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Evaluation of Vitamin C effect on murine colitis in *Gulo* knock-out mouse

by

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ABSTRACT

Intestinal mucosal damage in the inflammatory bowel diseases (IBD), Crohn's disease (CD) and ulcerative colitis (UC), involves the dysfunctional immunoregulation of the gut. Among the immunoregulatory factors, reactive oxygen species (ROS) are produced in abnormally high levels in IBD, and their destructive effects may contribute to the initiation or propagation of the disease. Vitamin C not only scavenges free radicals as an antioxidant but also has anti-inflammatory effects. Therefore,I investigated the effect of vitamin C on dextran sulfate sodium (DSS)-induced colitis in *gulo(-/-)* mice which cannot synthesize vitamin C. Vitamin C-insufficient *gulo(-/-)* mice showed decreased survival and lowered recovery efficacy. It was accompanied with more severe colitis such as epithelial erosion, infiltration of inflammatory cells and contraction of colon. The production of pro-inflammatory cytokine, interleukin (IL)-6, was remarkably higher in DSS-treated vitamin C-insufficient *gulo(-/-)* mice than vitamin C-sufficient *gulo(-/-)* mice and wild type mice. In contrast, the production of anti-inflammatory cytokine, IL-22, was significantly decreased in vitamin C-insufficient *gulo(-/-)* mice compared to vitamin C-sufficient *gulo(-/-)* mice and wild type mice after DSS treatment. In addition, the phosphorylation of signal transducer and activator of transcription (STAT)3, a downstream signaling of IL-6 and IL-22, was increased in both epithelial cells and lamina propria cells, and immunoblot also showed increased phospho-STAT3 in KO group. To summarize, vitamin

C deficiency was associated with more severe DSS induced colitis, and was related to increased pro-inflammatory IL-6 production and oxidative stress, resulting in activated STAT3 signaling, and decreased production of anti-inflammatory IL-22. Taken together, it suggests that vitamin C represents a protective effect on DSS-induced colitis by regulating the production of cytokine and the induction of inflammation.

Keywords: Inflammatory bowel diseases, Vitamin C, Anti-oxidants

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CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA: analysis of variance

CD: Crohn’s disease

DAI: disease activity index

DSS: dextran sulfate sodium

ELISA: enzyme linked immunosorbent assay

GULO: L-gulono-γ-lactone oxidase

HTAB: hexadecyltrimethylammonium bromide

IBD: inflammatory bowel diseases

IL: interleukin

MCP: monocyte chemotactic protein

MPO: myeloperoxidase

NO: nitrogen oxide

NT: nitrotyrosine

PFA: paraformaldehyde

ROS: reactive oxygen species

STAT: signal transducer and activator of transcription

TAC: total antioxidant capacity

UC: ulcerative colitis

INTRODUCTION

Inflammatory bowel diseases (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), are characterized by bloody diarrhea, abdominal pain, and weight loss, generally begin in young adulthood and last through life. These chronic diseases result in significant morbidity and mortality and confer increased risk of gastrointestinal malignancy. There is no cure for these diseases, thus quality of life and life expectancy in IBD patients are compromised .[1]

A rising trend in the incidence and prevalence of IBD in Asia has been reported, and it is postulated that this increase may be related to the westernized lifestyles, including changes in diet and environmental changes such as improved sanitation and industrialization.[2] During the past two decades in Korea, the mean annual incidence rates of CD and UC increased significantly, from 0.05 and 0.34 per 100,000 inhabitants, respectively, in 1986-1990 to 1.34 and 3.08 per 100,000 inhabitants, respectively, in 2001-2005. In addition, the adjusted prevalence rates of CD and UC per 100,000 inhabitants in 2005, were 11.24 and 30.87. Although, the incidence and prevalence of CD and UC in Korea are still low compared with those in Western countries, but they are rapidly increasing .[3]

IBD are thought to be caused by an inappropriate and exaggerated immune responses directed toward commensal microbiota in a genetically susceptible individual.[4] Although, major advances have been made in our

understanding of the biology of intestinal inflammation and how it is related to the pathogenesis of IBD for the last two decades, their etiology is still largely unknown.[1]

Oxidative stress and depletion of antioxidants may play an important role in the initiation or propagation of IBD.[5] It has been suggested that intestinal damage in IBD is related both to increased free radical production and to decreased concentration of endogenous antioxidant defenses.[6] Reactive oxygen species (ROS), which form as natural byproducts of the normal metabolism of oxygen, are highly reactive molecules as a result of the presence of unpaired electrons.[5] In patients with IBD, excessive oxidized molecules in a variety of organic systems, such as gastrointestinal tract and respiratory tract, were demonstrated compared with healthy control, although some conflicting results exist.[7-9]

The serum or plasma levels of both total antioxidant capacity (TAC) and corrected TAC (cTAC) in patients with UC and CD were significantly lower compared to healthy controls. This reduced cTAC levels suggest that exogenous antioxidants are seriously impaired in IBD.[8] In addition expression of antioxidants has been investigated in several studies in various organs including colonic tissue. Although the change in antioxidant level is conflicting among the studies, imbalance in antioxidant concentration substantiates the fact that vital organs in IBD patients are in oxidative stressed state.[5]

Vitamin C (L-ascorbic acid) is a reducing agent, donating electrons to various enzymatic and non-enzymatic reactions. As an electron donor,

vitamin C is a potent water-soluble antioxidant in humans, and this antioxidant effects have been demonstrated in many experiments *in vitro.* In addition, it is also a cofactor in many enzymatic reactions including several collagen synthesis reactions. Human cannot synthesize the vitamin C, hence it is an essential nutrient that should be supplied in the diet. Deficiency in vitamin C leads to scurvy in humans.[10] Vitamin C not only scavenges free radicals as an antioxidant but also is suggested to have anti-inflammatory effects.[11]

Some mammals, including human and other primates, cannot synthesize vitamin C due to mutations in the *GULO* gene encoding L-gulono-γ-lactone oxidase (GULO), rate-limiting enzyme in vitamin C biosynthesis. There are few studies that have attempted to address the effect of vitamin C deficiency on intestinal inflammation. Therefore, I aimed to investigate the effect of vitamin C deficiency in a murine model for DSS-induced acute colitis using *gulo*(-/-) mice.

MATERIALS AND METHODS

Animal C57BL/6 wild type (WT) mice and *gulo*(-/-) mice were maintained in a specific pathogen free condition at the animal facility in the Seoul National University College of Medicine. Five to six weeks old male mice were used for experiments. For *gulo*(-/-) mice, vitamin C (3.3 g/L) was supplemented in the water to prevent death by deficiency of vitamin C until 5-6 weeks. After that, vitamin C was depleted in the vitamin C deficiency group (KO group) for 3 weeks to induce vitamin C-deficient state. However, for the vitamin C supplemented group (KO+VC group), vitamin C was maintained in the drinking water (Figure 1).

Induction of acute colitis and assessment of colitis activity Mice were given 3% dextran sulfate sodium (DSS, MP Biochemicals, Irvine, CA, USA) dissolved in the drinking water for 7 days to induce acute colitis, as described in figure 1. The mice were checked daily for behavior, water and food consumption, body weight, stool consistency, and the presence of gross blood in stool or at the anus. Weight change was calculated as the percentage change in weight compared with body weight at day1. Colonic damage was quantified by a clinical score assessing weight loss, stool consistency, and gross rectal bleeding. The calculated disease activity index (DAI) was mean score of the three parameters. After mice were sacrificed, colon length was measured from anus to ileocecal valve. All experiments using animals were

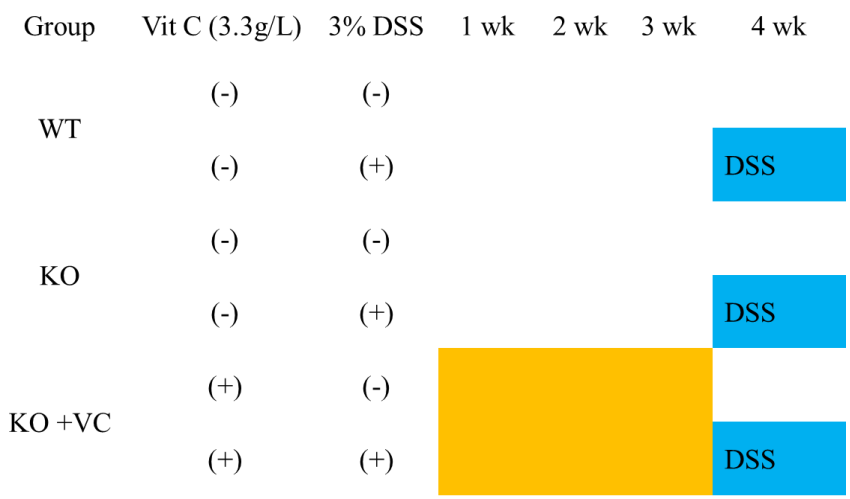


Figure 1. Experimental Scheme

Five to six weeks old male C57BL/6 wild type (WT) mice and *gulo*(-/-) mice were used for experiments. For *gulo*(-/-) mice, vitamin C (3.3 g/L) was supplemented in the water until 5-6 weeks. Vitamin C was depleted in the vitamin C deficienct group (KO group) for 3 weeks to induce vitamin C-deficient state, while vitamin C was maintained in drinking water for the vitamin C supplemented group (KO+VC group). Then, mice were given 3% dextran sulfate sodium dissolved in the drinking water for 7 days to induce acute colitis.

reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University.

Histological evaluation of colonic damage Colon samples were fixed in 4 % paraformaldehyde (PFA), paraffin-embedded, sectioned, and stained with with hematoxylin and eosin. Histologic assessment was performed by a trained pathologist who was blinded to the treatment. All histological quantification was performed in a blinded fashion using a scoring system. Briefly, cellularity, edema, erosion, or ulceration of mucosa, and loss of mucosal architecture were evaluated as follows. 0, Normal; 1, Slight increase in cellularity (primarily lymphocytes in the lamina propria); 2, Increase in cellularity, neutrophils present, mild edema; 3, Diffuse increase in cellularity, focal erosions, or ulcerations of mucosa; 4, Increased cellularity, large and/or multifocal mucosal ulcerations; 5, Diffuse ulceration, loss of mucosal architecture.

Flow cytomix Blood was collected from intra-orbital plexuses of mice with capillary tube and readily centrifuged with 14,000 rpm for 30 min at 4℃. The serum was stored until use at -70℃. The CBA Mouse Th1/Th2/Th17 Cytokine kit was purchased from BD Bioscience (San Jose, CA, USA) and performed according to manufacturer's instructions. Cytokines were analyzed by FACS Calibur (BD Bioscience, San Jose, CA, USA).

Enzyme linked immunosorbent assay (ELISA) Blood was collected from intra-orbital plexuses of mice with capillary tube and readily centrifuged with 14,000 rpm for 30 min at 4℃. Sera of upper layer were collected to new tubes, and stored at -70℃ until use. Colonic tissues were homogenized with lysis buffer and quantified with BCA method. ELISA kit for IL-6 and IL-22 were purchased from R&D system (Minneapolis, MN, USA). ELISA was performed according to the manufacturer's instructions. The final concentration of IL-6 and IL-22 in colon was normalized with the amounts of total protein in colonic tissue lysates.

Myeloperoxidase (MPO) assay Weighted colons were homogenized in 0.5 ml of ice-cold 0.5% hexadecyltrimethylammonium bromide (HTAB, Sigma, St. Louis, MO, USA) in 50 mM of phosphate buffer (pH 6.0). HTAB was used to negate the pseudoperoxidase activity of hemoglobin and to solubilzed the membrane-bound MPO. The homogenate was centrifuged at 18,000g for 20 min at 4℃. Supernatant was reacted with a mixture containing 0.167 mg/ml O-dianisidine dihydrochloride (Sigma, St. Louis, MO, USA) and 0.005 % H_2O_2 and the amount of myeloperoxidase for 10 min at room temperature. The reaction was terminated by adding 0.02 % sodium azide. The absorbance was measured at 460 nm, and amount of MPO was normalized with the weight of colon.

Immunoblotting Colonic tissues were homogenized with lysis buffer and quantified with BCA method. 50 μg of protein was mixed with 5x SDS sample buffer and loaded onto each lane of 10 % SDS-PAGE gel. Proteins were separated by eletrophoresis and transferred from the gel to a nitrocellulose membrane with an electroblotting apparatus. Nonspecific sites of membranes were blocked with 5 % skim milk for 1 hr and then membranes were incubated with primary antibody against phospho-signal transducer and activator of transcription (STAT)3, STAT3, 3-nitrotyrosine (NT) (1:500, Santa Cruz, Palo alto, CA, USA), or β-actin (1:4000, Sigma, St. Louis, MO, USA) at 4℃ overnight. Followed by washing with PBS-T (0.05 % Tween-20 in PBS), they were subsequently incubated with horseradish peroxidase-conjugated secondary antibody (1:4000, cell signaling, Danvers, MA, USA) and detected with the ECL detection kit (Amersham, Piscataway, NJ, USA). Product form western blot analysis was quantified using a densitometry analysis program (SCION Image program, Frederick, MD, USA).

Immunohistochemistry Colon tissues were freshly isolated and fixed in 4 % paraformaldehyde (PFA) at 4℃ overnight. Paraffin-embedded tissues were sectioned with 4 μm thickness. After deparaffinization and hydration, epitope of antigen was retrieved by heating with 0.1 M citrate buffer (pH 6.0) under microwave. Followed by blocking endogenous peroxidase with H_2O_2 and inhibiting nonspecific signals with 5 % horse serum, sections were incubated with primary antibodies against phospho-STAT3, (1:50, Santa Cruz, Palo alto,

CA, USA), at 4℃ overnight in a humidified chamber. Then, sections were incubated with a matching biotinylated secondary antibody (1:150, Vector laboratories, Burlingame, CA, USA) for 1 hr at room temperature. ABC solution (Vector laboratories, Burlingame, CA, USA) was loaded on sections for 30 min, and DAB kit (Vector laboratories, Burlingame, CA, USA) was used for chromogenic detection. Subsequent to dehydration and clearing, the sections were mounted with DPX mountant (Fluka, St. Louis, MO, USA) and observed with light microscope (Olympus, Center Valley, PA, USA).

Statistics Data were expressed as mean±SD of each group in independent experiments. For comparison of three or more groups, data were analyzed by one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparison test. A value of $P < 0.05$ was considered statistically significant. Statistical tests were carried out using GraphPad InStat (GraphPad Software, San Diego, CA, USA).

RESULTS

1. Severity of colitis

Three groups did not show any mortality without DSS treatment. However, 30% of mortality was observed in KO group in the presence of DSS treatment, although no morality was observed in WT or KO+VC group treated with DSS (Figure 2). Body weight gain was observed in WT group without DSS treatment, but KO+VC and KO group did not show normal body weight gain. Mean body weight of the KO group was significantly lower than that of WT and KO+VC group after 7 days of DSS treatment ($P < 0.05$) (Figure 3). Also, the severity of colitis in the KO group assessed by DAI was more severe compared to WT or KO+VC group, though statistical significance did not reach between KO and KO+VC group (Figure 4).

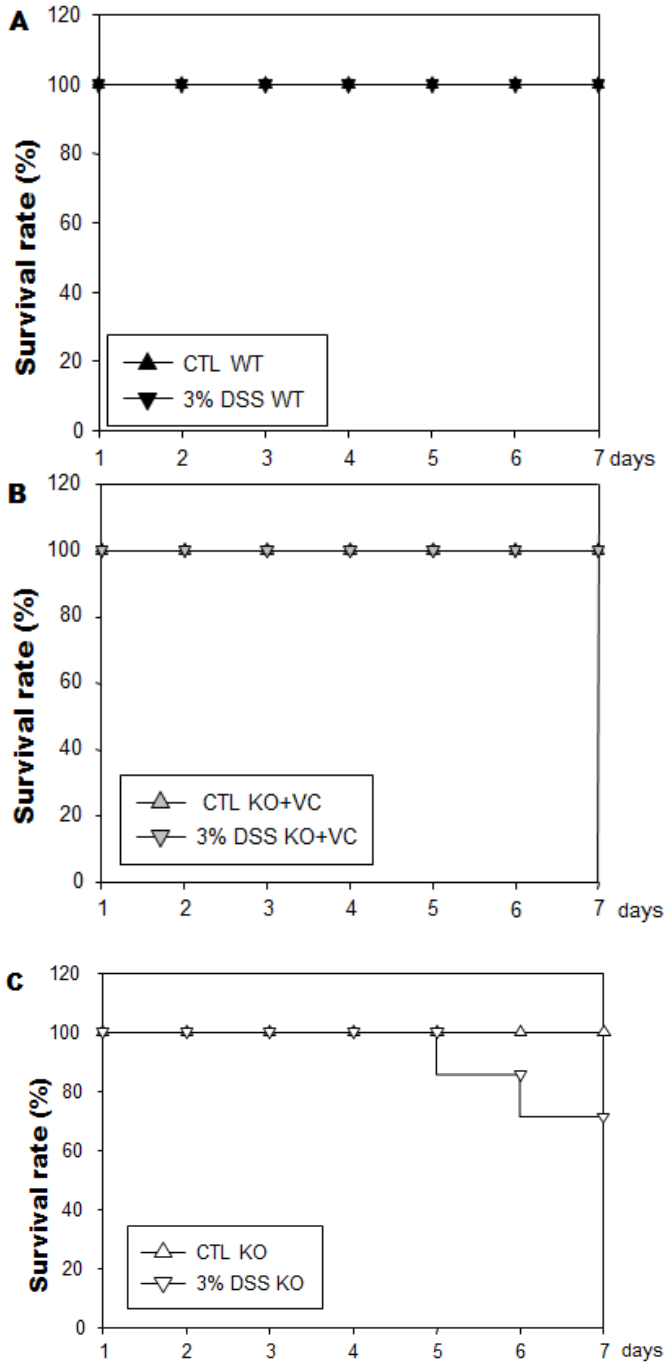


Figure 2. Survival rate of WT/KO/KO+VC treated with or without DSS

After mice were treated with 3% DSS, survivals of three groups were traced

during experimental period.

(A), (B) After 7days of DSS treatment, there was no mortality in WT or KO+VC group.

(C) KO group showed no mortality without DSS treatment, but 30% of mortality was observed when they were treated with DSS.

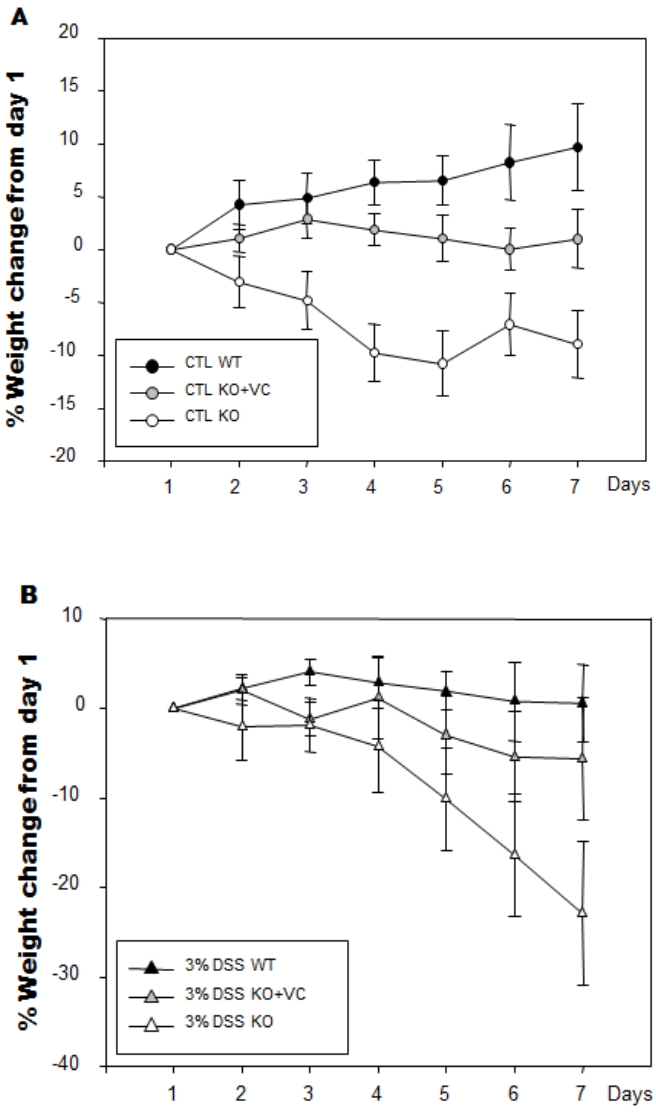


Figure 3. Body weight change in WT, KO, and KO+VC mice treated with or without DSS

(A) KO+VC and KO group did not show normal body weight gain in the absence of DSS treatment, while WT group had continued weight gain.

(B) Seven days of DSS treatment with DSS induced significantly more body weight loss in KO group than that in WT and KO+VC group ($P<0.05$).

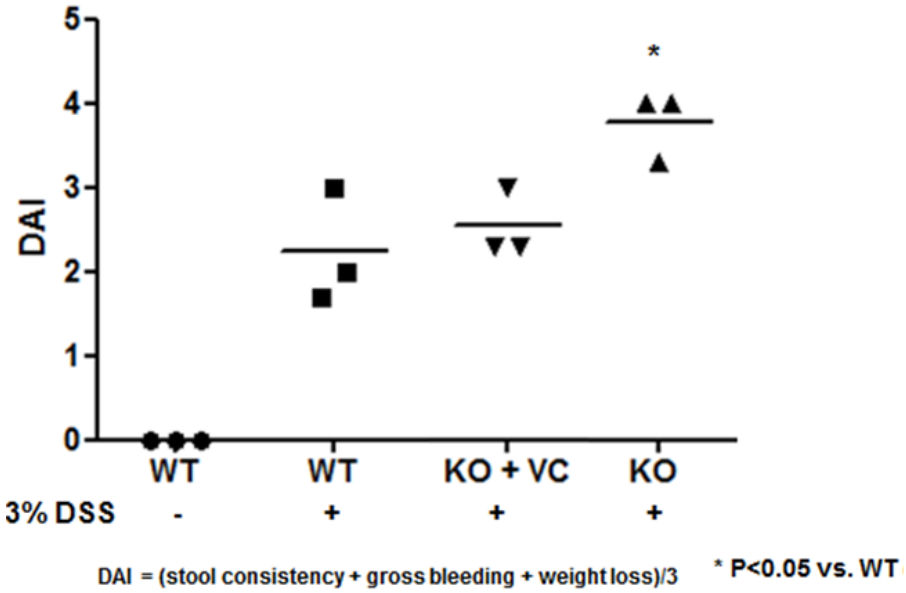


Figure 4. Disease activity index in WT, KO, and KO+VC mice treated with or without DSS treatment

Severity of colitis in the KO group assessed by DAI was more severe compared to WT or KO+VC group, but the difference did not reach statistical significance.

2. Colon length and histologic grade

To evaluate the severity of colitis histologically, we measured colon length from anus to ileocecal valve and inflammation was evaluated by blinded histological scoring as previously described in the method. DSS treatment induced significant colonic shortening in all three groups as shown in Figure 5. Shortening was more severe in KO group than WT and WT+VC groups, and the difference was statistically significant ($P < 0.05$). The colon of DSS-treated mice showed complete destruction of epithelial architecture with loss of crypts and epithelial integrity, submucosal edema, and intense inflammatory cellular infiltration in all layers compared with that of controls. Histological grading of KO group showed more severe inflammation compared to that of the other groups (Figure 6).

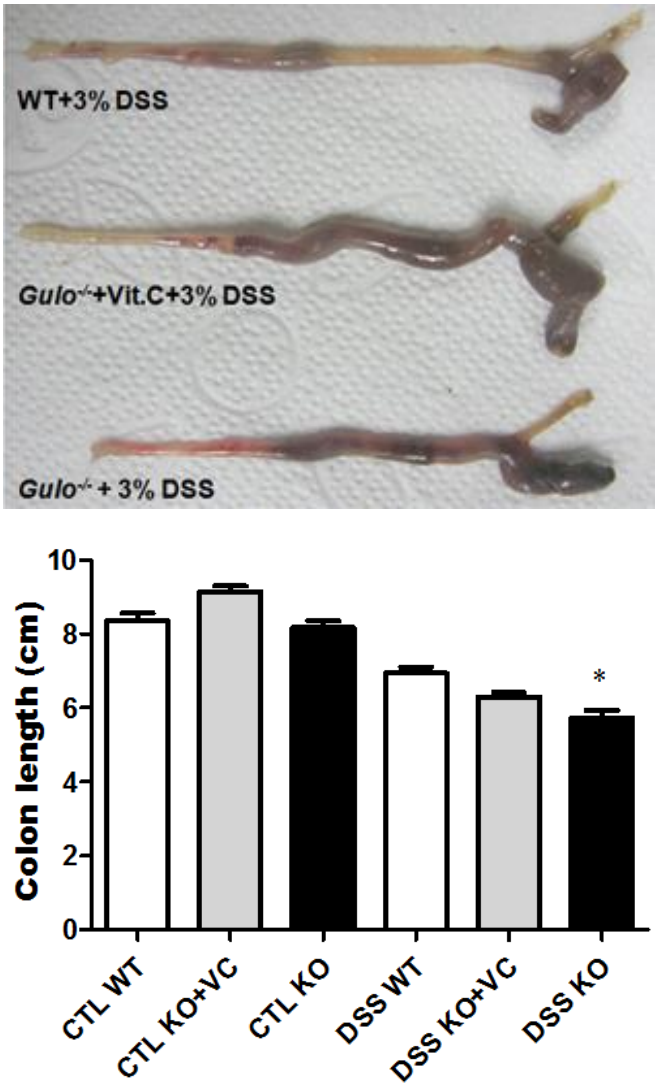
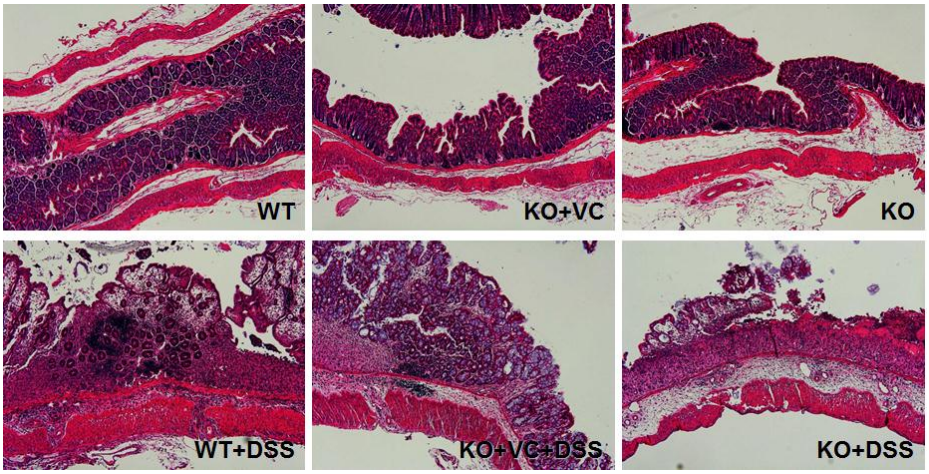


Figure 5. Colon length in WT, KO, and KO+VC groups treated with or without DSS treatment

Colon was isolated after mice were sacrificed and length was measured between anus and ileocecal valve. DSS treatment induced more sever shortening in KO group compared with WT and WT+VC groups, and the difference was statistically significant (*P<0.05 vs. WT and KO+VC)



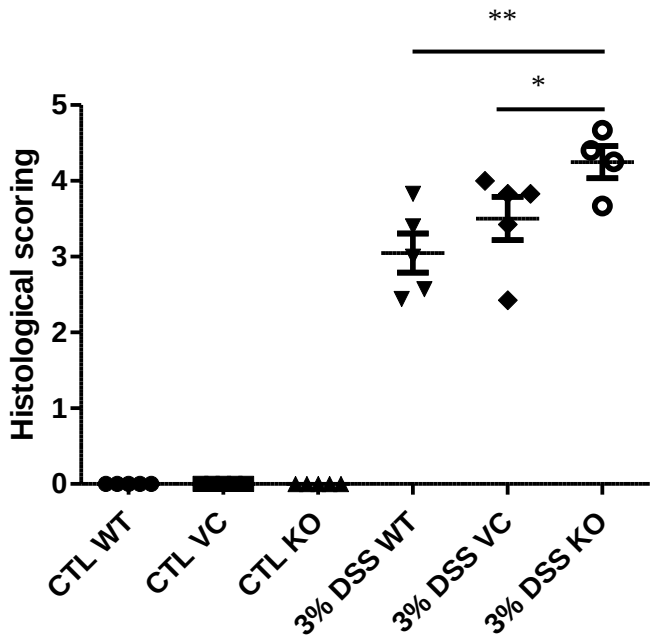


Figure 6. Histology and degree of inflammation in WT/KO/KO+VC treated with or without DSS.

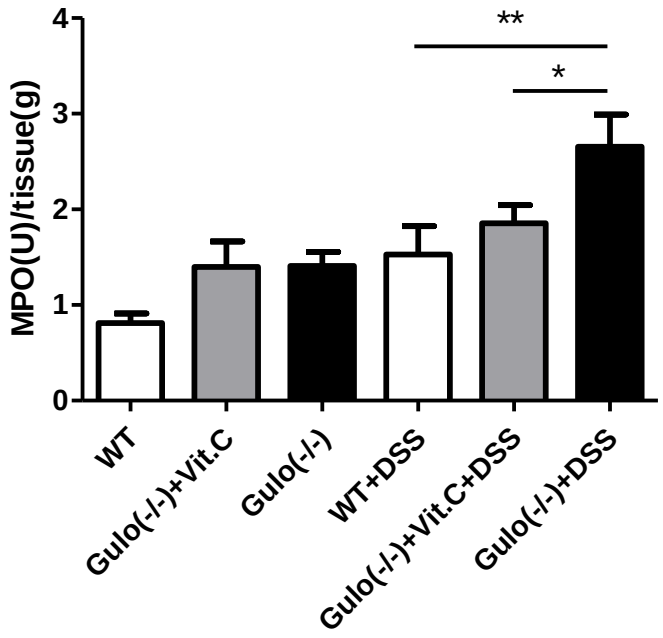
Isolated colons from each group were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. All histological quantification was performed in a blinded fashion using a scoring system as described in

materials and method. Histological grading of KO group showed more severe inflammation compared to that of the other groups (*P<0.05 vs. KO+VC, **P<0.01 vs. WT)

3. Tissue MPO activity and 3-NT expression

In addition to the clinical and histological severity, the level of MPO activity, as a parameter of neutrophil accumulation, was significantly increased in colons of KO group compared with those of WT and KO+VC groups (Figure 7A). NT is known as a marker of nitrogen oxide (NO) production, cellular damage, and inflammation. This marker is known to reflect degree of cell damage induced by NO, and is reported to be increased in a variety of inflammatory diseases such as atherosclerosis and rheumatoid arthritis.[12] To evaluate the effect of vitamin C deficient state on tissue oxidative stress, we examined the 3-NT in colonic tissue using immunoblotting. As shown in figure 7B, 3-NT expression was increased in KO group treated with DSS, but we could not detect 3-NT in other groups even in the presence of DSS.

A



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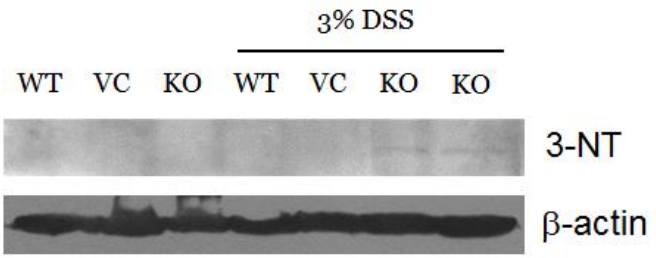


Figure 7. MPO activity and Western-blot for 3-nitrotyrosine in colonic tissue

(A) The level of MPO activity was increased in colons of mice with DSS-induced colitis. Increased MPO activity was significantly higher in KO group than in KO+VC(*$p<0.05$) and WT (**$P<0.01$).

(B) The expression of 3-NT in the colon from mice in each group was examined by western blot analysis. 3-NT was expressed only in KO group treated with DSS.

4. Serum IL-6 and IL-22 concentration

I evaluated the changes of various pro- and anti-inflammatory cytokines, including IL-1α, IL-2, IL-5, IL-6, IL-10, IFN-γ, TNF-α, GM-CSF, IL-4, and IL-17 using CBA Mouse Th1/Th2/Th17 flow cytomix kit, and found that IL-6 was increased in KO compared to WT or KO+VC with DSS treatment. Based on the flow cytomix results, I measured the serum concentrations of IL-6 by ELISA. DSS treatment induced IL-6 production in all groups, as shown in figure 8A. IL-6 production was more pronounced in KO groups compared to WT and KO+VC groups and the difference was statistically significant ($P<0.001$).

IL-22 belongs to the IL-10 family of cytokines, and is bifunctional in that it has both pro-inflammatory and protective effects on tissues depending on the inflammatory context.[13] Recently, it was reported that IL-22 stimulates mucus production and goblet cell restitution, and also contributes to the rapid attenuation of inflammation in a mouse model of UC.[14] Therefore, I measured the serum concentration of IL-22 by ELISA. As shown in figure 8B, we found than IL-22 production was induced both in WT and KO+VC by DSS treatment, but IL-22 production was impaired in KO group. Notably, serum level of IL-22 in KO group treated with DSS was significantly lower than that in DSS-treated WT and KO+VC groups ($P<0.01$).

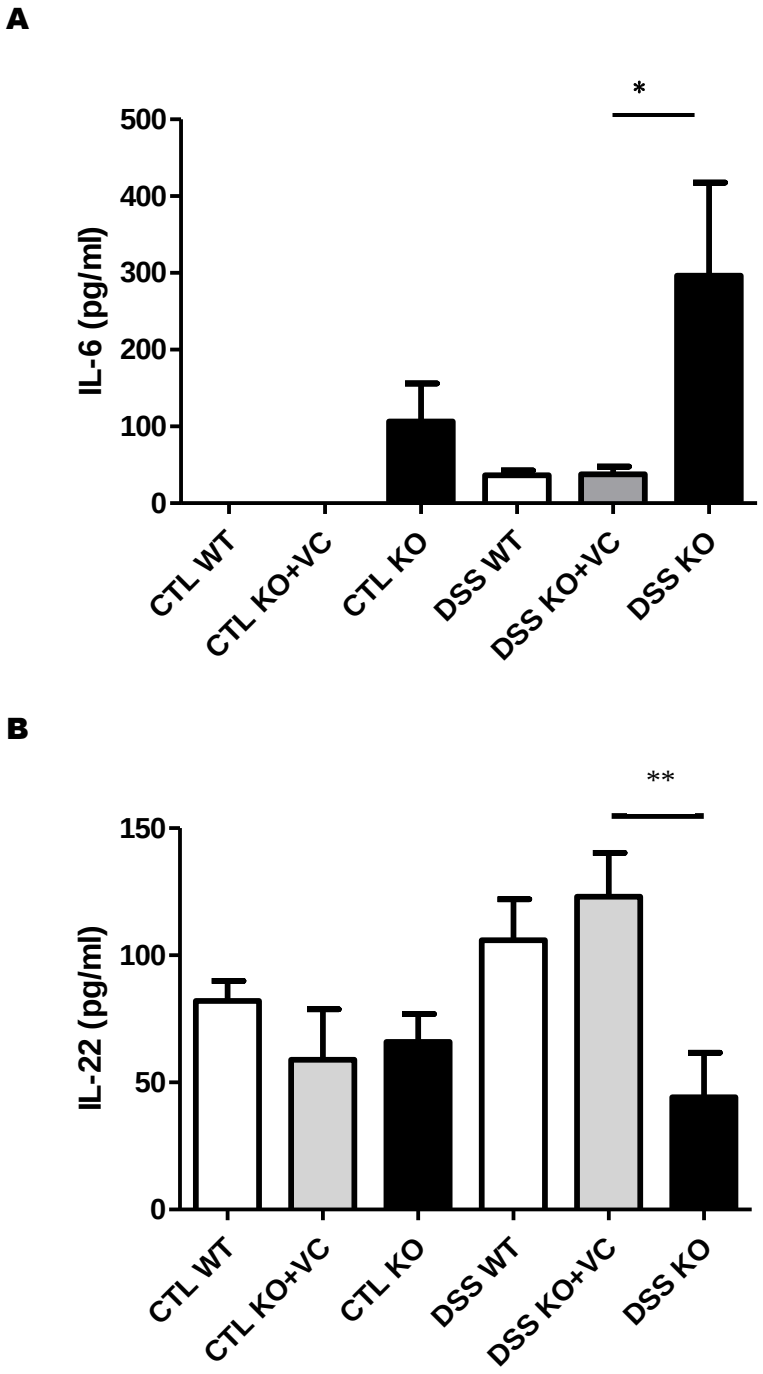


Figure 8. Serum level of IL-6 and IL-22 in WT, KO, and KO+VC with or without DSS treatment

(A) Based on the flow cytomix results, serum concentrations of IL-6 were

measured by ELISA. IL-6 production was induced in all groups after DSS treatment. IL-6 production was more pronounced in KO groups compared to WT and KO+VC groups and the difference was statistically significant (*P<0.001).

(B) IL-22 production was induced both in WT and KO+VC by DSS treatment, but IL-22 production was impaired in KO group. Serum level of IL-22 in KO group treated with DSS was significantly lower than that in DSS-treated WT and KO+VC groups (**P<0.01).

5. Tissue IL-6 and IL-22 concentration

To examine that increased IL-6 and IL-22 in serum can reflect tissue level of IL-6 and IL-22, I measured colonic IL-6 and IL-22 by ELISA. As shown in figure 9A, tissue level of IL-6 in KO group treated with DSS was higher than that in WT and KO+VC group, although I could not find statistical significance. However, IL-22 expression was significantly decreased in KO group compared with WT and KO+VC group when treated with DSS (figure 9B), as observed in serum.

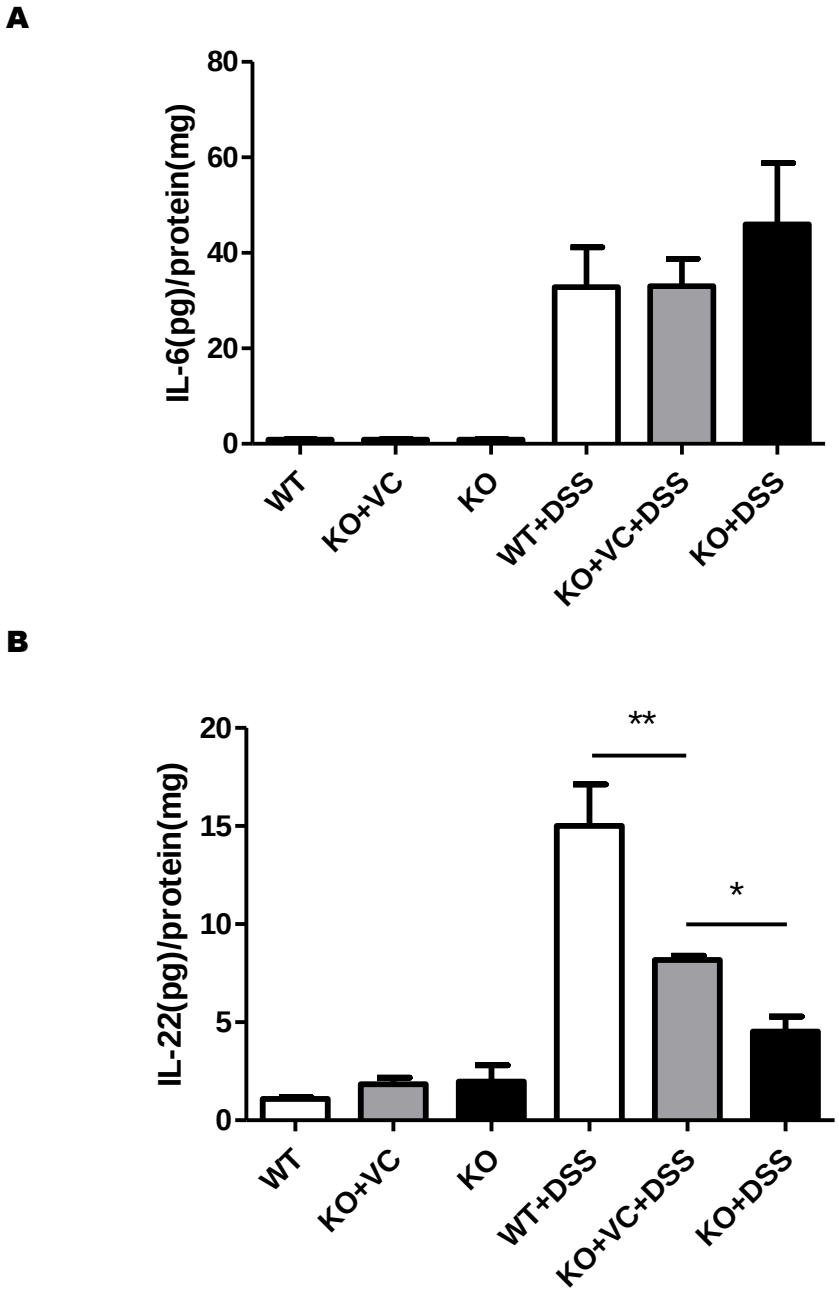


Figure 9. Tissue level of IL-6 and IL-22 in WT, KO, and KO+VC with or without DSS treatment.

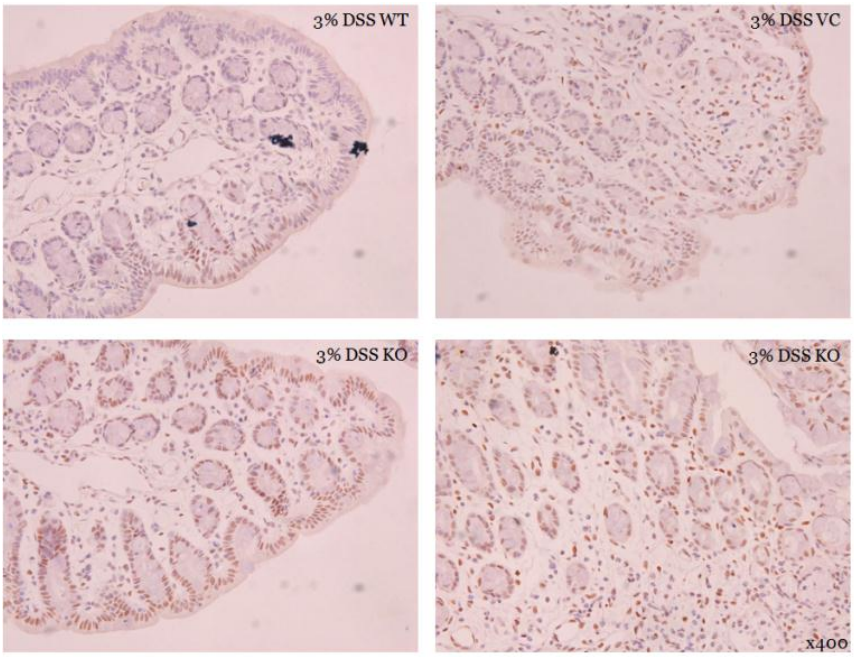
(A) After mice treated with DSS, IL-6 production was increased in all groups. Tissue level of IL-6 in KO group was higher than that in WT and KO+VC group.

(B) IL-22 production after DSS treatment in colonic tissue was significantly higher in WT and KO+VC groups than KO group (*P<0.01, WT vs. KO+VC, **P<0.05, KO+VC vs. KO).

6. Phosphorylation of STAT3

Both IL-6 and IL-22 has been demonstrated to efficiently activate STAT3, thus results in increased transcription of various pro-inflammatory cytokines.[15] To evaluate phosphorylation of STAT3, I performed immunohistochemistry and found that STAT3 activation in KO group was increased in both intestinal epithelium and infiltrating immune cells in lamina propria compared with WT and KO+VC groups (figure 10A). As shown in figure 10B, phospho-STAT3 was significantly increased in KO group compared to other two groups with DSS treatment.

A



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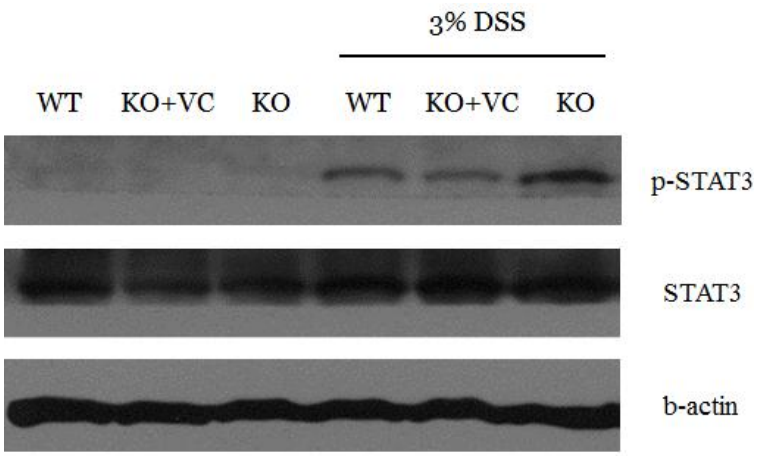

Figure 10. Immunohistochemical stain and Western blot for phospho-STAT3

The activation of STAT3 in the colon from mice in each group was examined by immunohistochemical stain(A) and western blot analysis(B) as described in *materials and method*.

DISCUSSION

Here, I showed that vitamin C deficiency was associated with more severe DSS induced colitis assessed by body weight change, clinical index, colon length, and histologic grade. In addition, more severe inflammation in vitamin C deficient state was related to increased pro-inflammatory IL-6 production and oxidative stress, resulting in activated STAT3 signaling, and decreased production of anti-inflammatory IL-22.

Although the exact pathogenesis of IBD remains unclear, recent evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host.[16] Amongst many immunoregulatory factors, ROS are produced in abnormally high levels in patients with IBD.[5] In addition, TAC and cTAC used as a marker of oxidative stress are significantly reduced in IBD patients irrespective of disease activity.[8] Increased oxidative stress and decreased antioxidant defense can lead to increased DNA damage in patients with IBD, and this may explain the increased risk of developing colon cancer in these patients.[7,17]

Vitamin C is synthesized in the liver or kidneys and transported to the tissues via the circulation. The terminal rate-limiting enzyme in the synthetic pathway, gulonolactone oxidase, is lost in some species, including humans, thus, vitamin C is an essential dietary component.[18] Increased vitamin C uptake into keratinocytes by UVB effectively suppressed inflammatory response via the downregulation of IL-8 and monocyte chemotactic protein (MCP)-1 production, suggesting anti-inflammatory effect

of vitamin C.[11] Considering pathogenesis of IBD and the role of vitamin C as an anti-oxidant and anti-inflammatory action, this essential micronutrient may have supplementary role in the management of IBD.

In addition to well-known scavenging effect as a reducing agent, vitamin C is an important cofactor in the hydroxylation of lysine and proline in collagen synthesis and cross-linkage. In animal model, vitamin C supplementation poses as a beneficial treatment in the context of collagen accumulation, inflammatory response, and anastomotic strength.[19,20] Recently, mucosal healing as well as clinical remission and steroid independence are considered to be important prognostic factors in IBD management. Hence, vitamin C can be an option to enhance mucosal healing for better clinical outcome in selected population of IBD patients.

Underweight and specific nutrient deficiencies are frequently observed in adult patients with IBD, because of loss of appetite, decreased intake due to abdominal pain and absorption defect, and chronic gastrointestinal loss.[21,22] Of particular, 70% of inpatients and 30% of outpatients with CD are reported to have clinically significant weight loss. Patients with active stage of UC are also accompanied by various nutritional deficiency including hypoalbuminemia, iron deficiency anemia and electrolyte imbalance.[21,22] In addition, patients with IBD are at increased risk of developing nutritional deficiencies, because of restrictive diets even in inactive stage, especially micronutrients including vitamin C.[9,21,23,24]

GULO acts at the terminal step in vitamin C biosynthesis, and *gulo* knock-out mice cannot synthesis vitamin C like human. Two weeks of

vitamin C depletion reduce the tissue and serum concentration of vitamin C by 10-15%, weight loss and anemia are accompanied by 5^{th} week of depletion, and total depletion finally leads mice to death. In this study, there were mortalities only in KO group by DSS treatment. Additionally, KO group showed body weight loss after 3 weeks of vitamin depletion in the absence of DSS treatment. Inflammation assessed by DAI including weight loss, colon shortening, and histologic grading was more severe in KO group when treated with DSS as compared with other groups. This demonstrates that vitamin C deficiency results in more susceptible state to DSS induced colitis.

In IBD, various pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, and IFN-γ, are increased, and anti-inflammatory cytokines including IL-10 and IL-22 are known to be decreased. I screened change of cytokines using cytokine flow cytomix, and IL-6 was found to be increased in KO group even in the absence of DSS treatment, implicating that vitamin C deficiency itself causes pro-inflammatory state. I also demonstrated that both serum and tissue level of IL-6 in KO group were increased compared to other groups, and this reflects vitamin C deficiency state is associated with more severe systemic and local inflammation in a murine colitis model.

To evaluate innate immune response in colonic tissue, MPO activity was measured. In consistent with IL-6 results, MPO activity, as a parameter of neutrophil accumulation, was increased in KO group with DSS-induced colitis. This result suggests that vitamin C has an anti-inflammatory role in a murine colitis model. In addition, 3-NT, reflecting oxidative stress in tissue, was also increased in KO group when treated with DSS, while I could not

detect any 3-NT expression in WT and KO+VC groups. Increased oxidative stress was observed in various tumor tissues as well as inflammatory disease, and is known to be associated with DNA damage.[12] Increased expression of 3-NT in vitamin C deficient group treated with DSS might be associated with increased risk of colitis associated cancer in patients with longstanding and severe IBD, and further studies are warranted in a murine colitic cancer model.

IL-22 is a member of the IL-10 family, which are induced during chronic inflammation. It is proposed to protect the tissue damage during inflammation just like IL-10 via a STAT3-mediated mechanism. IL-22 has recently been shown to be protective during acute inflammation in a mouse colitis model.[13,14] I, therefore, measured the serum and tissue level of IL-22, and found that there was increased production of both serum and tissue IL-22 in WT and KO+VC groups when treated with DSS. However, DSS-induced production of IL-22 was impaired in KO group. Both serum and tissue levels of IL-22 in KO group were significantly lower than those of the other groups. These results suggest that vitamin C deficiency could lead to impaired production of colonic IL-22, which results in more severe colitis, and mechanisms involved should be sought in the future studies.

To summarize, vitamin C deficiency was associated with more severe DSS induced colitis, and was related to increased pro-inflammatory IL-6 production and oxidative stress, resulting in activated STAT3 signaling, and decreased production of anti-inflammatory IL-22. These results suggest potential role of vitamin C in management of IBD patients with anti-oxidant and anti-inflammatory effect, and further studies are warranted to elucidate

the underlying mechanism and to evaluate another possible role in colitis-associated cancer model.

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국문 초록

궤양성 대장염과 크론병으로 대별되는 염증성 장질환은 비정상적인 면역반응에 의해 만성적인 염증이 위장관에 반복적으로 지속되는 질환으로 아직 그 원인은 명확히 밝혀져 있지 않다. 염증성 장질환 환자에서 비정상적으로 reactive oxygen species (ROS)가 증가되어 있으며, 이는 염증성 장질환의 발병 및 진행에 기여할 것으로 알려져 있다. 비타민 C 는 항산화 효과 뿐만 아니라 항염증 효과도 있는 것으로 보고된다. 이에 비타민 C 를 합성하지 못하는 *gulo(-/-)* 마우스를 이용하여 dextran sulfate sodium (DSS)-유발 장염에서의 비타민 C 의 영향을 알아보고자 하였다. 5-6 주 령의 C57B/6 wild type 또는 *gulo(-/-)* 수컷 마우스를 이용하여, DSS 를 7 일간 음용수로 투여하여 급성 대장염을 유발하였다. 마우스는 DSS 투여 후 7 일 째 cytokine 의 변화를 보기 위한 혈액 채취를 하며, 희생한 후 대장을 적출하여 면역조직화학염색과 Immunoblotting 을 시행하였다. 비타민 C 결핍군에서는 DSS 투여 후 생존율이 감소하였으며, 장염에서의 회복 정도도 감소하였다. 비타민 C 결핍군에서 대조군에 비하여

조직학적으로 장상피세포의 탈락, 염증 세포의 침윤 등 더욱 심한 조직학적 염증 소견을 보였다. DSS 를 처치한 비타민 C 결핍군의 혈액과 대장 조직에서의 IL-6 농도가 비타민 C 보충군이나 정상 대조군에 비하여 높았다. 혈액과 대장조직에서의 IL-22 는 비타민 C 결핍군에서 유의하게 감소되었다. 비타민 C 결핍군에서 산화 스트레스와 연관된 3-nitrotyrosine 이 증가되었고, signal transducer and activator of transcription(STAT)3 의 인산화가 장상피세포층과 고유층에서 증가되었다. 결론적으로, 비타민 C 는 염증성 싸이토카인의 생성과 염증의 발현을 조절함으로써 DSS 유발 대장염을 약화시키는 효과를 나타내었다.

주요어 : 염증성 장질환, 비타민 C, 항 산화제

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