG increases circulating bile acid concentrations to increase TGR5 signaling, which contributes to improved metabolic regulation after VSG. Therefore, we investigated the effect of VSG on chemically-induced colitis development and the role of TGR5 in this context.

Methods: VSG or sham surgery was performed in high fat diet-fed male *Tgr5*$^{+/+}$ and *Tgr5*$^{-/-}$ littermates. Sham-operated mice were food restricted to match their body weight to VSG-operated mice. Colitis was induced with 2.5% DSS in water post-operatively. Body weight, energy intake, fecal scoring, colon histopathology, colonic markers of inflammation, goblet cell counts and colonic microRNA-21 levels were assessed.

Results: VSG decreased body weight independently of genotype. Consistent with previous work, genetic ablation of TGR5 increased the severity of DSS-induced colitis. Notably, despite the effect of VSG to decrease body weight and increase TGR5 signaling, VSG increased the severity of DSS-induced colitis. VSG-induced increases in colitis were associated with increased colonic expression of TNFα, IL-6, MCP-1 and microRNA-21.

Conclusions: While our data demonstrate that TGR5 protects against colitis, they also demonstrate that VSG potentiates chemically-induced colitis in mice. These data suggest that

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individuals undergoing VSG may be at increased risk for developing colitis; however, further study is needed.

Keywords

VSG; colitis; TGR5

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory condition encompassing both Crohn's disease and ulcerative colitis (1). The mechanisms underlying IBD are complex and involve both genetic and environmental factors (2). Recent work demonstrates that obesity is positively correlated with IBD, suggesting that obesity may contribute to IBD pathogenesis (3–5). Obesity is known to induce chronic low-grade inflammation and it is possible that obesity-related inflammation may promote IBD development and progression (6–8). While bariatric surgery, such as vertical sleeve gastrectomy (VSG), is arguably the most effective long-term treatment for obesity and produces numerous cardiometabolic benefits (9–11), there is limited and conflicting literature on the effects of bariatric surgery on IBD.

Several small case reports describe the development of inflammatory bowel disease (IBD) after bariatric surgery (12–14). While some small case studies suggest that bariatric surgery is safe in patients with pre-existing IBD (15, 16), and may even improve IBD-related symptoms (17, 18), the largest case series reported to date demonstrate that bariatric surgery increases the risk of developing IBD (19, 20). IBD can predispose patients to the development of colorectal cancer (21, 22). Consistent with this, bariatric surgery has been reported to increase the risk of developing colorectal cancer (23–25). Clinical studies have analyzed IBD risk in cohorts of patients undergoing a variety of different bariatric surgery types; therefore, the relative impact of different bariatric surgery types on IBD risk is unknown. VSG is the most commonly performed bariatric procedure in the U.S. (26). Therefore, we focused our study on the effect of VSG on colitis development and progression in a mouse model of chemically-induced colitis.

Bariatric surgery, including VSG, increases circulating bile acid concentrations in humans and rodent models (27–29). While bile acids are best known as steroid molecules that help digest dietary cholesterol and lipids, they also play an important immunomodulatory role. For example, increased signaling through the bile acid receptor, TGR5, on immune cells reduces inflammatory cytokine secretion (30, 31). Furthermore, TGR5, has been shown to protect against chemically-induced colitis in mice (32). We and others have previously demonstrated that increased signaling through the bile acid receptor, TGR5, contributes to improved glucose regulation after VSG (27, 33). Therefore, we investigated the role of TGR5 on the effect of VSG in a chemically-induced mouse model of colitis.

Materials and Methods

Animals and Diets

Heterozygous *Tgr5*$^{+/-}$ breeding pairs generated on a C57BL/6J background were obtained from the UC Davis Knockout Mouse Project Repository and a breeding colony established. *Tgr5*$^{+/+}$ and *Tgr5*$^{-/-}$ male littermates were placed on 60% high fat diet at 2 months of age (D12492, Dyets, Bethlehem, PA) for 2 months to produce an obese and insulin resistant phenotype. At 4 months of age mice underwent sham surgery or VSG, as previously described (27, 34). The following groups were studied: sham-operated TGR5 wild-type (Sham WT), sham-operated TGR5 knockout (Sham KO), VSG operated TGR5 wild-type (VSG WT), and VSG operated TGR5 knockout (VSG KO). Groups were matched for body weight at the time of surgery. Sham-operated mice were food restricted to match body weight to their respective VSG-operated groups to control for body weight. Food intake and body weight were measured twice per week. 21 days after surgery mice received 2.5% dextran sodium sulfate (DSS) in water for 5 days (TdB Consultancy; Upsala, Sweden). We chose a short low-dose DSS dosing paradigm to study a mouse model with a mild colitis phenotype to ensure that the potential differential effects of VSG and TGR5 were not overwhelmed by the severity of disease. 6 hour fasted mice were euthanized 25 days after surgery by an overdose of pentobarbital (200 mg/kg i.p.). The experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Colitis Evaluation

During DSS treatment, fecal scores were performed daily to assess disease severity *in vivo*. Specifically, diarrhea score was assessed by measuring the softness and appearance of the stool on a scale of 0–3. Bleeding score was measured by detection of heme in stool using the Hemoccult Sensa test (Beckman Coulter; Fullerton, CA) or evidence of gross bleeding on a scale from 0 to 3.

At sacrifice, colons were removed, weighed and length measured. A small full-thickness section of colon was removed from the proximal colon and frozen in liquid nitrogen for immunoblotting analysis. Remaining tissue samples were prepared as spiral “Swiss rolls” and fixed in 4% formalin for 24 hours followed by paraffin embedding. Paraffin blocks were sectioned on a microtome at 5μm and slides stained with hematoxylin and eosin (H&E). Stained sections were analyzed for the presence and severity of colitis in a blinded fashion by a board-certified veterinary pathologist. Briefly a pathological score from 1–4 was given to each sample: 1) none; 2) mild; 3) moderate; 4) severe. Scoring was based on the presence and severity of colitis, inflammatory infiltration, the presence of erosion/ulcers, and epithelial changes.

Histologic quantification of crypt density, crypt depth and goblet cell numbers

H&E stained slides were used for histomorphometric measurements of colonic crypt density and crypt depth. For both measurements, multiple non-overlapping fields were evaluated per slide at 200x magnification. Fields involving at least 5 adjacent, clearly discernible, individual crypts were enumerated over a measured linear distance for determining crypt density. Crypt depth was assessed by measuring the linear distance of clearly delineated

crypts spanning the crypt base and the epithelial surface junction. Linear measurements were made using ImageJ software. Alcian blue stained sections were used to enumerate the number of goblet cells per colonic crypt. Distinguishable goblet cells from clearly delineated crypts from multiple fields at 200x magnification were numbered.

Immunoblotting

For immunoblotting, radio immunoprecipitation assay (RIPA) buffer (10mM Tris-HCL, pH 7.4, 150mM NaCL, 0.1% sodium dodecyl sulfate (SDS), 1% Triton-X-100, 1% sodium deoxycholate, 5mM EDTA, 1mM NaF, 1mM sodium orthovanadate and protease inhibitors) was used to lyse tissues in liquid nitrogen. Lysates were separated by centrifugation at 10,000 rpm for 10 minutes and protein concentrations determined by bicinchoninic acid protein assay (Pierce Chemical, IL). Proteins (40μg) were resolved by SDS-PAGE (10%) and transferred to PVDF membranes. Immunoblots were performed with relevant antibodies and proteins visualized with Luminata Forte (Millipore; Billeorica, MA). The actin antibody (0.002μg/ml) was from Santa Cruz Biotechnology (Dallas, TX), the antibodies for TNF-α (1μg/ml) and MCP-1 (1μg/ml) from Abcam (Cambridge, MA), and the antibody for IL-6 (1μg/ml) from Cell Signaling (Danvers, MA). Horseradish peroxidase (HRP)-conjugated mouse secondary antibody (0.04μg/ml) was from Santa Cruz Biotechnology (Dallas, TX) and horseradish peroxidase (HRP)-conjugated rabbit secondary antibody (0.04μg/ml) was from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence was detected using the Chemidoc MP imaging system and protein expression quantified using ImageLab 5.1 software.

miR-21 quantification

Total RNA was extracted from 5 mm sections of frozen proximal colon samples with the Total RNA Purification kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the manufacturer's instructions. To remove potentially contaminating DSS, purified RNA samples were precipitated with 2.5M lithium chloride (Thermo Fisher Scientific, Waltham, MA), as described previously (35). Reverse transcription and real time-PCR were performed with specific TaqMan primers for miR-21 (#000397, Thermo Fisher Scientific, Waltham, MA) and U6 snRNA (#001973, Thermo Fisher Scientific, Waltham, MA). Fold change of expression was calculated based on the $\Delta\Delta C_t$ method.

Statistics and Data Analysis

Data are presented as mean ± SEM. All statistical analysis were performed with GraphPad Prism 6.00 for windows (GraphPad Software, San Diego, CA). Data were analyzed by two-factor ANOVA with Bonferroni's post-test unless otherwise indicated. Differences were indicated by an α of 0.05.

Results

VSG decreases body weight independently of TGR5 in DSS-treated mice

To test the impact of TGR5 and VSG on colitis development, we treated sham and VSG-operated *Tgr5*$^{+/+}$ and *Tgr5*$^{-/-}$ mice with 2.5% DSS for 5 days, starting 21 days after surgery. Sham-operated mice were food restricted to match body weight to VSG-operated mice to

control for this variable. Therefore, cumulative energy intake, body weight and adiposity did not differ between sham and VSG-operated groups throughout study (Figure 1A–C). VSG lowered body weight relative to baseline in both VSG WT and VSG KO groups (Figure 1B, $P<0.05$). Energy intake, body weight and adiposity did not differ between genotype in VSG or sham-operated mice throughout study (Figure 1A–C). This is consistent with previous work showing that VSG decreases body weight independently of TGR5 in non-DSS treated mice (27).

Genetic ablation of TGR5 and VSG potentiate histologic injury following DSS-induced colitis

To assess disease severity *in vivo*, diarrhea and colitis-associated bleeding were scored daily during DSS administration. Diarrhea score did not differ between Sham WT and VSG WT (Figure 2A). However, diarrhea score was elevated 2.5-fold in VSG KO compared with Sham KO on day 4 of DSS treatment (Figure 2A, $P<0.05$). Bleeding score, colon length and colon weight did not differ between groups (Figure 2B–D).

Histologic examination of H&E stained colons revealed a trend for a higher incidence of erosion, hyperplasia, lymphocytic infiltration and colitis in VSG WT compared with Sham WT, and Sham WT compared with Sham KO (Figure 3A and Table 1). Specifically, 63% of Sham WT colons were positive for histopathological changes consistent with colitis, compared with 86–90% in all other groups (Table 1). Interestingly, the incidence of neutrophilic infiltration was significantly elevated in VSG KO compared with Sham KO, but not in VSG WT compared with Sham WT ($P<0.05$, Table 1). To gain more detailed insight into the histologic changes induced by VSG and genetic ablation of TGR5, we quantified colonic crypt depth, crypt density and goblet cell number per crypt. Crypt density and crypt depth did not differ between groups (Figure 3B–C). There was a trend for goblet cell number per crypt to be elevated in VSG compared with Sham in both $Tgr5^{+/+}$ and $Tgr5^{-/-}$ mice; however, this did not reach significance (Figure 3D). If we set a threshold of average goblet cell count per crypt at 10 (based on the Sham WT data), only 50% of mice exceeded this threshold in the sham-operated group whereas 75% of mice exceeded this threshold in the VSG WT groups. Histopathologic scoring revealed that colitis severity was higher in VSG KO compared with Sham WT (Figure 3E, $P<0.05$). Direct comparison of VSG WT with Sham WT revealed a 45% increase in colitis severity score in VSG WT compared with Sham WT (Figure 3E, $P<0.05$). Direct comparison of Sham WT with Sham KO revealed that genetic ablation of TGR5 increases colitis severity score in sham-operated mice (Figure 3E, $P<0.05$). Colitis severity score did not differ between VSG WT and VSG KO (Figure 3E). Overall, these data suggest that genetic ablation of TGR5 and VSG exacerbate histologic evidence of colitis and its severity.

Genetic ablation of TGR5 and VSG enhance colonic inflammation

To quantify the degree of colonic inflammation, immunoblotting was performed on colon tissue for the following inflammatory markers: monocyte chemotactic protein 1 (MCP-1), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6). MCP-1, TNF-α and IL-6 expression were higher in VSG WT compared with Sham WT, demonstrating that VSG increases colonic inflammation in DSS-treated mice (Figure 4A–D, $P<0.05$). MCP-1 was

elevated in VSG KO compared with Sham KO (Figure 4A–B, $P<0.05$). Furthermore, MCP-1, TNF-α and IL-6 expression were elevated in Sham KO compared with Sham WT, demonstrating that TGR5 ablation augments DSS-induced colonic inflammation in mice (Figure 4A–D, $P<0.05$). Finally, IL-6 and MCP-1 expression were elevated in VSG KO compared with VSG WT, suggesting that TGR5 ablation aggravates DSS-induced colonic inflammation after VSG (Figure 4A–B and D, $P<0.05$). Thus, these data are consistent with the histologic finding that Sham KO and VSG-operated mice had higher colitis severity. Unlike our histologic analysis, these data suggest that VSG in combination with genetic ablation of TGR5 has an additive effect on indices of colonic inflammation. Together, these data demonstrate that genetic ablation of TGR5 and VSG increase colonic inflammation.

VSG enhances colonic microRNA-21 expression

MicroRNAs (miRNAs) are critical regulators of gastrointestinal health through the post-transcriptional regulation of gene expression. In particular, colonic miR-21 expression has been shown to be upregulated in patients with Crohn's disease and ulcerative colitis (36–39). Furthermore, recent studies show that genetic ablation of miR-21 protects against DSS-induced colitis (38, 40). Therefore, we hypothesized that miR-21 would be aberrantly elevated in the context of TGR5 ablation and/or VSG, both of which aggravate DSS-induced colitis according to our data. To test this hypothesis, we carried out real-time quantitative PCR and found that VSG significantly increased colonic expression of miR-21 (Figure 4E, 3-fold, $P<0.05$). On the other hand, genetic ablation of TGR5 did not significantly increase miR-21 (Figure 4E). Therefore, increased colonic miR-21 expression may contribute to the effect of VSG to promote DSS-induced colitis, but does not appear to be related to the effect of genetic ablation of TGR5 to increase colitis severity.

Conclusion

Herein, we report that VSG is associated with worse DSS-induced colitis in mice. We have previously reported that increased TGR5 signaling contributes to improved glucose regulation after VSG and our findings herein demonstrate that TGR5 protects against colitis. This is consistent with previous reports that genetic ablation of TGR5 promotes DSS-induced colitis in mice (30, 41). Nevertheless, our data demonstrate that VSG promotes DSS-induced colitis in mice despite increased TGR5 signaling. Our findings are consistent with data in human patients reporting worsening of colitis after bariatric surgery and may help explain findings of *de novo* IBD in patients that undergo bariatric surgery (12).

There is conflicting information in the literature regarding the impact of bariatric surgery on IBD. There are reports that bariatric surgery is effective in IBD patients and can improve their IBD status (16, 17). However, IBD increases the complexity and post-operative care of patients after bariatric surgery (42). For example, chronic steroid administration, such as that used for IBD treatment, is associated with increased morbidity and mortality after bariatric surgery (43). In addition, recent reports suggest that bariatric surgery can lead to the development of *de novo* IBD. One retrospective study reports 44 cases of *de novo* IBD from electronic medical records from two institutions over the past 20 years, with an incidence rate of 26.7 cases per 100,000 persons per year (19). This study demonstrates a higher

incidence of IBD development in patients with bariatric surgery compared with the normal population. Our data suggest that VSG worsens the severity of experimental colitis which may explain the increased incidence of IBD development observed in human patients after bariatric surgery.

It is likely that the impact of bariatric surgery on colitis varies between surgery types. One previous study has investigated the effect of colitis in a rodent model of bariatric surgery. Interestingly, they found that duodenojejunal bypass decreased colitis severity compared to both sham and VSG-operated mice (44). Our data also demonstrate that VSG exacerbates colitis in a DSS-induced colitis model. Thus, it appears that there may be differences between surgery types on the impact on colitis development and/or progression and that VSG may have a greater impact on IBD than other bariatric surgery types.

One potential contributor to the effect of VSG to promote the development of colitis is through changes in gut microbial composition. Bariatric surgery results in dramatic alterations in gut microbial composition in both rodent models and human patients (45–49). Surprisingly, these gut microbial shifts are remarkably similar between vastly different bariatric surgery types (45–47, 49, 50), demonstrating that the relevant microbial changes occur irrespective of differences in the specific gastrointestinal anatomic alterations. These shifts in the gut microbiome have been suggested to play an important role in the metabolic benefits of bariatric surgery. For example, transplantation of gut microbiota derived from RYGB-operated humans or mice to germ-free mice produces lower adiposity than control transplants (47, 50). However, the changes in gut microbial composition include increased relative abundance of bacterial genera considered pathogenic and associated with the development of colitis. Indeed, these gut microbial shifts are remarkably similar to those observed in human IBD patients. One of the most consistently reported gut microbial changes after various types of bariatric surgery in both humans and rodents is an increase in the class, Gammaproteobacteria, and its associated family, Enterobacteriaceae (45, 47, 49–51). We have previously reported that our mouse VSG model exhibits a significant increase in the relative abundance of Gammaproteobacteria and Enterobacteriaceae up to 6.5 months after surgery (27). Enterobacteriaceae are increased in patients with IBD and in mouse models of IBD (52). In particular, *Escherichia* and *Shigella*, subdivisions of Enterobacteriaceae, are increased in patients with IBD and treatment with anti-inflammatory drugs decreases the relative abundance of these genera (53, 54). *Escherichia* and *Shigella* are elevated in human patients and rodents, including our rodent model, after bariatric surgery (45, 47, 49–51). Therefore, while metabolically beneficial, these gut microbial changes may contribute to increased colitis risk after bariatric surgery.

Our data also reveal that increases in colonic miR-21 expression may contribute to the effect of VSG to promote colitis development. MiR-21 is among the most significantly upregulated miRNAs in the colon of adult and pediatric patients with Crohn’s disease (37, 38). Other studies have demonstrated that colonic miR-21 levels are elevated in ulcerative colitis and that this may increase risk for colitis-associated colon cancer (36, 39, 55). Moreover, it has been reported that the levels of miR-21 in colonic CD3+ T cells are rescued during remission of ulcerative colitis (40). Very recent studies show that genetic ablation of miR-21 induces a healthy shift in the gut microbiome and protects against DSS-induced colitis (38,

40). Therefore, it is possible that postoperative increases in colonic miR-21 expression contribute to pathogenic shifts in gut microbial composition to promote colitis development; however, further work is needed to evaluate this connection.

Our work demonstrates that VSG potentiates DSS-induced colitis in mice. The current results are consistent with prior reports in humans demonstrating worsening of IBD symptoms in patients after surgery and may help explain *de novo* colitis development in patients who undergo bariatric surgery. Further work is needed to confirm these findings in human patients and differentiate the relative impact of different bariatric surgery types on IBD risk in humans. Overall, these data suggest that caution should be exercised in performing VSG on patients with increased risk of colitis development.

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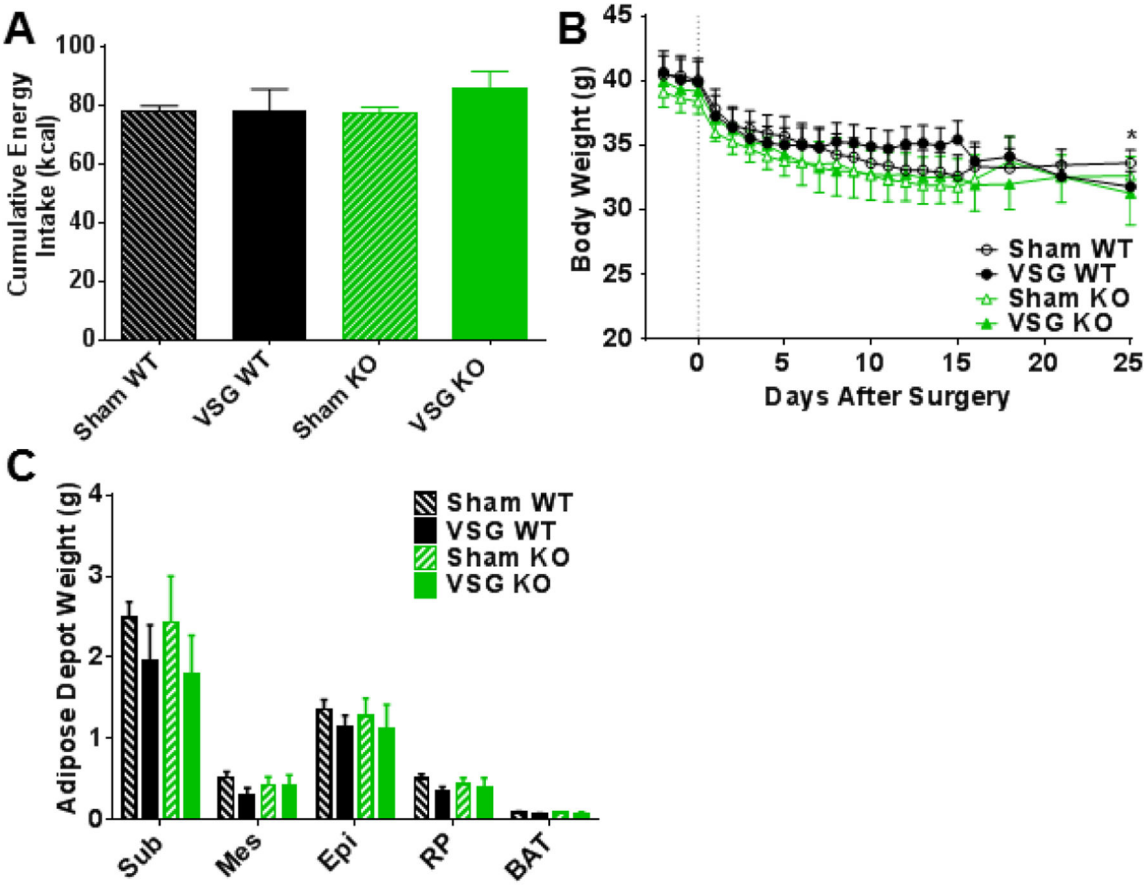

Figure 1. VSG decreases body weight independently of TGR5.

Cumulative energy intake (A) and body weight (B). Adipose depot weight (Subcutaneous adipose tissue (Sub), mesenteric adipose tissue (Mes), epididymal adipose tissue (Epi), retroperitoneal adipose tissue (RP), and brown adipose tissue (BAT)) (C). Data are presented as mean ± SEM. n=7–10 per group. *P<0.05 for all groups compared with baseline body weight by two-factor ANOVA.

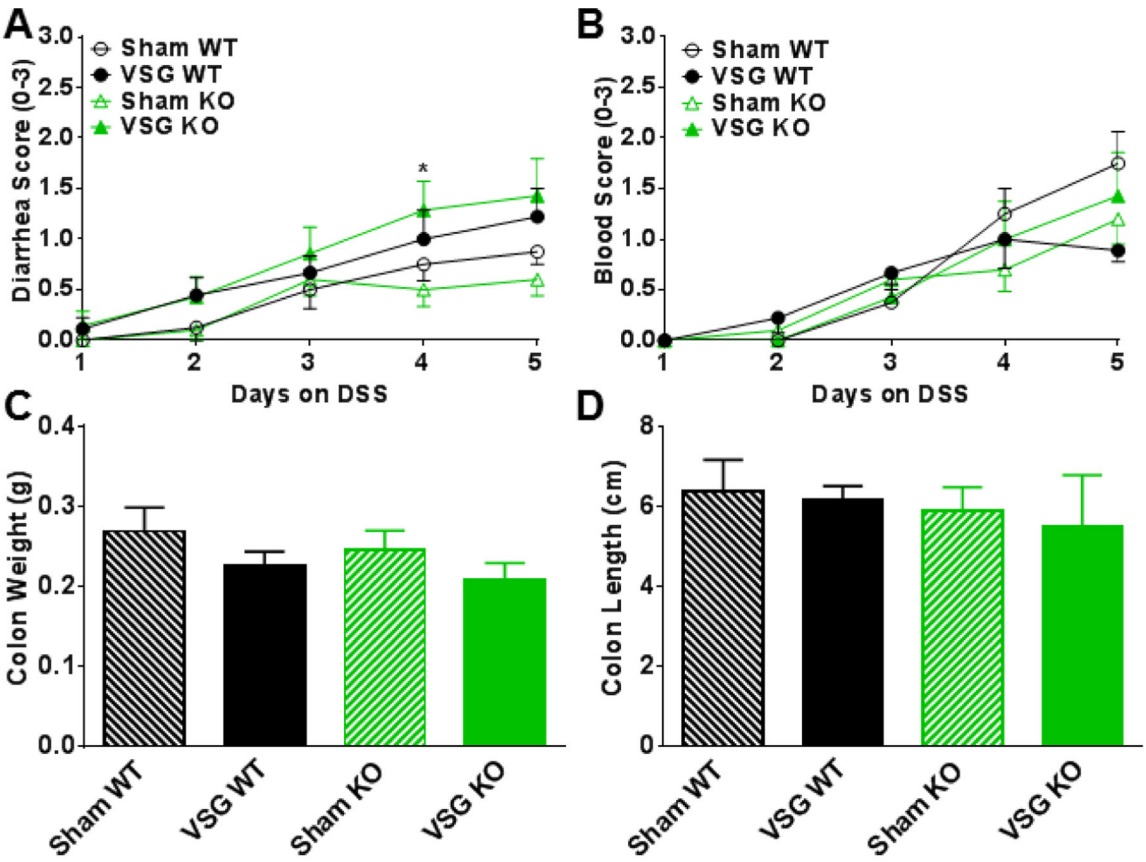

Figure 2. ***In vivo*** **assessment of DSS-induced disease severity.**

Diarrhea score (A) and fecal blood score (B). Colon weight (C) and colon length (D). Data are presented as mean ± SEM. n=7–10 per group. *P<0.05 for VSG KO compared with Sham KO by two-factor ANOVA.

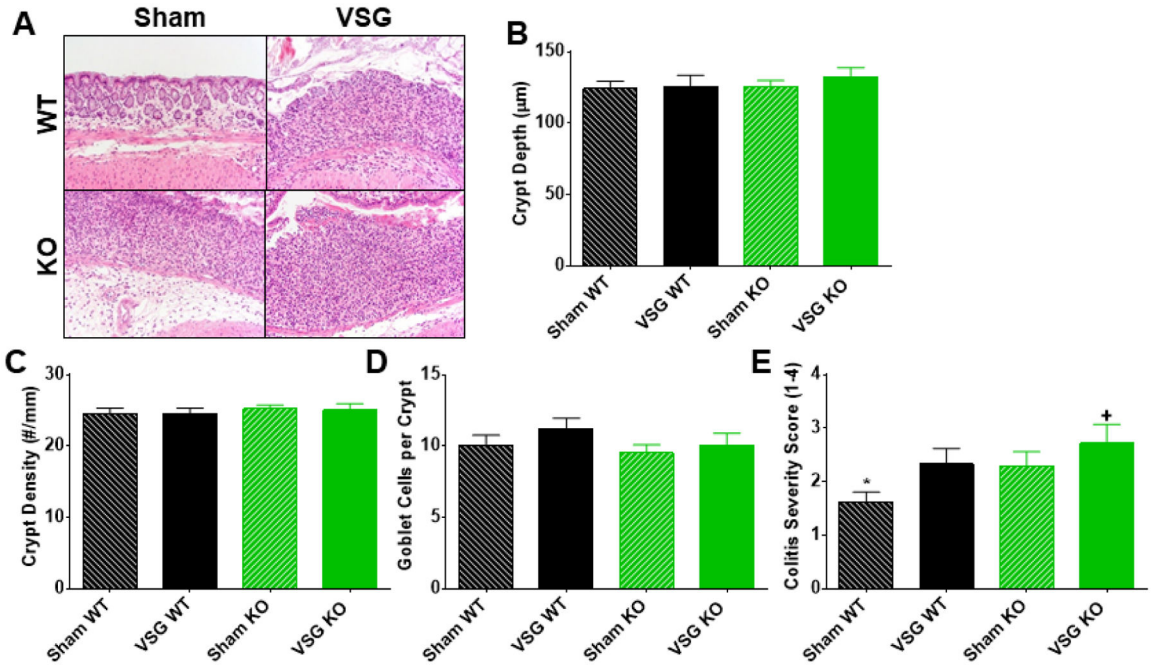

Figure 3. VSG and genetic ablation of TGR5 potentiate histomorphologic signs of DSS-induced colitis.

Representative H&E stained colon sections (A). Quantification of colonic crypt depth (B), crypt density (C), number of goblet cells per crypt (D) and colitis severity score (E). Data are presented as mean ± SEM. n=7–10 per group. *P<0.05 Sham WT compared with VSG WT and Sham KO by Student's t-test. ^{+}P<0.05 Sham WT compared with VSG KO by two-factor ANOVA.

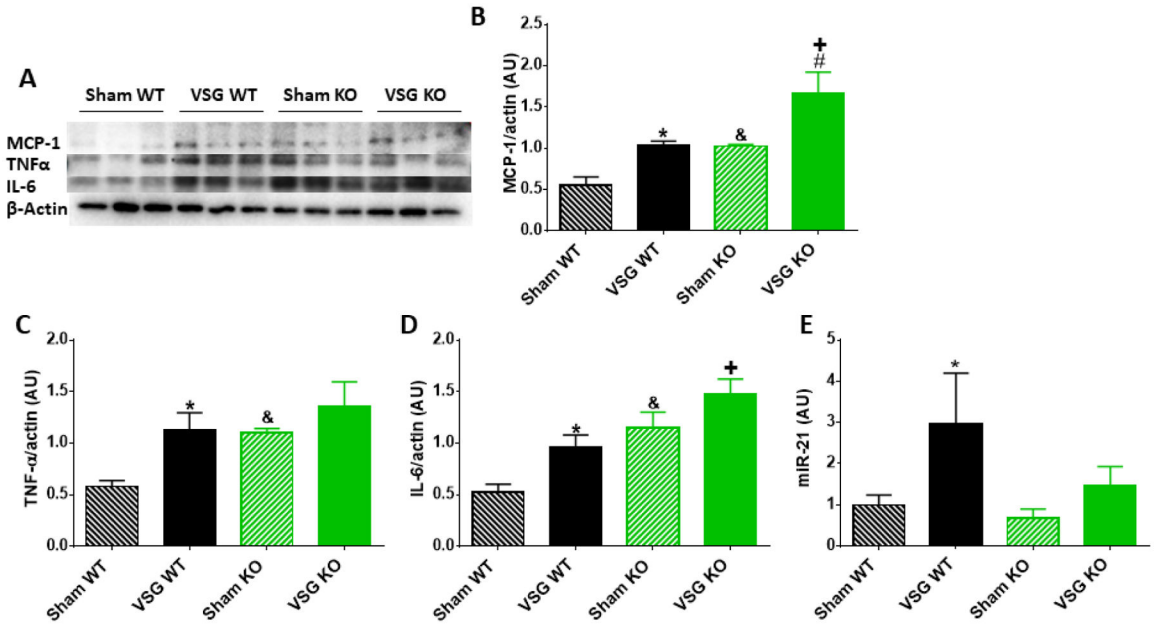

Figure 4. VSG and genetic ablation of TGR5 increase colonic inflammation.

Representative immunoblots for monocyte chemotactic protein 1 (MCP-1), tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and β-actin (A). MCP-1 relative to β-actin (B), TNF-α relative to β-actin (C) and IL-6 relative to β-actin (D). Results were quantified in densitromic units. n=6 per group. Colonic miR-21 expression (E). n=4–7 per group. Data are presented as mean ± SEM. $^{*}P \leq 0.05$ Sham WT compared with VSG WT, $^{\#}P<0.05$ Sham KO compared with VSG KO, $^{\&}P<0.05$ Sham KO compared with Sham WT, $^{+}P<0.05$ VSG KO compared with VSG WT by two-factor ANOVA.

Table 1.

Effect of VSG and genetic ablation of TGR5 on incidence of colitis, inflammatory infiltrates and hyperplasia.

% of mice with	Sham WT	VSGWT	Sham KO	VSGKO
Colitis	63%	89%	90%	86%
Erosion/Ulcer	25%	56%	50%	57%
Neutrophilic Infiltration	38%	44%	30%	86% *
Lymphocytic Infiltration	63%	89%	90%	86%
Hyperplasia	13%	44%	30%	57%

Percent of mice with colitis, erosion/ulcer, neutrophilic infiltration, lymphocytic infiltration, and hyperplasia. n=7–10 per group.

* $P<0.05$ VSG KO compared with Sham KO by Chi-square test.