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MyD88 mediates the protective effects of probiotics against the arteriolar thrombosis and leukocyte recruitment associated with experimental colitis

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Abstract

Several studies in IBD patients and in animal models of IBD have revealed a protective effect of probiotics in reducing clinical symptoms of disease and in blunting the gut inflammation that accompanies this condition. However, the mechanism underlying the therapeutic effect of probiotics is currently unknown. Furthermore, the ability of probiotics to influence the enhanced thrombus development that accompanies IBD has not been studied. This study addresses whether the enhanced extra-intestinal thrombosis (induced by light/dye injury) associated with experimental colitis is altered by oral treatment with the probiotic preparation VSL#3 or by the absence of microbiota. Colitis was induced by DSS 3% in Swiss Webster mice, germ free mice, C57BL/6 WT or *Myd88*^{-/-} mice. In some experiments, mice received VSL#3 for 8 days before and during DSS feeding. Swiss Webster mice were also subjected to a chronic model of DSS colitis and the effect of VSL#3 was evaluated. VSL#3 treatment significantly attenuated the accelerated thrombus formation observed in both acute and chronic models of colitis. VSL#3-treated mice also exhibited attenuated inflammatory response and injury in the colon. The protective effects of VSL#3 on colitis-associated thrombogenesis and inflammation were not evident in *MyD88*-deficient mice. Our results suggest that improved control of the enteric microflora in IBD may afford protection against the hypercoagulable, prothrombotic state that follows this condition.

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Authorship Contributions

DGS and DNG designed the experiments, DGS, ES and JR performed experiments, DGS, ES and DNG wrote the manuscript.

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Keywords

Thrombosis; Probiotics; Colitis; Microbiota; MyD88

Introduction

The inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are chronic disorders characterized by rectal bleeding, severe diarrhea, abdominal pain and weight loss^{1,2}. There is also a variety of extra-intestinal manifestations that contribute to the morbidity and mortality of IBD^{3,4}. These include pathological alterations in the mouth, eyes, skin, liver, joints, lungs and blood. A life-threatening hematological consequence of IBD is thrombosis. Patients with IBD exhibit a 3–6 fold higher risk for development of thromboembolism (TE) than the general population, with clinical studies suggesting an incidence of TE of approximately 6%, while autopsy studies predict an incidence as high as 40%.^{5,6} Hematologic studies of IBD patients suggest that abnormalities in coagulation, fibrinolysis, and platelet function are likely to contribute to the prothrombotic state that related to this disease⁷. These hematological changes as well as the enhanced thrombus formation reported in IBD patients have also been demonstrated in animal models of colitis^{8,9}.

While the mechanisms that underlie the initiation and perpetuation of the gut inflammation and extra-intestinal complications of IBD have not been fully elucidated, there is a large and growing body of clinical and experimental evidence that implicates genetic, immunological, and environmental factors in IBD pathogenesis^{1,2}. Indeed, it has been proposed that IBD may result from an aggressive immune response to gut microbiota in genetically susceptible individuals¹⁰. The critical role for gut bacteria in IBD is supported by clinical and experimental evidence showing that antibiotic treatment, to eliminate aggressive bacterial species, can reduce the symptoms of IBD^{11–13}. An alternative therapeutic strategy that is gaining widespread attention is the use of probiotics, which are orally taken living microorganisms that benefit the host by altering the microbial balance in the gut lumen in favor of protective bacterial species¹⁴. Several studies in IBD patients and in animal models of IBD have revealed a protective effect of probiotics in reducing clinical symptoms of disease and in blunting the gut inflammation that related to this condition^{15–16}. For example, commercially available probiotic preparations, such as VSL#3 (which includes different strains of *Bifidobacterium*, *Lactobacillus*, and *Streptococcus*) have been shown to reduce gut inflammation and disease activity in both mouse and rat models of colitis^{17,18}. VSL#3 treatment also appears to blunt neutrophil accumulation elicited by acute models of inflammation in the gut, such as ischemia-reperfusion¹⁹. It remains unclear whether protection against IBD afforded by probiotics or antibiotics that alter the gut microbiota extend beyond the gut. This appears likely in view of reports describing beneficial effects of oral probiotic treatment in clinical and animal studies of arthritis and cardiovascular diseases, including myocardial infarction and atherosclerosis^{20–22}. The overall objective of this study was to determine whether the enhanced extra-intestinal thrombus development associated to experimental colitis (induced by dextran sodium sulfate) is altered by oral treatment with the probiotic preparation. We also evaluated whether probiotic-induced

protection against thrombus development was dependent on Toll-like receptors (TLR) signaling by employing mice that were genetically deficient for MyD88, an adaptor molecule involved in TLR signaling. The findings of this study reveal significant protection against colitis-induced extra-intestinal thrombosis by VSL#3 and implicate MyD88 in this protective response.

Methods

Animals

Male Swiss Webster (SW)/Germ free (GF) mice (6–8 weeks old) were obtained from Taconic Farms (Germantown, NY). Experiments with gnotobiotic mice were carried out in sterile micro-isolators. Water and a commercial autoclavable diet were sterilized by steam and administered *ad libitum* to all the animals. To confirm sterility of germ free mice, fecal samples were cultured using a thioglycolate test every two days. Male C57BL/6J, and MyD88^{-/-} (6–8 weeks) were purchased from Jackson Laboratory (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions in standard cages and fed standard laboratory chow and water. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Louisiana State University Health Sciences Center-Shreveport. All animal experiments were performed according to the criteria outlined by the National Institutes of Health.

Induction of Colonic Inflammation

Acute colitis was induced as previously described^{8,23}. Briefly, mice were fed *ad libitum* with 3% (w/v) DSS (40,000 molecular weight; MP Biomedicals, Solon, OH) dissolved in filter-purified drinking water. The first day of DSS feeding is day 0. SW and GF mice were maintained on DSS feeding for 2, 4, 6 or 9 days. In a separate SW mice group (called recovery group), animals were fed 3% DSS for 9 days, followed by 5 days of water. C57BL6 and MyD88^{-/-} mice were fed with 3% DSS for 6 days.

Chronic colitis was induced by exposing SW mice to 2% DSS dissolved in filtered-purified drinking water for 1 week, followed by exposure to filtered-purified drinking water alone for 1 week. This cycle of DSS exposure was repeated for up to 4 times (8 weeks in total). Control SW, GF, C57BL6 or MyD88^{-/-} mice received filtered water *ad libitum*.

All mice were weighed every day for determining the body weight (BW) change. This was calculated as the difference between BW on the day of measurement and day 0. On the day of euthanasia, mice were first submitted to intravital microscopic analysis, then after euthanasia the colon was excised to measure: 1) colon length, 2) leukocyte recruitment (EPO/MPO activity), 3) and cytokine concentrations. A colon sample was also used for histologic analysis. A blood sample was obtained to determine platelet and leukocyte counts and for measurement of hematocrit.

Probiotic VSL#3 administration protocol

The probiotic preparation VSL#3 was obtained from Sigma-Tau Pharmaceuticals (Gaithersburg, MD). This preparation includes *Lactobacillus plantarum*, *Lactobacillus*

delbrueckii subsp. *bulgaricus*, *Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Streptococcus salivaris* subsp. *Thermophilus*. VSL#3 (1 billion CFU/mice diluted in PBS) was administered daily by gavage. VSL#3 treatment began 8 days prior to the initial induction of colitis and continued until the time of euthanasia. In some groups, VSL#3 treatment began on day 0 of the experiment (2 hours before DSS exposure) or 4 days after the beginning of DSS administration.

Antibiotic treatment

A combination of large spectrum antibiotics was used to sterilize the intestinal microbiota of conventional mice as described previously²⁴. The antibiotic mix includes: Metronidazole, Ciprofloxacin, Vancomycin, and Imipenem (Sigma Aldrich) (all at 50 mg/kg body weight/day). Animal bedding was changed daily and all experimental procedures were conducted under aseptic conditions to avoid reinfection. To assess whether the antibiotic treatment was adequate to deplete the intestinal microbiota, fecal samples were cultured using a thioglycolate test after 7, 14, and 21 days of antibiotic treatment²⁵. On day 21, animals were submitted to 3% DSS exposure.

Conventionalization of germ-free mice

Some germ-free mice were colonized with microbiota obtained from conventional mice as described previously²⁴. Briefly, fecal samples removed from the large intestine of conventional mice were homogenized in saline (10% w/v) and administered by oral gavage to germ-free mice. 21 days later, these animals were submitted to DSS exposure, as described above. To assess whether there was adequate conventionalization of germ-free mice, fecal samples were cultured using a thioglycolate test²⁵. Another group of germ-free mice was recolonized with microbiota from conventional mice previously treated with VSL#3 and submitted to same protocol described.

Assessment of colitis activity (clinical score) – Diseases Activity Index (DAI)

Mice were monitored clinically every day using a disease activity index, as described previously²⁶. Briefly, mice were left alone in a box for 10 minutes to determine the consistency of feces and to obtain samples for further evaluation. Fecal blood was tested using guaiac paper (ColoScreen®; Helena Laboratories). DAI scores ranged between 0 to 8, and included stool consistency, presence of fecal blood, macroscopic evaluation of rectal bleeding and signs of morbidity.

Intravital microscopy

The microvasculature was observed using an upright microscope (BX51WI; Olympus, Tokyo, Japan) with a 40× water immersion objective lens (LUMPlanFI/IR 40×/0.80 w, Japan). The light and fluorescent microscopic images were projected onto a monitor (TRINITRON PVM-2030; Sony, Tokyo, Japan) through a color video camera (Hitachi VK-C150; Hitachi, Tokyo, Japan) or a charge-coupled device video camera (Hamamatsu XC-77; Hamamatsu, Tokyo, Japan), respectively. The images were recorded by using a DVD recorder (JVC SR-MV50, NJ). A video timer (Panasonic Time-Date Generator WJ-810;

Panasonic, Tokyo, Japan) was connected to the monitor to record time and date. The diameters of the blood vessels were measured by video analysis software (ImageJ 1.37v; NIH, Public Domain software) on a personal computer (G4 Macintosh; Apple, Cupertino, CA). Red blood cell velocity (V_{RBC}) in the microvessels was measured by using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A&M University, College Station, TX). Blood flow was calculated from the product of mean red blood cell velocity ($V_{mean} = V_{RBC}/1.6$) and cross-sectional area, assuming cylindrical geometry. Wall shear rate was calculated based on the Newtonian definition: wall shear rate = $8 (V_{mean}/D_V)$.

Video analysis of leukocyte recruitment and microvascular thrombosis

Second- or third-order venules and arterioles (one to three per mouse, 35–50 μ m diameter and 100 μ m length, with wall shear rate 500 per second) were randomly selected in each cremaster muscle preparation to study thrombus formation or leukocyte recruitment (as described previously²⁷). Adherent leukocytes in venules were defined as cells remaining stationary for 30 seconds. The number of adherent leukocytes was expressed as number of cells per minute per millimeter of vessel area, calculated from diameter and length, assuming cylindrical vessel shape²⁷.

For induction of light/dye-induced thrombosis, 10 ml/kg of 5% FITC-dextran (150,000 MW; Sigma Chemicals) was injected intravenously and allowed to circulate for 10 minutes. Photoactivation of FITC-dextran (excitation: 495 nm; emission: 519 nm) within the microvessels was achieved by epi-illumination using a 175-W xenon lamp (Lambda LS, Sutter, CA) and a fluorescein filter cube (HQ-FITC, Chroma Technology Company, Rockingham, VT). The excitation power density was measured daily (ILT 1700 Radiometer, SED033 detector; International Light, Peabody, MA) and maintained within 1% of 0.74 W/cm², as previously described^{27,28}. Thrombus formation was quantified by determining (1) the time of onset of platelet deposition/aggregation within the microvessel (onset time), and (2) the time required for complete flow cessation for 60 seconds (cessation time). Epi-illumination was discontinued once blood flow ceased in the vessel under study.

Histological scoring

In each animal, three samples of the distal colon were evaluated histologically after staining with hematoxylin & eosin. Quantification of the histological changes was performed using a previously described scoring system²⁹.

Determination of the MPO and EPO activities

The extent of tissue eosinophil infiltration was assessed by measuring eosinophil peroxidase (EPO) activity, as described previously³⁰. Briefly, 100 mg of colon tissue were weighed and homogenized with 1.9 ml of PBS and centrifuged at $12,000 \times g$ for 10 minutes. The samples were then centrifuged, the supernatant was discarded, and the pellet was suspended in 1.9 ml of 0.5% hexadecyltrimethyl ammonium bromide in PBS, frozen three times in liquid nitrogen, and centrifuged at 4°C at $12,000 \times g$ for 10 minutes. The supernatant was used in the enzymatic assay by the addition of an equal amount of substrate (1.5 mmol/L *o*-phenylenediamine and 6.6 mmol/L H₂O₂ in 0.075 mmol/L Tris-HCl (pH 8)). The reaction was stopped with 50 μ l of 1 M H₂SO₄, and the absorbance was read at 492 nm.

The extent of neutrophil accumulation in colon tissue was determined by assaying tissue myeloperoxidase (MPO) activity, as described previously³¹. Briefly, a portion of the colon was removed and snapped frozen in liquid nitrogen. On thawing and processing, the tissue was assayed for MPO activity by measuring the change in optical density at 450 nm using tetramethylbenzidine.

Cytokine concentrations

TNF- α , IL-6 and IL-10 levels in colonic tissue were measured by cytometric bead array. Tissue samples were promptly mixed with PBS containing a protease inhibitor (Sigma Chemicals, St. Louis, MO) and thoroughly homogenized. The homogenate was centrifuged at 10,000 rpm $\times$ 5 minutes to separate the supernatant. Cytokine concentration in the supernatant was measured with the cytometric bead array as per the manufacturer's instruction (BD Biosciences, San Jose, CA). The detection limit of the cytometric bead array for mouse cytokines is 10 pg/ml.

Platelet count and hematocrit

Blood was collected in heparin-containing syringes at the indicated times under anesthesia. Briefly, 10 μ l of a solution containing 1% ammonium oxalate and blood (in a dilution of 1:100) was placed in a Neubauer chamber and platelets were visualized and counted under the microscope (Carton, Japan). Results are presented as number of platelets per μ l of blood. For determination of the hematocrit, a sample of blood was collected into heparinized capillary tubes (STATSPIN, Iris Sample Processing, Westwood, MA) and centrifuged for 10 min in a hematocrit centrifuge (Crit Spin, StatSpin Technologies).

Platelet life-span and reticulated platelet measurement

Platelet half-life ($t_{1/2}$) and number of reticulated platelets were determined as described elsewhere⁴¹. Mice were injected via the femoral vein with 1.2 mg of biotin (Sulfo-NHS-LC-biotin; ProteoChem, Denver, CO) dissolved in 300 μ l of saline. Every day thereafter (for 5 days) a fresh blood sample (5 μ l of tail blood) were incubated with 1 mg/mL thiazole orange (TO) dissolved in PBS (Sigma-Aldrich, St. Louis, MO), CD41-APC antibody (CD41-allophycocyanin (isotype control rat IgG1, k; eBioscience)), and streptavidin conjugated with phycoerythrin (eBioscience, San Diego, CA) for 15 minutes at room temperature, to detect immature (TO-positive) platelets and biotin-positive platelets. Formaldehyde (1%; Polyscience Inc., Warrington, PA) was used for cell fixation. Immediately after incubation samples were analyzed on an LSRII flow cytometer (BD Biosciences, San Jose, CA) with FACSDiva software version 6.1.3 (BD Biosciences). 10,000 to 20,000 events were collected.

Platelet aggregation

Platelet aggregation velocity to 0.63 u/ml (EC50) mouse α -thrombin was detected as previously described⁴¹. Briefly, arterial blood was collected from the carotid artery into a syringe loaded with anticoagulant citrate dextrose (ACD), 1 mM EDTA, and 0.2 u/ml apyrase (grade VII, Sigma-Aldrich) in 1/7 ratio. Platelet rich plasma (PRP) was obtained via centrifugation of blood samples at 300 g for 5 min. The PRP was centrifuged for additional

8 min at 80 g to separate PRP from red blood cell contamination. Platelet aggregation velocity was measured via light scattering analysis in 6 ml of modified HEPES buffer (140 mM NaCl, 10 mM HEPES, 10 mM NaHCO₃, 2 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5.5 mM D-glucose) via using a Laser Particle Analyzer (Lumex Ltd, St. Petersburg, Russia).

Statistical analysis

Results were analyzed using GraphPad Prism 4 (GraphPad Inc., San Diego, CA, USA). Statistical analyses between 2 experimental groups were performed by the Student *t* test. Statistical analyses between more than 2 experimental groups were performed using an analysis of variance (One Way ANOVA followed by Newman-Keuls multiple comparison test). P values <0.05 were considered significant. The results were expressed as mean ± standard error of the mean (SEM).

Results

Swiss Webster mice develop severe acute colonic inflammation with enhanced leukocyte recruitment and thrombus formation in the microvasculature of distant tissue

First, we evaluated the effect of 3% DSS exposure on parameters of body weight, disease activity index (DAI), colon length, leukocyte adhesion, and light/dye-induced thrombus formation in cremaster muscle microvessels. Figure 1 summarizes these results for 2, 4, 6, and 9 days of DSS 3%-induced colitis in Swiss Webster mice. On days 6 and 9, mice exhibited significant loss of body weight and increased disease activity (DAI) (Figure 1A-B). Hematocrit was reduced only on day 9 ($31.33 \pm 1.76\%$) of DSS feeding when compared to control mice ($42.33 \pm 0.5\%$). The length of the inflamed colon was reduced on days 4, 6, and 9 (Figure 1C). Significantly enhanced leukocyte adhesion in cremaster muscle venules was observed on day 9 (Figure 1D). DSS exposure induced a significant increase of neutrophil (MPO) and eosinophil (EPO) infiltration in the inflamed colon (Figure 1E) and in the lungs (data not shown). Significant histological changes, including inflammatory cell influx, hemorrhage and tissue erosion were noted in colon samples derived from colitic mice (data not shown).

DSS colitis was associated with enhanced thrombus formation in cremaster muscle arterioles following light/dye exposure (days 6 and 9), as evidenced by reduced times for thrombus onset and for flow cessation (Figure 1F). No differences in time of onset and time for complete blood flow cessation were detected in cremaster muscle venules of control and DSS fed SW mice (data not shown). Because peak inflammatory and thrombogenic responses were noted on the 9th day of DSS feeding in SW mice, we chose this time point for the subsequent experiments.

Pre-treatment with VSL#3 ameliorates clinical disease, inflammatory and thrombotic complication induced by DSS

Pre-treatment of DSS colitic mice (beginning 8-days prior to DSS feeding) with the probiotic preparation VSL#3 significantly attenuated the weight body loss (Figure 2A), disease activity (Figure 2B), colon shortening (Figure 2C) induced by DSS feeding. In addition, VSL#3 pre-treatment reduced adherence of leukocytes to cremaster muscle venules

elicited by DSS (Figure 2D). Similarly, the probiotic preparation reduced influx of neutrophils and eosinophils (Figure 2E), and blunted the histopathological changes (Figure 2F) in the colon following DSS feeding. Furthermore, VSL#3 treatment blunted the production/release of TNF- α and IL-6 induced by DSS in the colon (Figure 2G). VSL#3 also blunted the pro-thrombotic effects (reduced both the time of thrombus onset and time for blood flow cessation) of DSS treatment (Figure 2H). The enhanced thrombosis was not associated to change in total platelet count (Figure 3A). However, the DSS exposure in Swiss Webster mice was characterized for significant decreased of $\frac{1}{2}$ platelets lifespan (Figure 3B) it was associated to an increased release of newly produced platelets (Figure 3C). These changes were followed up by an increased (thrombin-induced) platelet aggregation velocity (Figure 3D). While VSL#3 treatment did not alter total platelet counts, it did reverse the $\frac{1}{2}$ lifespan (Figure 3B), the appearance of more immature platelets in blood (Figure 3C) and the faster platelet aggregation (Figure 3D). It is noteworthy that when VSL#3 therapy was initiated either at the onset of DSS treatment (beginning 2 hours before DSS feeding and maintained on DSS until the experiment) or 4 days after the initiation of DSS treatment, the probiotic preparation did not exert a beneficial effect on either pro-inflammatory or pro-thrombotic responses to DSS (data not shown).

We subsequently evaluated the effects of treatment with VSL#3 in the chronic model of DSS colitis. To this end, mice were subjected for 7 days of 2% DSS alternating with 7 days on normal water for a total of 4 cycles. The VSL#3 treated group received the probiotic preparation during the entire 4 cycles. This protocol yielded more significant changes in disease activity, colonic length, colon cytokines, as well as EPO and MPO activities (Figure 4A-E). Thrombus formation was also accelerated in this model (Figure 4F). VSL#3 therapy exerted a substantial protective effect in this model and was effective in blunting the loss of body weight (Figure 4A), DAI (Figure 4B), colonic shortening (Figure 4C) and recruitment of neutrophils and eosinophils into the colon (Figure 4D). VSL#3 treatment also prevented the increased production of TNF- α and IL-6 (Figure 4E) as well as the enhanced thrombus formation (Figure 3F). Platelet count was not altered in this model, with or without VSL#3 treatment.

Indigenous microbiota contribute to DSS induced inflammation and thrombosis

The role of indigenous bacteria in DSS-induced inflammation and thrombosis was assessed using germ-free (GF) mice placed on sterile drinking water (Figure 5). No differences were noted between GF and SW mice placed on normal drinking water. However, GF mice exhibit more dramatic changes in response to DSS treatment compared to conventionally raised (CNV) mice, as reflected by the more pronounced reduction in body weight (Figure 5A), increase in DAI (Figure 5B) and hemoconcentration (data not shown) on days 6 and 9 of DSS treatment. GF mice also exhibit larger changes in colon shortening (Figure 5C), tissue MPO (Figure 5D) and EPO (data not shown) activities, and tissue levels of IL-6 (Figure 5E) and TNF- α (data not shown). Similarly, GF mice show a more accelerated thrombosis response in cremaster arterioles than their conventionally raised counterparts (Figure 5F). Indeed, after 6 days on DSS, GF mice already exhibited a thrombotic response that is similar to that observed in SW mice fed DSS for 9 days. In contrast to SW mice, the DSS-accelerated thrombosis is followed up by thrombocytosis (increased platelet count) in

GF mice (Figure 5G). The more severe disease activity in GF mice were associated with increased mortality at > 7 days of DSS treatment. Consequently, subsequent experiments were carried out on day 6 of DSS treatment.

To confirm the participation of microbiota in the DSS model, we ablated the microbiota in conventional mice using a large spectrum antibiotic mix. The antibiotic mix resulted in no detectable bacteria on days 7, 14, and 21 after the start of treatment (data not shown). Interestingly, antibiotic administration did not alter indices of colonic inflammation (body weight loss, diseases activity or colon length) in DSS fed mice (Supplementary Figure 1A-C). On the other hand, antibiotic-treated DSS colitic mice exhibited a significantly accelerated thrombotic response in cremaster arterioles compared to DSS-treated conventional mice (Supplementary Figure 1 D), as reflected in the augmented onset of thrombus formation and more rapid cessation of blood flow. Collectively, these findings are consistent with the data from GF mice, which exhibit an exacerbation of DSS-induced inflammation and thrombosis.

Previous work has demonstrated that the normal composition of the gut microbiome is essential for physiological homeostasis and that its perturbation, or “dysbiosis”, is associated with inflammatory bowel disease. As GF mice develop more severe colitic disease, we hypothesized that reconstitution of the gut microbiota in GF mice would restore the potential of these mice to develop an inflammatory and thrombotic response to DSS treatment. To test this possibility, we used two groups of germ free mice, the first of which was re-colonized by gavaging with CNV feces (conventionalized group), while a second group was re-colonized with feces from CNV mice previously submitted to the probiotic treatment protocol (probiotic conventionalized group). Twenty-one days after re-colonization, both groups were treated with DSS. The results of these experiments revealed that recolonization of GF mice diminished disease severity (Figure 5B), weight loss (Figure 5A), colonic shortening (Figure 5C), MPO/EPO activities (Figure 5D) and cytokine production (Figure 5E). In addition, the conventionalized GF mice were significantly protected against thrombus development (Figure 5F) and thrombocytosis (Figure 5G). The mice that were conventionalized with feces from probiotic treated-conventional mice exhibited more protection against the deleterious responses to DSS treatment. The latter finding suggests that VSL#3 treatment yields a gut microbiota that, when transferred to GF mice, offers more protection against DSS induced inflammation and thrombosis.

Probiotic VSL#3 is not effective in colitic MyD88 deficient mice

Similar to SW mice, wild type C57BL/6 mice and MyD88 deficient mice develop severe disease after DSS exposure, which is characterized by body weight loss, colon shortening, increased MPO/EPO activities and enhanced cytokine production (Figure 6). As shown in Figure 6, DSS colitis in these mice was also associated with enhanced thrombus formation and thrombocytosis. While VSL#3 therapy largely prevented these DSS-induced responses in wild type C57BL/6 mice, the probiotic preparation failed to protect MyD88 deficient mice against the pro-inflammatory and pro-thrombotic effects of DSS treatment. These findings suggest that while MyD88 signaling does not contribute to the gut responses to DSS, it is critical for the protective effects of VSL#3 treatment.

Discussion

IBD patients are generally treated with anti-inflammatory, immunosuppressive, and/or biological agents (e.g., anti-TNF- α) and possibly surgery. While these strategies can provide relief of symptoms, they do not afford a cure for the periodic and life-long illness that characterizes IBD. An alternative therapeutic approach that has gained much attention in recent years is manipulation of the gut microbiota through the use of oral probiotics^{15,16}. There is growing evidence that oral probiotic therapy can confer protection against human and experimental IBD. VSL#3, a mixture of eight strains of lactic acid-producing bacteria, has been shown to reduce inflammation and maintain remission of ulcerative colitis in human subjects³². VSL#3 has also been shown to ameliorate colitis in the *IL-10*^{-/-} murine model³³ and in TNBS-induced chronic colitis in rats¹⁷. Despite the benefits offered by oral probiotic therapy, relatively little is known about the mechanisms that underlie the positive effects that are derived from this treatment. In this study, we provide evidence in support of the ability of probiotic therapy to attenuate the colonic inflammation and injury response to DSS exposure in both C57BL/6 and Swiss Webster mice. Furthermore, our analysis includes the novel observation that VSL#3-treated mice exhibit a significantly blunted thrombosis response in extra-intestinal tissue during DSS-induced acute and chronic colitis.

Our results indicate that the protective effect of VSL#3 treatment is associated with inhibition of critical components of the inflammatory response, including cytokine production and inflammatory cell recruitment. In humans, the accumulation of neutrophils and eosinophils has been described in rectal biopsies of ulcerative colitis patients³⁴. Murine colitis, including that induced by DSS, is also characterized by significant neutrophil and eosinophil infiltration in the inflamed colon. The importance of this infiltration is evidenced in animals that are deficient in neutrophils and eosinophils, which exhibit attenuated tissue pathology in response to DSS administration^{14,35}. Hence, inhibition of neutrophil and eosinophil influx in the colon may represent an important mechanism that underlies the beneficial effects of probiotic therapy in IBD. How the probiotic preparation exerts this effect on leukocyte recruitment remains unclear. However, it is noteworthy that we observed an attenuation of leukocyte-endothelial cell adhesion in the microcirculation of a tissue (cremaster) distant from the intestine in DSS treated mice receiving VSL#3. This may indicate that the probiotic diminishes cell-cell adhesion by blunting adhesion molecule expression and/or inhibiting chemokine production. Findings outlined in previous reports by others indicate that *Lactobacillus plantarum*³⁶ and *Lactobacillus casei*³⁷, both of which are found in VSL#3, inhibit the expression of ICAM-1 and MAdCAM-1 in a mouse model of experimental colitis. Chemokines (e.g., CXCL-1, CCL-3 and CCL-11) that mediate leukocyte recruitment are produced at an accelerated rate in experimental colitis¹⁴, and mice deficient in these chemokines or their receptors are protected against DSS-induced inflammation injury and tissue injury^{14, 35, 38}. Similarly, cytokines such as TNF- α and IL-6 have also been implicated in human³⁹ and experimental^{40–42} IBD, and interventions that blunt the accumulation of these cytokines offer protection against colitis. Since the protective effect of probiotics has been linked to chemokines and cytokines⁴³, then an attenuated production of these mediators could also explain how VSL#3 acts to reduce the recruitment of neutrophils and eosinophils in DSS colitis.

IBD is associated with an increased risk of extra-intestinal thrombosis. Animal studies have revealed contributions of tissue factor, activated protein C and thrombin in the colitis-induced enhancement of microvascular thrombosis⁸. Other studies have implicated the pro-inflammatory cytokines TNF- α , IL-1 β , and IL-6 as mediators of the distant organ thrombogenic response to DSS colitis^{27,41,42}. The enhanced thrombosis elicited by DSS in C57Bl/6 mice, like human IBD⁴⁴, is associated with an increased platelet count (thrombocytosis). While Swiss Webster exhibit an enhanced extra-intestinal thrombosis response similar to C57Bl/6 mice, the former do not develop thrombocytosis in response to DSS treatment. However, the number of immature platelets in blood is increased in Swiss Webster mice after DSS treatment, suggesting increased production or release from megakaryocytes. Since immature platelets are more reactive than mature platelets to agonist stimulation⁴⁵, this could contribute to the enhanced extra-intestinal thrombosis observed in Swiss Webster mice exposed to DSS.

The findings of the present study reveal the ability of VSL#3 in attenuating the thrombogenic response to DSS colitis. This protection by VSL#3 is reflected in a more prolonged time of thrombus onset (a measure of the initiation phase of thrombosis) and in the time to ceasing blood flow (a measurement of the propagation and stabilization phases of thrombosis). VSL#3 therapy was also associated with an attenuated release of immature platelets into blood during DSS feeding, which may explain (at least in part) the anti-thrombogenic effect of the probiotic preparation. While in vitro studies of human platelets indicate that the probiotic strains *Lactobacillus rhamnosus* and *Bifidobacterium lactis* do not affect homotypic platelet aggregation⁴⁶, *in vivo* studies using the DSS colitis model have shown that *Lactobacillus reuteri* reduces platelet-leukocyte and platelet-endothelial cell interactions in inflamed colonic venules⁴⁷. Hence, platelets may represent an important cellular target for the beneficial effects of probiotic therapy on thrombus development during colonic inflammation.

Commensal bacteria and vertebrate immune systems form a symbiotic relationship and have co-evolved such that proper immune development and function relies on colonization of the gastrointestinal tract by commensal bacteria. Our study confirms previous report⁴⁸ that describe GF mice developing more severe colitis than conventionalized mice and that bacterial re-colonization of colitic GF mice reverse disease severity. Furthermore, we report here the novel observation that DSS-colitis enhanced extra-intestinal thrombosis is more intense in GF mice. The deleterious effects of removing enteric bacteria on colitis-enhanced thrombogenesis is supported by our finding that treatment of conventional mice with a mixture of wide spectrum antibiotics also elicits a more severe thrombotic (and inflammatory) response. Collectively, these data indicate that the exacerbated thrombotic and inflammatory responses in germ free mice is related to lack of bacterial colonization of the gut and does not reflect a more primitive immune system in GF mice.

Previous studies have led to the proposal that the indigenous microbiota shapes the host's ability to respond properly to several inflammatory insults, ultimately leading to changes in the way the host perceives and reacts to environmental stimuli, leading to improved resilience of the host to environmental pressures. Maslowski et al⁴⁸ have ascribed the severe colitic disease observed in GF mice to an inability to produce short-chain fatty acids, which

are produced by fermentation of dietary fiber by intestinal microbiota and have the potential for clinical benefit in the treatment of colitis. It has also been suggested that global changes in microbiota composition (dysbiosis) are associated with inflammatory bowel diseases⁴⁹. These changes include reduced bacterial diversity, decreased levels of resident Firmicutes and/or Bacteroidetes, an overgrowth of Proteobacteria, as well as abnormal adherence of bacteria to the gut mucosa^{50, 51}. To avoid the microbiota disturbance in the present study, we re-colonized GF mice with feces derived from conventional mice that previously were treated with VSL#3. We hypothesized that VSL#3 alters the gut microbiota in a manner that is selective for specific strains of microorganisms that are important in maintaining a dynamic balance between intestinal homeostasis, the systemic immune system and hemostasis in healthy mice. Our hypothesis is supported by previous work demonstrating that VSL#3 alters the composition of the intestinal microbiota and these changes are correlated with VSL#3-induced disease protection in the TNBS model of colitis¹⁷. In our model, the hypothesis is supported by the ineffectiveness of VSL#3 in protecting mice when the probiotic preparation is not administered 8 days before DSS exposure, i.e., to exert protection VSL#3 requires enough time to appropriately alter the resident microbial population. Indeed, GF mice re-colonized with “adequate” microbiota developed less severe clinical, inflammatory and thrombotic alterations in response to DSS treatment.

In our experimental model, MyD88 signaling does not contribute to the clinical, inflammatory or thrombotic responses elicited by DSS treatment. The MyD88 adaptor molecule has been previously implicated in a different model (1.2% DSS) of murine colitis, wherein the MyD88 pathway appears to have a protective role against the development of colitis⁵². It is possible the higher dose (3 %) of DSS used in our study produced a response that is independent of MyD88 signaling. However, we did observe that MyD88 signaling is critical for the beneficial effects of the probiotic preparation, i.e., VSL#3 failed to reverse the effects of DSS in MyD88 deficient mice. Previous work has demonstrated that the probiotic *Bifidobacterium breve* suppresses intestinal inflammation via induction of IL-10-producing Tr1 cells that is dependent on MyD88 signaling⁵³. Collectively, our findings in MyD88 deficient mice suggest that the protective effects of VSL#3 on colitis associated thrombogenesis and inflammation are MyD88-dependent.

In conclusion, the results of this study reveal an important influence of the microbiota on the local inflammatory response and the enhanced extra-intestinal thrombosis that results from acute or chronic DSS exposure. Our findings raise the possibility that a disturbance in the microbiota exacerbates the gut inflammation and promotes the hypercoagulable, prothrombotic state that accompanies IBD. Furthermore, this study suggests that oral probiotics may be a relevant therapeutic approach for the prevention of thromboembolic events in patients with IBD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. DSS induces colitis in Swiss Webster mice

Swiss Webster mice were maintained on DSS for 2, 4, 6 or 9 days. All mice were weighed every day for determination of body weight (BW) change and body weight was expressed as the difference between BW on the day of the experiment and day 0 (A). Mice were monitored clinically every day using a disease activity index, DAI scores ranged between 0 to 8, and included stool consistency, fecal blood, macroscopic evaluation of rectal bleeding and signs of morbidity (B). 2, 4, 6 or 9 days of exposure of DSS mice were submitted to euthanasia and had the colon excised to measure of colon length (C). Adherent leukocytes in venules were defined as cells remaining stationary for 30 seconds. The number of adherent leukocytes was expressed as number of cells per minute per millimeter of vessel area, calculated from diameter and length, assuming cylindrical vessel shape (D). To assess the presence of leukocytes on colitis colon, the assays of EPO/MPO activity were performed, as indirect measure of neutrophil and eosinophil, respectively (E). Thrombus formation was

quantified by determining the time of onset of platelet deposition/aggregation within the microvessel (onset time), and the time required for complete flow cessation for 60 seconds (cessation time) (F). Results are the mean $\pm$ SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group.

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Figure 2. Pre-treatment with VSL#3 improves clinical disease, inflammatory and thrombotic complications induced by the acute DSS colitis model

VSL#3 treatment induced attenuation of body weight loss (A) and DAI (B) on the days 7, 8 and 9 after DSS exposure compared to vehicle treated-mice. On day 9, VSL#3 treated-mice presented less intestinal shortening (C) induced by DSS. VSL#3 was associated to decrease of adherent cells in cremaster muscle (D). In colonic colon tissue, VSL#3 treatment resulted in a reduction of neutrophil and eosinophil influx (E), less mucosal damage and ulceration, these alterations resulted in decrease of histological score (F) when compared to vehicle group mice. Control mice did not present any histopathological alteration and its score is zero. VSL#3 induced decrease in TNF- α and IL-6 production (G). Time to onset thrombus formation and time to cessation of flow were increase in VSL#3-treated mice (H). Results are the mean $\pm$ SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group and # $p < 0.05$; when compared to vehicle treated mice.

Figure 3. VSL#3 treatment was associated to decrease of new platelets releasing and decrease of platelets aggregation velocity

DSS feeding, in the presence or absence of VSL#3, did not induce increase of platelets counts in SW mice (A). In DSS treated SW mice platelets exhibit shorter life span (B), increase of immature platelet numbers (C). VSL#3 treatment reversed both decrease of life span and immature platelet count. VSL#3 decrease high aggregation velocity to thrombin of platelet induced by DSS exposure (D). Results are the mean ± SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group and # $p < 0.05$; when compared to vehicle treated mice

Figure 4. Pre-treatment with VSL#3 improves clinical disease, inflammatory and thrombotic complications induced by the chronic DSS colitis model

VSL#3 treatment induced attenuation of body weight loss (A) and DAI (B) after DSS chronic exposure (A) compared to vehicle treated-mice. After 4 cycles of DSS/Water (1 week each one 4 times) exposure, VSL#3 treated-mice presented less intestinal shortening (C) reduction of neutrophil and eosinophil influx (D) and decrease in TNF- α and IL-6 production (E) when compared to vehicle group mice. Time to onset thrombus formation and time to cessation of flow were increased in VSL#3-treated mice (F). Results are the mean $\pm$ SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group and [#] $p < 0.05$; when compared to vehicle treated mice.

Figure 5. Germ free mice exhibit more severe disease after DSS feeding

When compared to conventionally raised (CNV) mice, Germ free mice on days 6 and 9 of DSS treatment presented more pronounced reduction in body weight (A), increase in DAI (B), larger changes in colon shortening (C), increase of tissue MPO (D) and levels of IL-6 (E), decrease of time to onset thrombus formation and time to cessation of flow (F) and increase of platelets count (G). Reposition of normal microbiota was associated to partial improving of all parameters, while microbiota from VSL#3-treated mice protects germ free mice of disease induced by DSS. Black bars represent germ free mice that did not receive DSS feeding. The dotted line represents Swiss Webster mice exposure to DSS for 9 days. Results are the mean $\pm$ SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group and # $p < 0.05$; when compared to vehicle treated mice.

Figure 6. The protective effect of VSL#3 in colitis model is dependent on MyD88 signaling
 Wild type C57BL/6 mice and MyD88 deficient mice develop severe disease after DSS exposure. VSL#3 treatment in C57/BL6 mice, but not in Myd88 mice, induced attenuation of body weight loss (A) and DAI (B), decrease colon shortening (C), MPO activity (D) and IL-6 production (E). Furthermore, VSL#3 increased in C57/BL6 mice time to onset thrombus formation and time to cessation of flow (F) and decrease of platelets count (G). In Myd88^{-/-} mice, VSL#3 did not alter thrombus formation time or systemic platelets number. Results are the mean $\pm$ SEM of 5–7 animals in each group. * $p < 0.05$ compared to the control group and # $p < 0.05$; when compared to vehicle treated mice. IBD-14-0794.R1