NAD^+ modulation of intestinal macrophages renders anti-inflammatory functionality and ameliorates gut inflammation

Young-In Kim[1], Inseok Ko[2], Eun-Je Yi[1], Jusik Kim[2], Yong Rae Hong[2], Wheeseong Lee[2], and Sun-Young Chang[1]

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Abstract

Background and Purpose: Macrophages not only can maintain gut immune homeostasis by driving clearance of infection, but also can prevent chronic inflammation and induce tissue repair. Macrophages are a heterogenous group of cells whose characteristics are determined by tissue microenvironment and metabolism. Since macrophages play an important role in inflammatory disorders such as inflammatory bowel disease (IBD), they can be a potential therapeutic target. Experimental Approach: Here we show an IBD therapeutic candidate LMT503, a substrate which modulates NADH quinone oxidoreductase 1, which enhances NAD^+ and induce anti-inflammatory macrophage polarization. To determine the anti-inflammatory effect of LMT503, a dextran sulfate sodium (DSS)-induced colitis mouse model was used in this study. Key Results: Treatment of bone marrow derived macrophages (BMDMs) with LMT503 increased IL-10 and Arg1 levels but decreased levels of TNF-α, iNOS, and IL-6. LMT503 also increased levels of SIRT1, SIRT3, and SIRT6, suggesting that macrophages were driven to an anti-inflammatory character. In a murine DSS-induced colitis model, oral treatment with LMT503 ameliorated colonic inflammation and decreased infiltrating monocytes and neutrophils. Although NAD^+ enhancement did not alter $CX_3CR1^{int}CD206^-$ or $CX_3CR1^{hi}CD206^+$ colon macrophage population, it decreased levels of TNF-α and iNOS and increased IL-10 level, with colonic macrophages showing an anti-inflammatory character shift. Depletion of CX_3CR1 expressing gut resident macrophages abrogated the immune regulatory effect of LMT503 in the colon. Conclusion and Implications: These data suggest that LMT503 is a therapeutic candidate that can target macrophages to drive polarization with an immunosuppressive character and ameliorate IBD.

NAD^+ modulation of intestinal macrophages renders anti-inflammatory functionality and ameliorates gut inflammation

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Abstract

Background and Purpose:

Macrophages not only can maintain gut immune homeostasis by driving clearance of infection, but also can prevent chronic inflammation and induce tissue repair. Macrophages are a heterogenous group of cells whose characteristics are determined by tissue microenvironment and metabolism. Since macrophages play an important role in inflammatory disorders such as inflammatory bowel disease (IBD), they can be a potential therapeutic target.

Experimental Approach:

Here we show an IBD therapeutic candidate LMT503, a substrate which modulates NADH quinone oxidoreductase 1, which enhances NAD^+ and induce anti-inflammatory macrophage polarization. To determine the anti-inflammatory effect of LMT503, a dextran sulfate sodium (DSS)-induced colitis mouse model was used in this study.

Key Results:

Treatment of bone marrow derived macrophages (BMDMs) with LMT503 increased IL-10 and Arg1 levels but decreased levels of TNF-α, iNOS, and IL-6. LMT503 also increased levels of SIRT1, SIRT3, and SIRT6, suggesting that macrophages were driven to an anti-inflammatory character. In a murine DSS-induced colitis model, oral treatment with LMT503 ameliorated colonic inflammation and decreased infiltrating monocytes and neutrophils. Although NAD^+ enhancement did not alter $CX_3CR1^{int}CD206^-$ or $CX_3CR1^{hi}CD206^+$ colon macrophage population, it decreased levels of TNF-α and iNOS and increased IL-10 level, with colonic macrophages showing an anti-inflammatory character shift. Depletion of CX_3CR1 expressing gut resident macrophages abrogated the immune regulatory effect of LMT503 in the colon.

Conclusion and Implications:

These data suggest that LMT503 is a therapeutic candidate that can target macrophages to drive polarization with an immunosuppressive character and ameliorate IBD.

Keywords: Inflammatory bowel disease, NAD^+modulation, Gut macrophage, Macrophage polarization, Colon

Abbreviations:

Arg1: arginase 1

BMDM: bone marrow derived macrophage

DSS: dextran sulfate sodium

IBD: Inflammatory bowel disease

IL: interleukin

iNOS: inducible nitric oxide synthase

MLN: mesenteric lymph node

NQO1: NADH quinone oxidoreductase 1

PBMC: peripheral blood mononuclear cell

PP: Peyer's Patches

SIRT: Sirtuin; silent information regulator

TNF-α: tumor necrosis factor α

What is already known

Distinct metabolic pathways determine macrophage subtype and function.

What does this study add

Enhancement of NAD^+ in gut macrophages shifted to an anti-inflammatory character and ameliorated inflammation in a murine colitis model.

What is the clinical significance

Modulation of NAD^+ in macrophages may be a promising therapeutic target to treat inflammatory bowel disease.

Introduction

The intestine not only serves as a barrier against external harmful agents and pathogen infection, but also interacts with commensal microbiome and food antigens. Therefore, the intestinal immune system reacts to both inner and external environments to maintain homeostasis. When this balance is disrupted, chronic inflammatory disorders can occur in the intestine, leading to inflammatory bowel disease (IBD) (Maloy & Powrie, 2011). There are two main forms of IBD: Crohn's disease, which includes inflammatory disorder throughout the gastrointestinal tract, and ulcerative colitis, which is restricted in the colon (Kaser, Zeissig, & Blumberg, 2010). Causes of IBD is complicated. It involves multiple factors such as genetic factors, failure of the host immune system, and disruption of gut microbiota (Hill & Artis, 2010; Podolsky, 1991).

Macrophages are innate immune cells well distributed in almost all tissues and are highly heterogenous to achieve niche-specific functions. Gut macrophages not only can maintain immune homeostasis by driving clearance of infection, but also can control oral tolerance and tissue repair (Mazzini, Massimiliano, Penna, & Rescigno, 2014; Murray & Wynn, 2011). Gut macrophages initiate inflammation in response to stimuli such as pathogen or cell damage. After an inflammatory response takes place, complete resolution must take place to maintain homeostasis. Gut macrophages are key players initiating inflammation to stimuli such as infection, but also in resolution of inflammation afterwards. Dysfunction of macrophages may result in failure of resolution, leading to chronic inflammation (Na, Stakenborg, Seok, & Matteoli, 2019). Alternatively activated macrophages can produce anti-inflammatory cytokines IL-10 and growth factors such as TGF-β, prostaglandin E_2 (PGE_2), bone morphogenetic protein 2 (BMP2), and WNT ligands (Lin et al., 2010; Wynn & Vannella, 2016). IL-10 production is known to promote immunosuppressive $CD4^+$ regulatory T cells (Treg).

Gut macrophages contain self-maintaining resident macrophages derived from embryonic birth and bone marrow derived macrophages matured from circulating monocytes (Viola & Boeckxstaens, 2021). Freshly supplied bone marrow derived $Ly6C^{hi}$ monocytes undergo differentiation into CX_3CR1^+macrophages in the lamina propria (Bain et al., 2014). They rapidly upregulate major histocompatibility complex type II (MHCII) and downregulate Ly6C. During colitis, CX_3CR1^{int} expressing monocytes are recruited to the gut. They show a pro-inflammatory phenotype. Mature CX_3CR1^{hi} macrophages are rather anti-inflammatory. They can maintain homeostasis (Diehl et al., 2013; Geissmann, Jung, & Littman, 2003; Viola & Boeckxstaens, 2021).

IBD patients show altered differentiation of macrophages compared to healthy individuals. Since functional macrophage defect can lead to chronic inflammatory disorders, macrophages can be considered a potential target for IBD treatment. Previous studies have shown that macrophages have distinct phenotypes through metabolic regulation. Inflammatory macrophages show enhanced glycolysis while anti-inflammatory macrophages show enhanced oxidative phosphorylation (Russell, Huang, & VanderVen, 2019; Wculek et

al., 2023). Distinct pathways of arginine metabolism determine macrophage characters. Inflammatory macrophages express nitric oxide synthase which leads to production of nitric oxide and citrulline, while anti-inflammatory macrophages express arginase which leads to production of urea and ornithine (Rath, Muller, Kropf, Closs, & Munder, 2014). Also, inflammatory macrophages can be induced by accumulation of citrate or succinate due to impairment of the TCA cycle (Harber et al., 2020; Palmieri et al., 2020). Thus, utilizing metabolic modulation to induce macrophage polarization can be a therapeutic approach to treat IBD.

In this study, we introduce LMT503, an IBD therapeutic candidate, which act as a substrate for NADH quinone oxidoreductase 1 (NQO1), a multi-functional protein that can increase cellular NAD^+, drive oxidation-reduction reaction, and stabilize proteins such as p53 (Nebert, Roe, Vandale, Bingham, & Oakley, 2002; Ross & Siegel, 2021). Increased NAD^+ by NQO1 can activate sirtuins and drive metabolic modulation of macrophages to have an anti-inflammatory character (S. Y. Park et al., 2017). The objective of this study was to determine whether LMT503 could induce polarization of macrophages to an immune-suppressive type to alleviate colitis in a DSS-induced murine model.

Methods

Cell culture and stimulation

To prepare bone marrow derived macrophages (BMDMs), marrow was flushed out from femurs and tibiae of hind legs using complete RPMI medium (Invitrogen, Carlsbad, CA, USA). After red blood cells were lysed, cells were seeded onto cell culture plates in complete RPMI medium with 20 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA) and cultured for 6 days. To prepare monocytes from human peripheral blood mononuclear cells, whole blood was layered on top of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged at 400 x g for 30 mins at room temperature. The interface containing mononuclear cells was collected. Monocytes were then selected using a MagniSort Human Monocyte Enrichment Kit (Invitrogen, Carlsbad, CA, USA). Cells were stimulated with 200 ng/ml LPS for 4 hours and then treated with 10 or 100 μM LMT503 for 18 hours at 37°C. For IL-1β, BMDMs were stimulated with 200 ng/ml LPS for 3 hours followed by 5 mM ATP (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour, and then treated with 10 or 100 μM LMT503 for 18 hours at 37°C. For monocytes derived from human peripheral blood mononuclear cells, fresh blood was collected from healthy adult volunteers from Ajou University Hospital. This study was approved by the Human Research Protection Center of Ajou University Hospital (IRB #2022-0534-001).

NQO1 activity assay

BMDMs were cultured with 200 ng/ml LPS and 10 or 100 μM LMT503 for 18 hours at 37°C. Cells were washed with ice cold PBS. Cell pellet was then used for analysis with a NQO1 Activity Assay Kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Animals

Dextran sulfate sodium (DSS)-induced colitis experiment was approved by the Institutional Animal Care and Use Committee of Ajou University (IACUC #2023-0040). Animals were kept in the Laboratory Animal Research Center of Ajou University Medical Center under specific pathogen-free conditions. They received sterilized food and water *ad libitum* . Six-week-old wild-type (WT) C57BL/6 female mice were purchased from Orient Bio Inc. (Sungnam, Korea). CX_3CR1-DTR mice (Jackson Laboratories) were kindly provided by D.R. Littman (New York University Medical Center) (Diehl et al., 2013).

Colitis

Seven-week-old C57BL/6 female mice with initial body weight of 19 ± 1 g were used in this study. To prepare DSS-induced colitis mouse model, 2% DSS (molecular weight 36,000–50,000 Da; MP Biomedicals LLC, Solon, OH, USA) dissolved in autoclaved drinking water was provided to mice*ad libitum* for 5 days. Mice were then provided with normal drinking water until the end of the experiment. Each dose of LMT503 (Korean patent 10-2022-0125359, Lmito Therapeutics, Sungnam, Republic of Korea) or tofacitinib (Selleck

Chemicals, Houston, TX) in 0.5% methyl cellulose/0.2% Tween80 (Sigma-Aldrich, St. Louis, MO, USA) was used to treat mice by oral gavage once every day starting from 5 days after DSS treatment. During the whole period, body weight and disease activity index (body weight loss, rectal bleeding, and diarrhea) were monitored daily and scored as described previously (Kim et al., 2020). At day 10, colon lengths were measured, and colon histology studies were proceeded. To determined bacterial translocation, the spleen and mesenteric lymph node (MLN) isolated from mice at day 10 were homogenized and serially diluted. Serial dilutions were plated onto LB agar plates and incubated at 37°C overnight. CFU/g was then determined. For mice CX_3CR1^+ macrophage depletion, CX_3CR1-DTR mice received *i.p.* injection of 200 ng diphtheria toxin at day 1, day 4, and day 7 of DSS treatment.

Salmonella Typhimurium Challenge

Mice were challenged orally with 10^6 CFU of wild-type invasive *Salmonella* Typhimurium strain UK-1 at day 13 of DSS treatment. At 3 days after UK-1 challenge, mice were sacrificed and their spleens, Peyer's Patches and mesenteric lymph nodes were isolated to determine *Salmonella* Typhimurium strain UK-1 CFU. Tissue was homogenized and serially diluted. Serial dilutions were plated onto XLD agar plates with a selective growth medium for Salmonella (BD Biosciences, Franklin Lakes, NJ, USA). Red colonies with a black center were determined as *Salmonella* Typhimurium strain UK-1.

Gene expression

BMDM, THP-1 cells, or human monocytes were cultured with 200 ng/ml LPS for 4 hours and then treated with 10 or 100 μM LMT503 for 18 hours at 37°C. To investigate gene expression in colon macrophages, mononuclear cells were isolated from whole colon tissue as described previously (Kim et al., 2018). Macrophages were further purified with MagniSort Mouse F4/80 Positive Selection Kit (Invitrogen, Carlsbad, CA, USA). Whole RNAs were extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), followed by cDNA synthesis using a SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Gene expression was measured by StepOnePlus RT-PCR (Applied Biosystems, Waltham, MA, UNA). Primers used for PCR are listed in Supplementary Table 1. Each sample was run in triplicate and three independent experiments were performed for each sample.

Histology

Colon tissues fixed in neutral buffered formalin were trimmed, processed, and embedded in paraffin. Then 5-μm thick sliced tissues of the paraffin block were stained by hematoxylin and eosin (H&E). H&E stained colonic tissue sections were then scored according to the following semi-quantitative measurement system for three parameters: 1) severity of inflammation (0 = rare inflammatory cells in the lamina propria; 1 = increased numbers of granulocytes in the lamina propria; 2 = confluence of inflammatory cells extending into the submucosa; and 3 = transmural extension of the inflammatory infiltrate); 2) crypt damage (0 = intact crypts; 1 = loss of the basal one third; 2 = loss of the basal two thirds; 3 = entire crypt loss; 4 = change of epithelial surface with erosion; and 5 = confluent erosion); and 3) ulceration (0 = absence of ulcer; 1 = 1 or 2 foci of ulcerations; 2 = 3 or 4 foci of ulcerations; and 3 = confluent or extensive ulceration). Values from these three parameters were added to give a maximal histological score of 11.

Cytokine analysis

Whole colon tissues were homogenized using a tissue homogenizer (Minilys personal homogenizer, Bertin). Homogenates were analyzed for pro- and anti-inflammatory cytokines. TNF-α, IFN-γ, IL-6, MCP-1, and IL-10 were analyzed by flow cytometry using a BD Cytometric Beads Assay Mouse inflammation kit (BD Biosciences, Franklin Lakes, NJ, USA). IL-1β and IL-22 levels were detected using a mouse IL-1β ELISA Kit (Invitrogen, Carlsbad, CA, USA) and a mouse IL-22 ELISA Kit (Invitrogen, Carlsbad, CA, USA), respectively, following the manufacturer's instructions.

Statistics

All statistical analyses were performed using Graphpad Prism version 9 software (GraphPad Software, LLC,

San Diego, CA, USA). An unpaired two-tailed Student's t-test was used to compare differences between two groups when data had a Gaussian distribution with similar variances. To compare multiple groups, one-way analysis of variance (ANOVA) was performed followed by Tukey's post hoc test. Values of $p< 0.05$ were considered significant.

Results

NAD^+ enhancer modulates macrophage phenotype into an anti-inflammatory character

LMT503, a novel NAD^+ enhancer, was discovered by Lmito Therapeutics in an attempt to find IBD therapeutic candidates via modulation of macrophages (Figure 1a). NAD^+ synthesis in macrophages has been reported to contribute to immune suppressive functions by increasing oxidative phosphorylation (Minhas et al., 2019). LMT503 works as an efficient substrate for NQO1 which can increase cellular NAD^+/NADH ratio in BMDMs (Figure 1b). To investigate the effect of LMT503 on inflammatory macrophages, BMDMs were stimulated with LPS and then treated with LMT503. LMT503 treatment decreased levels of inflammatory signature molecules TNF-α and iNOS (Figure 1c) but increased anti-inflammatory signature molecules Arg1 and IL-10 (Figure 1d) in BMDMs. Sirtuins, a class III histone deacetylases dependent on NAD^+, can inhibit NLRP3-inflammatory pathways of macrophages and favor anti-inflammatory polarization of macrophages (Pan, Dong, Huang, & Fang, 2022; S. Y. Park et al., 2017). LMT503 treated BMDMs showed increased expression of Sirtuins 1, 3, and 6 (Figure 4e). Upregulated expression of GATA3 is known to induce anti-inflammatory polarization of macrophages (Faas et al., 2021). Treatment with LMT503 increased the expression of GATA3 in BMDMs (Figure 1f). However, pro-inflammatory cytokine IL-1β level was decreased after LMT503 treatment (Figure 1g). Human monocytic THP-1 cells and human monocytes also showed increased levels of anti-inflammatory markers Arg1 and IL-10 (Figure 1h,i). These data suggest that LMT503 treatment could induce shift of pro-inflammatory macrophages to an anti-inflammatory character.

NAD^+ enhancer ameliorates DSS-induced colitis in mice

To investigate the anti-inflammatory effect of LMT503 on IBD *in vivo* , mouse DSS-induced colitis model was used in this study. Mice received DSS for 5 days followed by oral treatment with LMT503 or tofacitinib from day 5 when DSS was replaced by normal drinking water. LMT503 treated mice showed alleviated body weight loss, which was comparable to an IBD therapeutic JAK-inhibitor tofacitinib (Figure 2a) (Sandborn et al., 2014). LMT503 treated mice also showed reduced disease activity index (Figure 2b), recovered colon length (Figure 2c), and bacterial translocation in mesenteric lymph nodes compared to mice in the vehicle control group (Figure 2d). H&E staining of colon tissues showed reduced inflammation in LMT503 treated groups (Figure 2e). Pro-inflammatory cytokines TNF, MCP-1, and IL-1β were also significantly reduced in LMT503 treated groups (Figure 2f).

To further evaluate inflammation in colon tissues, innate immune cell infiltration was analyzed. Percentage and absolute numbers of $CD11b^+Ly6G^+$ neutrophils and $CD11b^+Ly6C^+$ monocytes were decreased significantly in LMT503 treated groups (Figure 3a-d). However, macrophage and dendritic cell percentage did not differ between LMT503 treated groups and vehicle control groups (Figures 3e,f and Figure S1a,b).

NAD^+ enhancer modulates mouse colon macrophage shift to an anti-inflammatory character

During colonic inflammation, neutrophils and monocytes can be infiltrated into the colon, although they exist at a low level in steady state. These inflammatory immune cells can be summoned by tissue resident immune competent cells such as macrophages. We further analyzed colonic macrophages in mouse DSS-induced colitis model. Treatment with LMT503 did not alter the percentage or the number of pro-inflammatory $CX_3CR1^{int}CD206^{low}$ or anti-inflammatory $CX_3CR1^{hi}CD206^{hi}$populations (Figure 4a-c). However, TNF-α and iNOS levels were decreased while IL-10 levels were increased following LMT503 treatment in colon and small intestinal macrophages (Figures 4d,e and Figure S1d). Levels of chemokines such as MCP-1/CCL2, KC/CXCL1, and MIP2/CXCL2 were decreased after LMT503 treatment in colon macrophages (Figure 4f), suggesting that colon CX_3CR1^+macrophages contributed to reduced infiltration of neutrophils and monocytes to the intestine (Figure 3a-d). These data suggest that colon CX_3CR1^+ macrophage population

was not altered after LMT503 treatment, although their character shifted to an anti-inflammatory character to ameliorate DSS-induced colitis.

LMT503 treatment altered macrophages to an anti-inflammatory character to alleviate DSS-induced colitis. The anti-inflammatory environment might arouse susceptibility to infection. To investigate this issue, mice treated with LMT503 were challenged with *Salmonella*Typhimurium strain UK-1 at day 13 at the recovery phase of DSS-induced colitis. LMT503 treated group did not show significant body weight loss compared to the vehicle control group (Figure 5a). *Salmonella*Typhimurium trafficking to the MLN and spleen did not differ between vehicle control and LMT503 treated groups (Figure 5b). At the recovery phase of colitis, host defense including production of pro-inflammatory cytokines TNF, IL-6, and MCP-1 levels in the colon tissue did not differ significantly after LMT503 treatment (Figure 5c). These data suggest that LMT503 treatment did not increase vulnerability to bacterial infections.

Colonic CX_3CR1^+ macrophages play a crucial role in the anti-inflammatory effect of LMT503 in DSS-induced colitis

Although the ratio and absolute numbers of colon macrophage populations were not altered, LMT503 treatment resulted in polarization from inflammatory to an anti-inflammatory character. It is known that colon resident macrophages show high expression of CX_3CR1. During colitis, $Ly6C^+$ monocyte infiltration to the lamina propria can differentiate into CX_3CR1^{int} macrophages which are pro-inflammatory. However, afterwards, further maturation into CX_3CR1^{hi} regulatory macrophages drives resolution of inflammation (Bain et al., 2013). These gut CX_3CR1^{hi} macrophages can clear out apoptotic neutrophils and support tissue healing (Arandjelovic & Ravichandran, 2015; Castoldi et al., 2023). Thus, CX_3CR1^+ macrophages are crucial in maintaining gut homeostasis. They play a key role in IBD. To verify that macrophage character shift by LMT503 treatment contributed to DSS-induced colitis alleviation, we used a CX_3CR1^+ macrophage depletion system. For depletion of CX_3CR1^+ cells, CX_3CR1-DTR mice were *i.p.* injected with diphtheria toxin during DSS-induced colitis. Under DSS treatment, mice deficient of CX_3CR1^+ macrophages showed body weight loss and high score of disease activity index, similar to those of WT mice (Figure 6a,b). However, absence of CX_3CR1^+ macrophages abrogated alleviation of colitis including less body weight loss, low score of disease activity index, and recovered colon length after LMT503 treatment. At day 10, group comparison of body weight changes, disease activity index, and colon length suggested that the effect of LMT503 treatment could be abolished in absence of CX_3CR1^+ macrophages (Figure 6c-e). After CX_3CR1^+ macrophage depletion, LMT503 could not control pro-inflammatory cytokines IL-6 (Figure 6f). LMT503 treatment significantly increased IL-10 and IL-22 production in inflamed colon which could be diminished after depletion of CX_3CR1^+ macrophages (Figure 6g). These data suggest that LMT503 can exert its effect through targeted modulation of CX_3CR1^+ macrophages to alleviate DSS-induced colitis.

Discussion

Intestinal macrophages are known as "gatekeepers" for their ability to clear foreign pathogens by expressing pathogen-associated molecular patterns (PAMPs) and recognize tissue damage by damage-associated molecular patterns (DAMPs). They also show tolerance to food or commensal microbes. Macrophages are a heterogeneous population of innate immune cells that play diverse functions in immune response and tissue homeostasis. Although boundaries of the heterogeneous macrophage population are ambiguous, they can be functionally divided into pro-inflammatory versus anti-inflammatory macrophages. Macrophage polarization depends on their microenvironment. Disruption of the composition or function of macrophages can contribute to various gastro-intestinal diseases such as IBD. IBD patients show an altered population of macrophages where inflammatory macrophages are dominant. These inflammatory macrophages can accumulate in the intestine during IBD, which can release TNF-α, IL-1β, and IL-6. To overcome IBD, biologics such as anti-TNF therapy is commonly used since TNF-α is a key pathological cytokine in IBD. Anti-IL-12/23, anti-integrin, 5-aminosalicylic acids, and immunomodulators can also be used for IBD therapy. Despite recent advances in IBD therapy, limitations still exist since complete remission is difficult. Thus, the goal for overcoming IBD is shifting to targeting mucosal healing.

Since macrophages are deeply involved in regulating inflammation and mucosal healing, targeting these macrophages can be a good therapeutic strategy against IBD. Many previous studies targeting macrophages to induce an anti-inflammatory shift have shown the potential of an effective IBD therapy. Activation of SIRT1 can promote anti-inflammatory genes Mrc1 and IL-10 while decreasing pro-inflammatory genes iNOS, CCL2, IL-12 p35, and IL-12 p40. Anti-inflammatory macrophage polarization by the SIRT1/adenosine monophosphate-activated protein kinase pathway can reduce inflammatory responses in a rheumatoid arthritis mouse model (S. Park et al., 2020; Zhou, Zhang, & Ding, 2022). TLR2/1 agonist PAM3CSK4 can induce monocytes to differentiate into anti-inflammatory macrophages and reduce DSS-induced colitis in a mouse model (Horuluoglu, Kayraklioglu, Tross, & Klinman, 2020).

Metabolic pathways in macrophages are deeply involved in the fate of macrophage polarization. Therefore, reports targeting macrophage polarization to an anti-inflammatory character by metabolic modulation is arising as a promising strategy for IBD therapy. Pro-inflammatory macrophages depend on glycolysis, while anti-inflammatory macrophages are activated by mitochondrial oxidative phosphorylation (Liu et al., 2021). Different paths of arginine catabolism contribute to macrophage polarization. Generation of NO by iNOS favors inflammatory macrophage polarization, while generation of urea and orthinine by Arg1 favors anti-inflammatory macrophage polarization. Increased consumption of L-arginine by iNOS and decreased Arg1 have been shown in ulcerative colitis. Thus, dietary supplementation of L-arginine can be used as an IBD therapy (Andrade et al., 2018; Bourgonje et al., 2020).

LMT503 is an NAD^+ enhancer by working as an efficient substrate for NQO1. NQO1 has multiple roles in defending against cellular stress. NQO1 can detoxify quinones by reducing them into hydroquinones. At the same time, oxidation of NAD(P)H occurs, which increases NAD^+. Previous studies have shown that inflammation can be controlled by triggering macrophage shift by increasing NAD^+ level. NAD^+synthesis via the kynurenine pathway can shift aged inflammatory macrophages to an anti-inflammatory state with increased phagocytic activity (Minhas et al., 2019). Increasing NAD^+ by administration of NAD^+ precursor β-nicotinamide mononucleotide (β-NMN) can shift macrophages to an anti-inflammatory type during sepsis (Cros et al., 2022). Sirtuins are a family of class III histone deacetylases known to be dependent on NAD^+(Anderson, Madsen, Olsen, & Hirschey, 2017). They consist of seven members, SIRT1-7. Sirtuins can modulate inflammation via nuclear factor-kappa B (NF-ϰB) or activator protein 1 (AP-1). SIRTs except SIRT5 are known to inhibit NF-ϰB activation by deacetylation of p65 subunit. SIRT1 cam inhibit IL-1β secretion by down-regulating NLRP3 inflammasome (S. Park et al., 2020). SIRT1, SIRT3, and SIRT6 can decrease AP-1 transcriptional activity which controls pro-inflammatory cytokine production (Palomer et al., 2020; Zhang et al., 2010). Treatment of BMDMs with LMT503 increased SIRT1, 3, and 6.

In summary, this study found that NAD^+ enhancement alleviated intestinal inflammation by reinforcing immune regulatory character of tissue resident CX_3CR1^+macrophages. NAD^+ enhancement was achieved by LMT503, an efficient substrate for NQO1. Metabolic reprograming in macrophages treated with LMT503 down-regulated inflammatory markers TNF-α and iNOS but up-regulated anti-inflammatory IL-10 and Arg1 during colitis. NAD^+ enhancement of colonic CX_3CR1^+ macrophages was crucial for ameliorating DSS-induced colitis in mice since LMT503 treatment did not show any effect when CX_3CR1^+macrophages were depleted. Our study suggests that LMT503 is a potential IBD therapy candidate that can drive anti-inflammatory macrophage polarization via immuno-metabolic modulation.

Author Contributions

Young-In Kim, Yong Rae Hong, Wheeseong Lee, and Sun-Young Chang conceived and designed experiments. Young-In Kim, Inseok Ko, Eun-Je Yi, and Jusik Kim performed experiments and analysis. Inseok Ko and Wheeseong Lee provided critical materials. Young-In Kim and Sun-Young Chang wrote the manuscript and provided creative input.

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Conflict of Interest

The authors declare no conflicts of interest.

Figure Legends

Figure 1. NAD^+ enhancement shifts macrophage polarization into an anti-inflammatory character.

(a) LMT503 structure. (b-g) BMDM originated from wild-type B6 mice were treated with 200 ng/ml LPS for 4 h followed by treatment with 10 μM or 100 μM LMT503 for 18 h. (b) NQO1 activity measured by OD change per min. Gene expression of (c) inflammatory markers, (d) anti-inflammatory markers, (e) Sirtuins 1, 3, 6, and (f) GATA3 in BMDMs. (g) IL-1β expression in culture supernatant of BMDMs stimulated with 200 ng/ml LPS 3 h followed by 5 mM ATP 1h, afterwards 10 μM or 100 μM LMT503 for 18 h. (h) Human monocyte THP-1 cells or (i) human peripheral blood derived monocytes were treated with 200 ng/ml LPS 4 h followed by treatment with 10 μM LMT530 for 18 h. ns; not significant, *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, one-way ANOVA with multiple comparison.

Figure 2. NAD^+ enhancement ameliorates inflammation in a murine DSS-induced colitis model.

C57BL/6 female mice were given 2% DSS in drinking water for 5 days. Mice were then provided with normal drinking water afterwards (n = 9/group, n = 5 for healthy control). After DSS treatment, 50 mg/kg LMT503 or 30 mg/kg tofacitinib was then orally given to mice every day starting from Day 5.

(a) Body weight, (b) disease activity index (***, $p < 0.001$ one-way ANOVA with multiple comparison vs. vehicle control group), (c) colon length, and (d) bacterial translocation (CFU/g) in the MLN.

(e) H&E staining of mouse colon and histological score.

(f) Cytokine levels (TNF, MCP-1, and IL-1β) of colon homogenates. *p<0.05, **p<0.01, ***p<0.001 one-way ANOVA with multiple comparison.

Figure 3. NAD^+ enhancement reduces infiltration of innate inflammatory cells in colon of DSS-induced colitis mouse.

Percentage and absolute number of (a, b) $Ly6G^+CD11b^+$ neutrophils, (c, d) $Ly6C^+CD11b^+$ monocytes, and (e, f) $F4/80^+CD11b^+$ macrophages from colon lamina propria were analyzed (n = 5/group). ns, not significant; *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$ one-way ANOVA with multiple comparison.

Figure 4. Metabolic reprograming of colon macrophages shift to an anti-inflammatory character.

C57BL/6 female mice were given 2% DSS in drinking water for 5 days. Mice were then provided with normal drinking water (n = 6/group). Mice were given 50 mg/kg LMT503 orally everyday starting from Day 5. At day 10, macrophages isolated from colon lamina propria were analyzed for sub-population and gene expression.

(a) Colon macrophage population. CD206 and CX_3CR1 were analyzed among $CD11b^+$ gated cells.

(b) CX_3CR1^{hi} cell population and cell number among $CD11b^+$ gated cells.

(c) CX_3CR1^{int} cell population and cell number among $CD11b^+$ gated cells.

(d) Inflammatory cytokines, (e) anti-inflammatory cytokines, and (f) chemokine expression in colon macrophages. ns, not significant, *$p < 0.05$, **$p < 0.01$, ***$p< 0.001$ one-way ANOVA with multiple comparison.

Figure 5. Treatment with LMT503 does not increase vulnerability to*Salmonella* Typhimurium infection. At day 13 of DSS-induced colitis, mice (n = 6/group) were infected orally with 10^6 CFU of *Salmonella*

Typhimurium (UK-1). Bacteria count (CFU) and cytokine levels in colon tissues were analyzed at 3 days after infection.

(a) Body weight, (b) UK-1 (CFU/g) in the spleen, Peyer's Patches, and mesenteric lymph nodes. (c) Cytokine levels in colon homogenates.

Figure 6. CX_3CR1^+ macrophages are critical for immune regulation via metabolic reprograming.

For macrophage depletion, CX_3CR1-DTR mice were*i.p.* injected with 200 ng diphtheria toxin at Days 1, 4, and 7. At day 10, colon length and cytokines were analyzed (n = 6/group).

(a) Body weight, (b) disease activity index (*** $p < 0.001$, two-way ANOVA with multiple comparison), (c) body weight at day 10, (d) disease activity index at day 10, (e) colon length, and (f, g) cytokine gene expressions of colon homogenates. ns, not significant; *p <0.05, **p <0.01, ***p <0.001 one-way ANOVA with multiple comparison.

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Figure 1

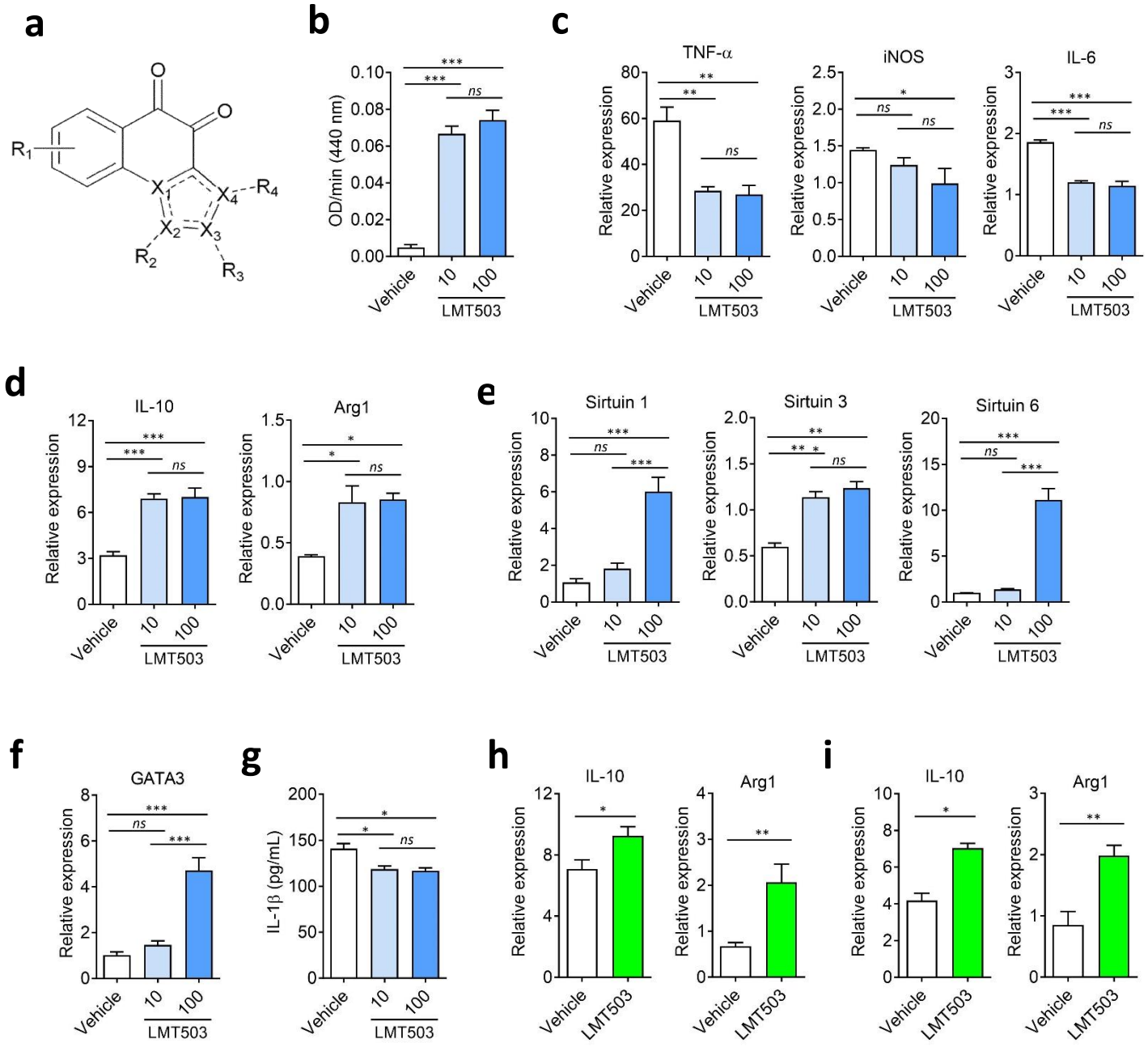

a
b
c
d
e
f
g
h
i
R1
R2
R3
R4
X1
X2
X3
X4
O
O
OD/min (440 nm)
TNF-α
iNOS
IL-6
IL-10
Arg1
Sirtuin 1
Sirtuin 3
Sirtuin 6
GATA3
IL-1β (pg/mL)
Relative expression
Vehicle
10
100
LMT503
ns

Figure 2

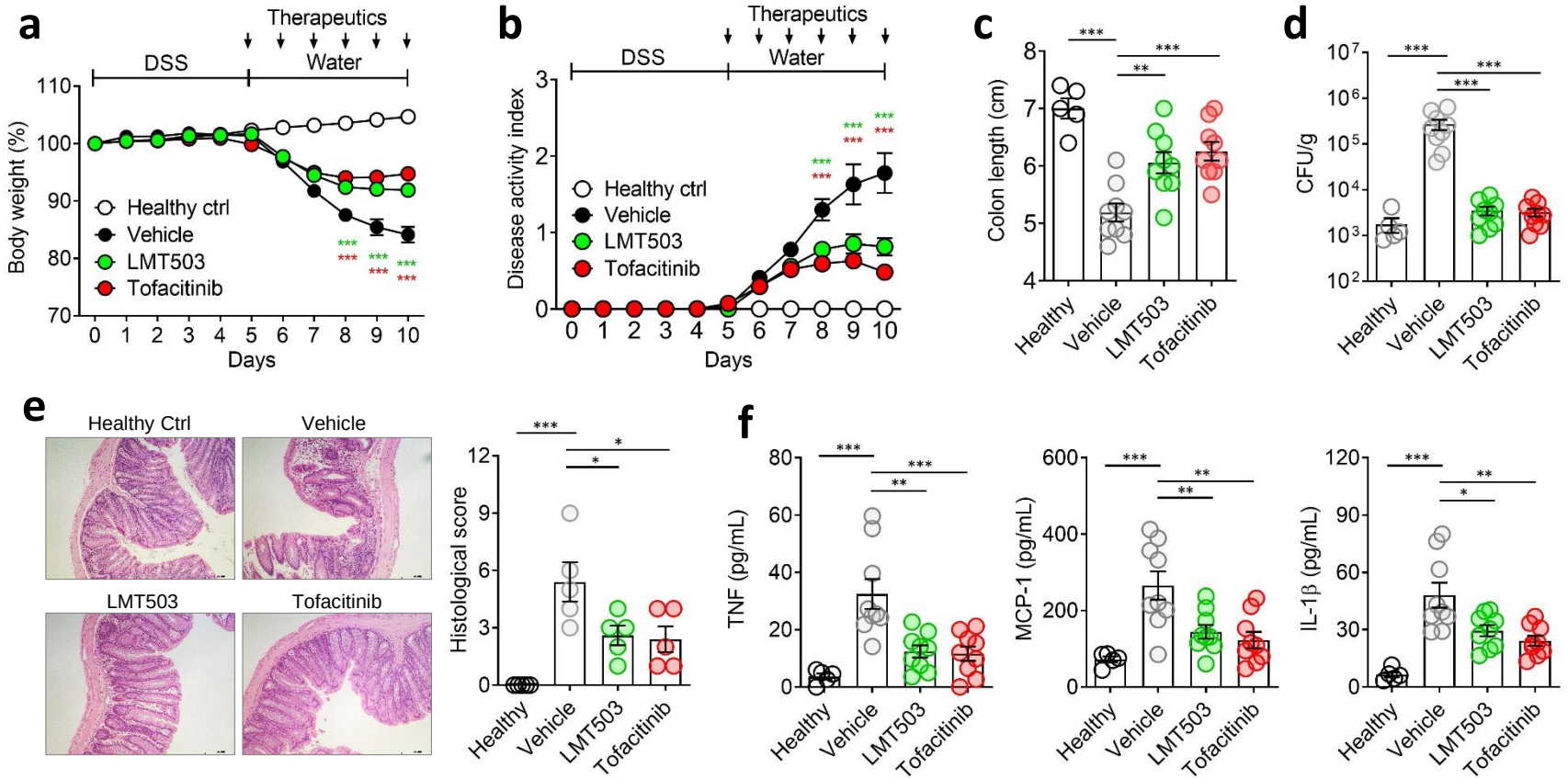

a
Therapeutics
DSS
Water
Body weight (%)
Healthy ctrl
Vehicle
LMT503
Tofacitinib
Days
b
Therapeutics
DSS
Water
Disease activity index
Healthy ctrl
Vehicle
LMT503
Tofacitinib
Days
c
Colon length (cm)
Healthy
Vehicle
LMT503
Tofacitinib
d
CFU/g
Healthy
Vehicle
LMT503
Tofacitinib
e
Healthy Ctrl
Vehicle
LMT503
Tofacitinib
Histological score
Healthy
Vehicle
LMT503
Tofacitinib
f
TNF (pg/mL)
MCP-1 (pg/mL)
IL-1β (pg/mL)
Healthy
Vehicle
LMT503
Tofacitinib

Figure 3

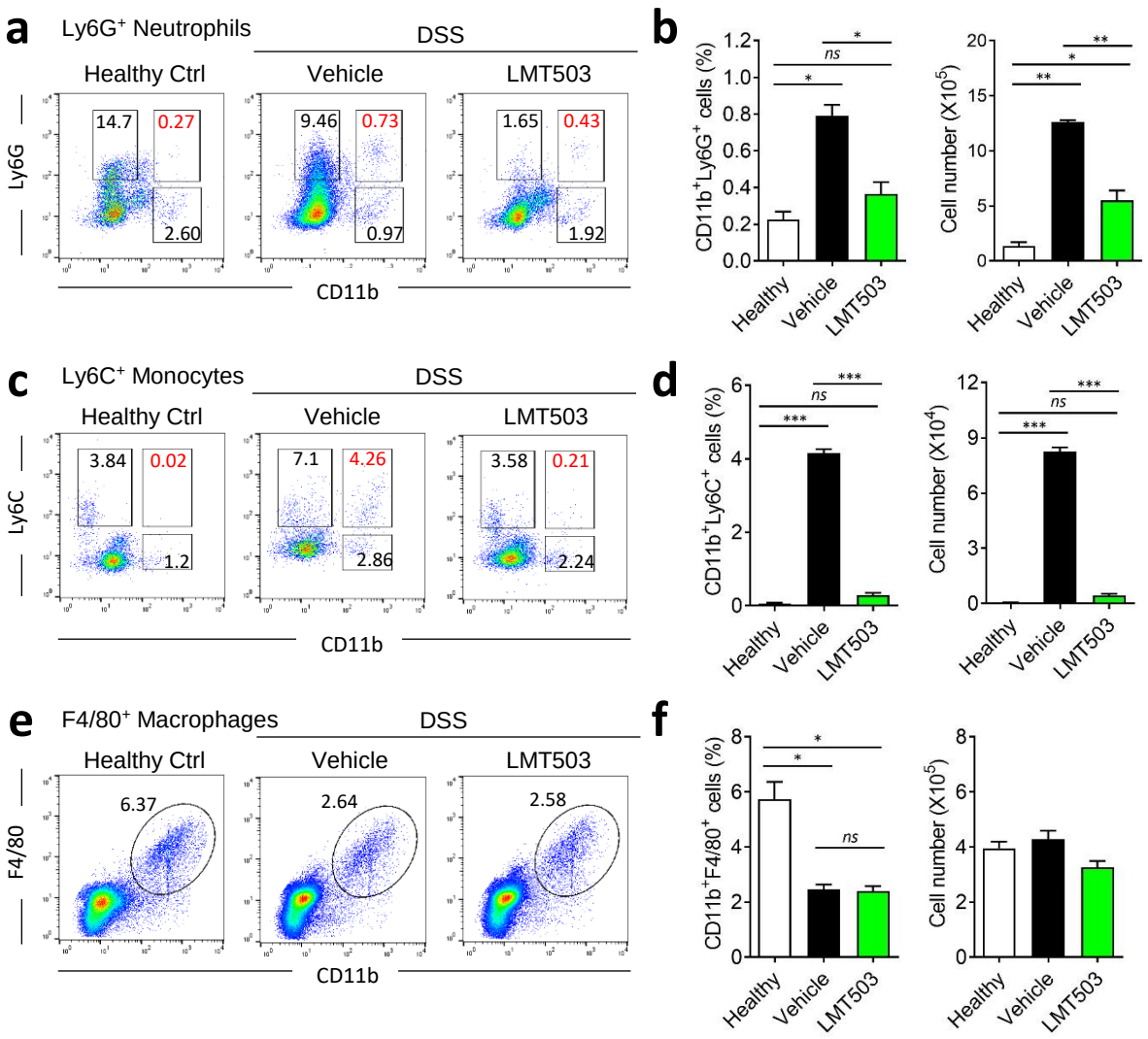

a
Ly6G+ Neutrophils
DSS
Healthy Ctrl
Vehicle
LMT503
Ly6G
CD11b
14.7
0.27
2.60
9.46
0.73
0.97
1.65
0.43
1.92
b
CD11b+Ly6G+ cells (%)
Cell number (X10^5)
Healthy
Vehicle
LMT503
ns
c
Ly6C+ Monocytes
Ly6C
3.84
0.02
1.2
7.1
4.26
2.86
3.58
0.21
2.24
d
CD11b+Ly6C+ cells (%)
Cell number (X10^4)
e
F4/80+ Macrophages
F4/80
6.37
2.64
2.58
f
CD11b+F4/80+ cells (%)

Figure 4

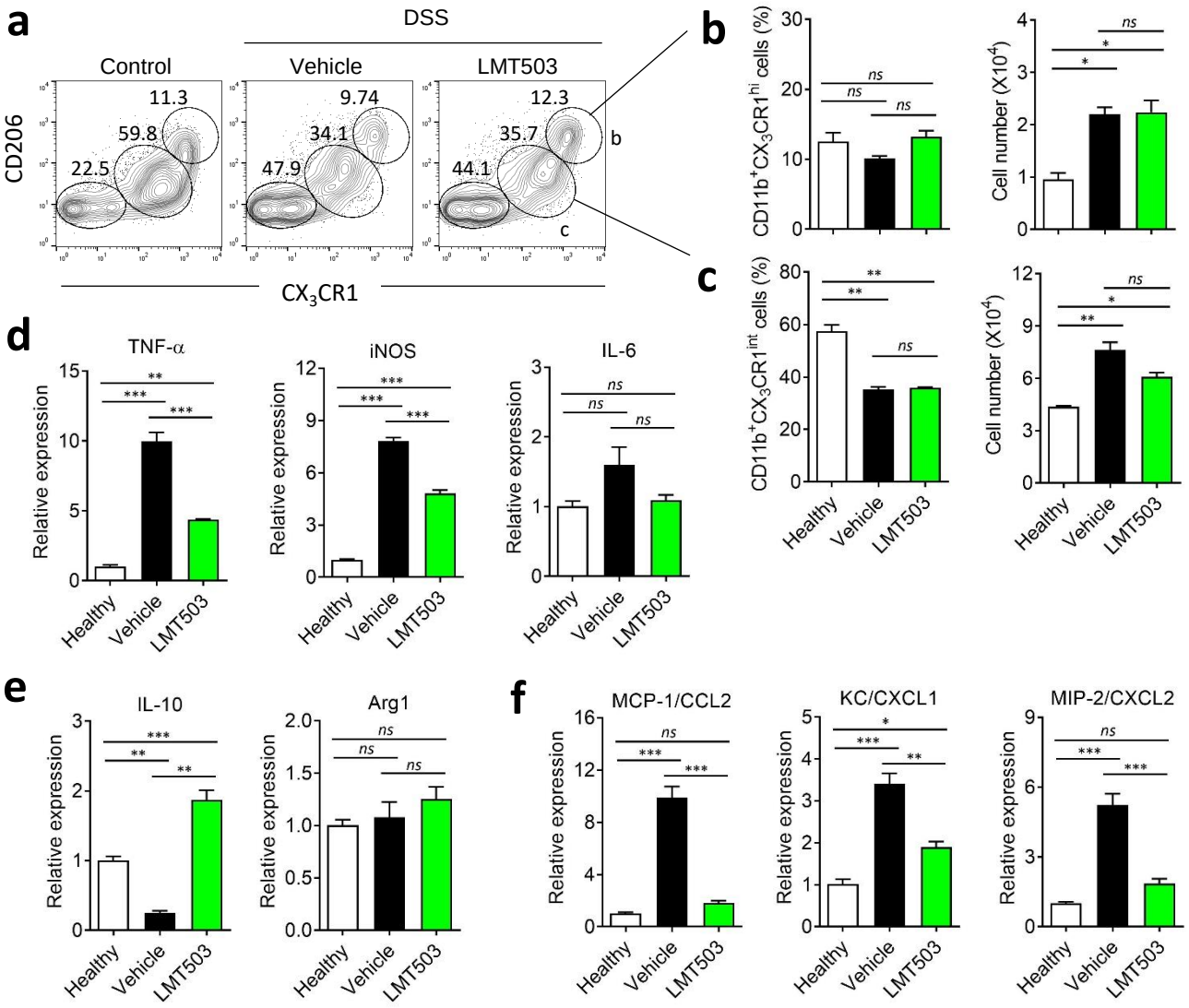

a
DSS
Control
Vehicle
LMT503
CD206
CX3CR1
11.3
59.8
22.5
9.74
34.1
47.9
12.3
35.7
44.1
b
c
b
CD11b+CX3CR1hi cells (%)
Cell number (X10^4)
ns
*
c
CD11b+CX3CR1int cells (%)
Cell number (X10^4)
**
ns
Healthy
Vehicle
LMT503
d
TNF-α
iNOS
IL-6
Relative expression
***
e
IL-10
Arg1
f
MCP-1/CCL2
KC/CXCL1
MIP-2/CXCL2

Figure 5

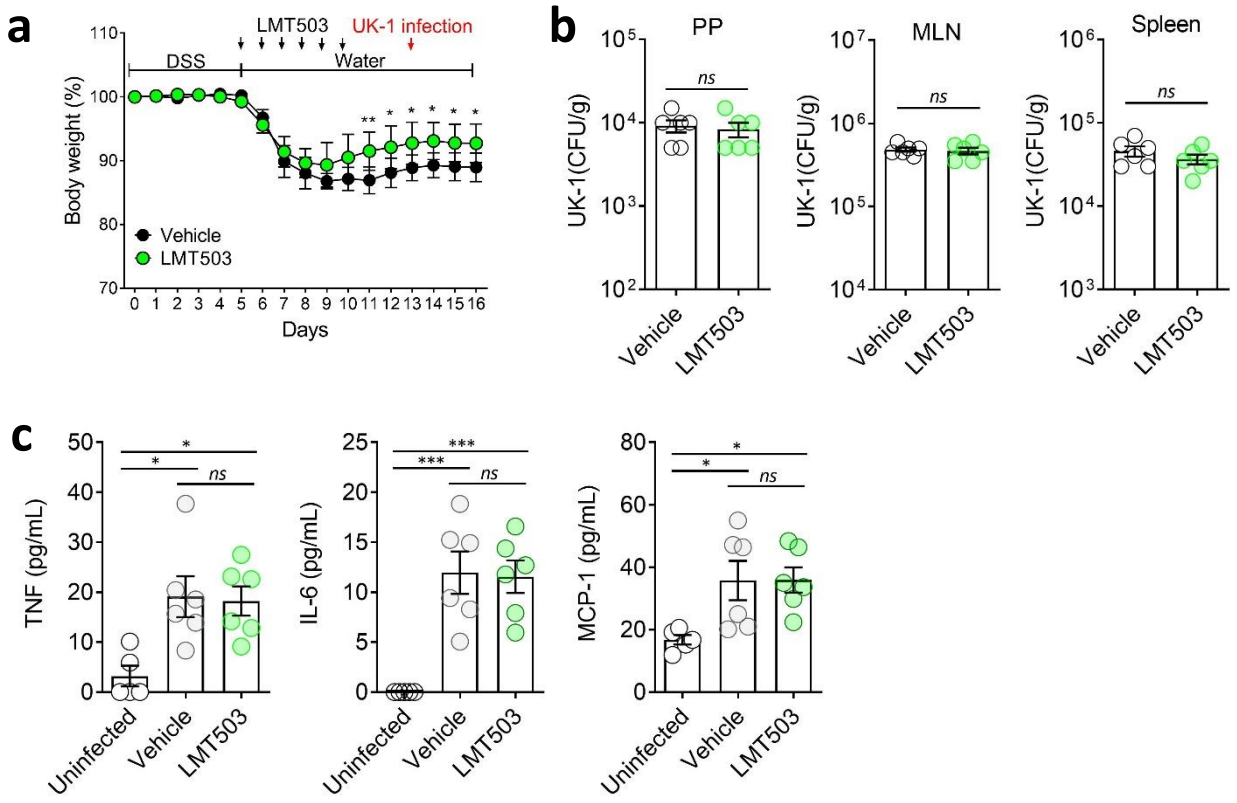
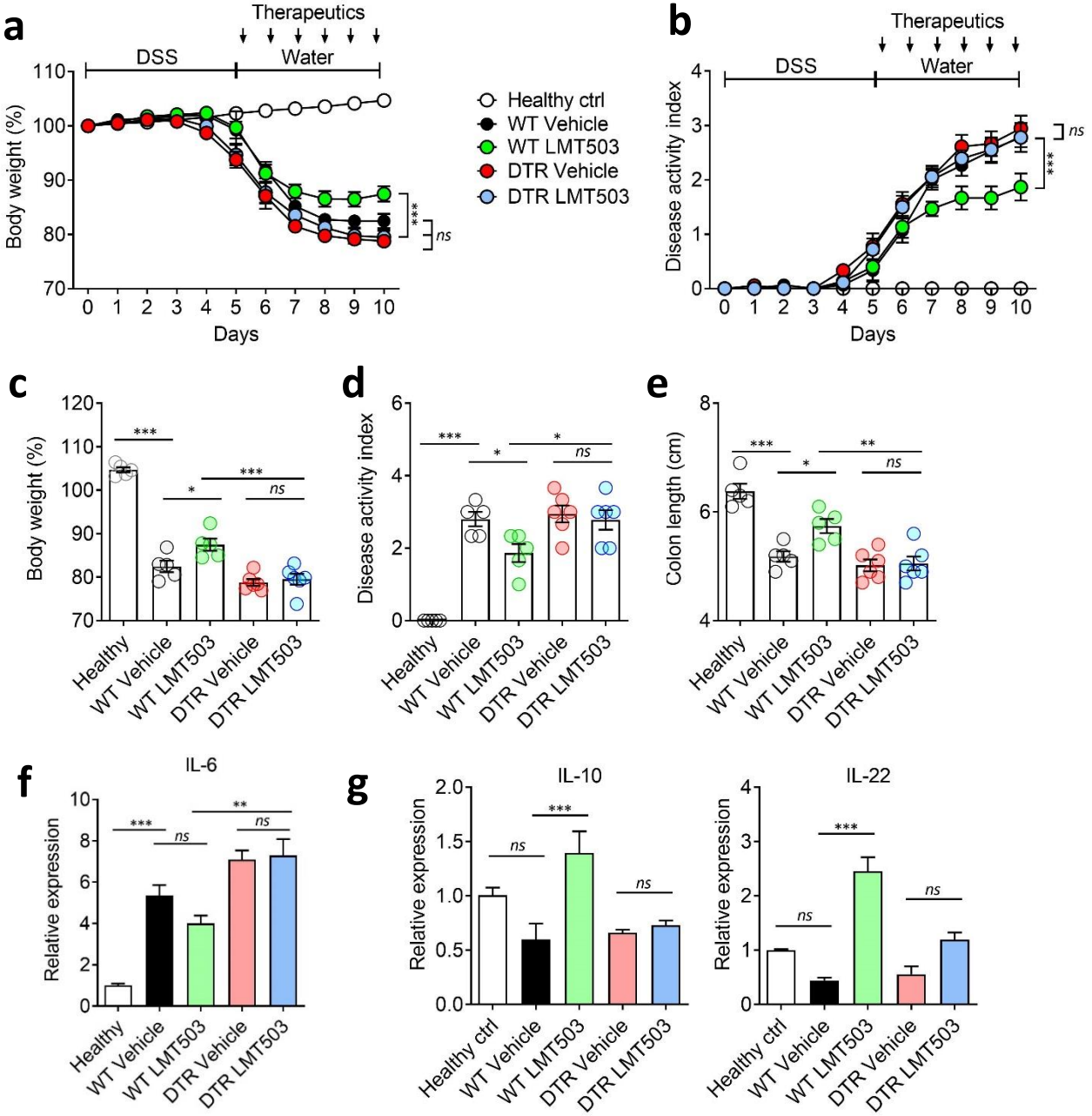

a
LMT503
UK-1 infection
DSS
Water
Body weight (%)
Vehicle
LMT503
Days
b
PP
MLN
Spleen
UK-1(CFU/g)
ns
Vehicle
LMT503
c
TNF (pg/mL)
IL-6 (pg/mL)
MCP-1 (pg/mL)
Uninfected
Vehicle
LMT503

Figure 6


a
Therapeutics
DSS
Water
Body weight (%)
Days
Healthy ctrl
WT Vehicle
WT LMT503
DTR Vehicle
DTR LMT503
***
ns
b
Therapeutics
DSS
Water
Disease activity index
Days
ns
***
c
Body weight (%)
Healthy
WT Vehicle
WT LMT503
DTR Vehicle
DTR LMT503
d
Disease activity index
e
Colon length (cm)
f
IL-6
Relative expression
g
IL-10
IL-22
Healthy ctrl