Glutamine Supplementation Attenuates Expressions of Adhesion Molecules and Chemokine Receptors on T Cells in a Murine Model of Acute Colitis

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1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn’s disease (CD) and ulcerative colitis (UC), is a relapsing and remitting disorder characterized by chronic inflammation of the gastrointestinal (GI) tract. The etiology of IBD remains unclear; however, both CD and UC are associated with enhanced leukocyte trafficking to the inflamed intestine [1, 2]. Previous studies indicated that effector T cells including CD4-positive T helper (Th) cells and CD8-positive cytotoxic T (Tc) cells play pivotal roles in the pathogenesis of mucosal lesions and chronic intestinal inflammation [3–5]. Blockage of lymphocyte migration to mucosal sites has therefore become a potential therapeutic strategy for IBD [6].

Lymphocytes migrate to specific tissues via a multistep process which is strictly regulated by adhesion molecules and chemokine receptors. Adhesion molecules present on the vascular endothelium and lymphocytes participate in the tethering, rolling, and adhesion of lymphocytes [7]. P-selectin and E-selectin appearing on activated endothelial cells interact with P-selectin glycoprotein ligand-1 (PSGL-1) expressed by lymphocytes, which promotes the initial tethering and subsequent rolling of lymphocytes over vessel walls. Integrins participate in rolling and firm adhesion of lymphocytes. Lymphocytes expressing α4β7 integrins roll on mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is required for homing of lymphocytes to intestinal sites [8]. Lymphocyte arrest mediated by integrins, such as lymphocyte function-associated antigen-1 (LFA-1) (also known as CD11a/CD18 and αLβ2 integrins) which interacts with their endothelial-cell ligands intercellular adhesion molecule-1 (ICAM-1), precedes extravasation into the underlying tissue [9].
Chemokines are small peptides which bind to chemokine receptors expressed on leukocytes and function as chemotactic agents. They regulate lymphocyte homing to secondary lymphoid organs and transmigration into tissues by forming a chemokine concentration gradient which attracts lymphocytes to move towards an increasing concentration [10]. Dysregulation of chemokines and chemokine receptors was implicated in various autoimmune diseases, including IBD [11,12]. Recent studies indicated that C-C chemokine receptor type 9 (CCR9) and α4β7 integrins are required for the localization of lymphocytes to the GI mucosa [13]. Agents developed for disrupting actions of CCR9 and α4β7 integrins showed promising results in IBD clinical trials [6].

Glutamine (Gln) is an immunomodulatory nutrient which is widely used in clinical practice [14]. Previous studies showed that Gln treatment has beneficial effects in different experimental models of colitis. Gln attenuates the expression of proinflammatory mediators [15] and improves outcomes which may be due to upregulation of heat shock proteins (HSPs) [16]. Recent work in our laboratory demonstrated that pretreatment with Gln suppresses cytokine expression of Th cells and ameliorates the severity of acute dextran sulfate sodium- (DSS-) induced colitis [17]. However, whether the beneficial effects of Gln are mediated by modulating lymphocyte trafficking in colitis is still unclear. Therefore, we investigated the influence of dietary Gln supplementation on T cell adhesion molecules and CCR9 expression in mice with DSS-induced acute colitis.

2. Materials and Methods

2.1. Study Protocols. Six-week-old male C57BL/6 mice were used in this study. Conventional mice were maintained in a temperature- and humidity-controlled room and were fed a standard chow diet ad libitum before the study. Care of laboratory animals was in full compliance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and protocols were approved by the Institutional Animal Care and Use Committee of Taipei Medical University.

2.2. Experimental Design. After 1 week of acclimation, 40 mice were randomly assigned to a control group or a Gln group in this study, with 20 mice in each group. Mice in the control group were fed a common semipurified diet, while the Gln group received a diet in which part of the casein was replaced with Gln. Gln provided 25% of the total amino acid nitrogen. This amount of Gln was proven to have an immunomodulatory effect in rodents [18–20]. Two diets were formulated to be isonitrogenous and isoenergetic (Table 1). After 5 d of being fed the diets, mice in the control and Gln groups were further divided into 2 respective subgroups. One subgroup was given distilled water, while the other subgroup received 1.5% (wt/vol) DSS (MW 40 kDa; MP Biomedicals, Solon, OH, USA) in the drinking water for 5 d to induce colitis. A flow diagram of the study design is shown in Figure 1. There were 4 groups in this study: control diet with distilled water (C group), Gln diet with distilled water (G group), control diet with DSS water (DC group), and Gln diet with DSS water (DG group). The respective experimental diets were given during the DSS exposure period. Body weights (BW) were recorded daily, and all mice had free access to food and water throughout the study. At the end of the experiment, mice were anesthetized and sacrificed by cardiac puncture. Fresh blood samples were collected in heparinized tubes for measurements of the leukocyte population. Mesenteric lymph nodes (MLNs) were removed and processed for further analysis by flow cytometry. The colon was cut close to the ileocecal valve, and its length and weight were measured. Sections (1 cm) of the distal colon were cut. Colon tissues were fixed with buffered 4% paraformaldehyde for an immunohistochemical analysis.

2.3. Blood Leukocyte Distribution. A five-color flow cytometric analysis was performed to determine the distribution of peripheral blood leukocytes. Antibodies against mouse leukocyte surface antigens were added to 100 μL aliquots of whole blood. The antibodies used to detect different subsets of leukocytes were as follows: PerCP-conjugated anti-CD45 (Biolegend, San Diego, CA, USA) for leukocytes, PE-conjugated anti-F4/80 (eBioscience, San Diego, CA, USA) for

| Component          | Control diet | Gln diet |
|--------------------|--------------|----------|
| Soybean oil        | 100          | 100      |
| Casein             | 200          | 150      |
| Glutamine          | 0            | 41.7     |
| Salt mixture<sup>a</sup> | 35            | 35       |
| Vitamin mixture<sup>b</sup> | 10            | 10       |
| Methyl cellulose   | 31           | 31       |
| Choline bitartrate | 2.5          | 2.5      |
| Methionine         | 3            | 3        |
| Corn starch        | 626.8        | 618.5    |

<sup>a</sup>The salt mixture contained the following (mg/g): calcium phosphate dibasic: 500; sodium chloride: 74; potassium sulfate: 52; potassium citrate monohydrate: 20; magnesium oxide: 24; manganese carbonate: 3.5; ferric citrate: 6; zinc carbonate: 1.6; cupric carbonate: 0.3; potassium iodate: 0.01; sodium selenite: 0.01; and chromium potassium sulfate: 0.55.

<sup>b</sup>The vitamin mixture contained the following (mg/g): thiamin hydrochloride: 0.6; riboflavin: 0.6; pyridoxine hydrochloride: 0.7; nicotinic acid: 3; calcium pantothenate: 1.6; D-biotin: 0.05; cyanocobalamin: 0.001; retinyl palmitate: 1.6; DL-α-tocopherol acetate: 20; cholecalciferol: 0.25; and menaquinone: 0.005.

![Figure 1: Flow diagram of the study design.](image-url)
monocytes/macrophages, FITC-conjugated anti-Ly6G (BD Biosciences, San Jose, CA, USA) for neutrophils, APC-conjugated anti-CD3ε (eBioscience) for T cells, and Pacific blue-conjugated anti-CD19 (Biologend) for B cells. Antibodies were used at the concentration recommended by manufacturer. After a 30 min incubation at 4°C in the dark, red blood cells were lysed, and cells were suspended in staining buffer and then analyzed with a FACS Canto II flow cytometer (BD Biosciences). CD45-positive cells were gated, and results are presented as a percentage of specific CD-marker-expressing cells in blood leukocytes. Representative flow cytometry plots are shown in Figure 2(a).

2.5. Expressions of Adhesion Molecules and Chemokine Receptors by T Cells. Whole blood and MLNs were obtained by passing the tissues through a nylon cell strainer with a 40 μm pore size (BD Biosciences) in RPMI1640 medium (Biological Industries, Kibbutz Beit Haemek, Israel). After centrifugation at 300×g for 10 min, pelleted MLN cells were suspended in 1 mL of staining buffer. One hundred microliters of cell suspension was incubated with APC-conjugated anti-CD3ε (eBiosience) and Pacific blue-conjugated anti-CD19 (Biologend) for 30 min at 4°C in the dark. Stained cells were washed and resuspended in staining buffer to measure the lymphocyte population by flow cytometry. Percentages of T and B lymphocytes were determined by CD3ε- and CD19-expressing cells in MLN cells. Representative flow cytometry plots are shown in Figure 2(b).

2.6. RNA Extraction and Real-Time PCR. Total RNA was isolated from colon tissue using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA (1 μg) was reverse-transcribed with a complementary (c)DNA synthesis kit (Fermentas, Glen Burnie, MD, USA) according to standard protocols. For real-time PCR, 5 μL of 1/10 diluted cDNA was amplified in a 25 μL PCR volume containing 12.5 μL of 2X SYBR green master mix reagent (Applied Biosystems, Foster City, CA, USA). The reaction was performed with ABI 7300 Real-Time PCR System (Applied Biosystems) according to the thermocycling protocol recommended by the PCR system. Primer sequences were as follows: mouse ICAM-1 (5'-AGCACCTCCCCCCCTACTTTT-3' and 5'-AGCTTGACAGCCCTTCTAA-3'), mouse P-selectin (5'-TCCAGGAAGCTCTACTCGTTACTG-3' and 5'-GCAGGTTAGTGGAAGACTCGATGT-3'), mouse E-selectin (5'-TGAACTGAGGTAACAGGAAGCACTCGAT-3' and 5'-GCCAGGGGACATCATCACAT-3'), and mouse 18S rRNA (5'-GGCGTTCTATTTTGTGTTGTG-3' and 5'-AGTCGGCATCGTTATATGTC-3'). All samples were analyzed in triplicate, and fold change for each target gene was calculated by the equation 2−ΔΔCt (ΔΔCt indicates the difference in threshold cycles between the test gene and 18S rRNA, and ΔΔCt indicates the difference of ΔCt between the experimental and C groups).

2.7. Immunofluorescence Staining. Double-staining combinations CD3-CD4 and CD3-CD8 were performed on 5 μm paraffin-embedded colon sections. After antigen retrieval, sections were incubated with an antibody against CD3ε (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and amplified with a rabbit anti-goat immunoglobulin G (IgG) secondary antibody conjugated with FITC (Santa Cruz Biotechnology). For colocalization, sections were then costained overnight at 4°C with secondary antibodies against CD4 (Abcam, Cambridge, UK) or CD8 (Novus Biologicals, Littleton, CO, USA) and amplified with the respective appropriate secondary antibodies: goat anti-mouse IgG or goat anti-rabbit IgG conjugated with rhodamine (Santa Cruz Biotechnology). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO, USA) for 10 min at room temperature. Digital images at 400x magnification per section were acquired using appropriate filters of a Zeiss AxioPhot fluorescence microscope (Carl Zeiss MicroImaging LLC, Thornwood, NY, USA) fitted with a Nikon DIX digital camera (Tokyo, Japan). Cells containing both FITC and rhodamine labels appeared yellow. These images were then overlaid with DAPI-staining images to determine the infiltration of T lymphocyte subpopulations in the colon mucosa.

2.8. Statistical Analysis. All data are expressed as the mean ± standard error of the mean (SEM). Differences among groups were analyzed by an analysis of variance (ANOVA) with Tukey’s test. A two-way ANOVA with Bonferroni correction was used to analyze differences in BW changes. A P value of <0.05 was considered statistically significant.

3. Results

3.1. BW and Weight/Length Ratio of the Colon. Initial BWs ranged 21–25 g and did not differ among the 4 groups. There was no significant difference in BWs during the study between the C and G groups. At 4 d (d9) and 5 d (d10) after DSS administration, weight loss was observed in the DG group compared to the C group, whereas mice with Gln supplementation maintained their BWs during the DSS exposure period. At the end of the study, BWs were significantly higher in the DH group than the DC group (Figure 3(a)).
Figure 2: Representative flow cytometry plots. Blood leukocytes (a) were defined by gating on CD45-positive cells. The percentage of Ly6G-positive neutrophils from an individual representative mouse per group is listed. For analyzing the lymphocyte population in MLNs (b), MLN cells were first gated to exclude debris. Numbers indicate the percentage of CD3ε-positive lymphocytes in MLN cells. For analyzing the expression of adhesion molecules and chemokine receptors by T cells (c), lymphocytes were first identified based on low FSC and SSC characteristics. CD4- or CD8-positive lymphocytes were gated to analyze the percentages of adhesion molecule- and chemokine receptor-expressing CD4^+ and CD8^+ lymphocytes. Representative dot plots of leukocytes in blood are shown.
The weight/length ratio of the colon, an indicator of colonic edema, was significantly higher in the colitis groups than the C group (Figure 3(b)). Treatment with Gln attenuated colonic edema associated with DSS-induced inflammation.

3.2. Leukocyte Populations in Blood and MLNs. There was no significant difference in blood or MLN leukocyte subpopulations between the C and G groups. Compared to the C group, the DSS colitis groups had a higher percentage of blood neutrophils and lower T-cell population in MLNs. DSS exposure did not alter the blood monocyte and lymphocyte distributions. Also, subsets of effector T cells in the blood and MLNs did not change. Gln supplementation had no influence on blood leukocyte or MLN lymphocyte populations in normal or colitic mice (Table 2).

3.3. Adhesion Molecule and CCR9 Expressions by Th Cells. Percentages of adhesion molecules and CCR9 expressed by blood and MLN Th cells did not differ between the C and G groups. DSS administration resulted in higher PSGL-1, CD11a (LFA-1 αL subunit), and CCR9 expressions by Th cells in both blood and MLNs, whereas no difference in α4β7 integrin expression was detected among the control and DSS groups. Mice in the DG group had lower percentages of PSGL-1−, CD11a−, and CCR9−expressing Th cells in blood (Figures 4(a)−4(d)) and MLNs (Figures 5(a)−5(d)). The expression level of CCR9 on α4β7−positive Th cells was also suppressed in the DG group (Figures 4(e) and 5(e)).

3.4. Adhesion Molecule and CCR9 Expressions by Tc Cells. No differences in adhesion molecules and CCR9 expressed by blood and MLN Tc cells were observed between the C and G groups. The DSS colitis groups had higher percentages of PSGL-1−, CD11a−, and CCR9−expressing blood and MLN Tc cells, whereas expression levels of α4β7 integrins did not differ among the 4 groups. Compared to the DC group, the DG group had lower expression of CD11a by blood (Figures 6(a)−6(d)) and MLN Tc cells (Figures 7(a)−7(d)). There was no difference in expression levels of CCR9 on α4β7−positive Tc cells between the 2 DSS colitis groups (Figures 6(e) and 7(e)).
3.5. Gene Expression of Endothelial Adhesion Molecules in Colon Tissues. There was no difference in mRNA levels of ICAM-1, P-selectin, and E-selectin between the C and G groups in colon tissues. DSS-induced colitis greatly upregulated the adhesion molecule genes expressed by activated endothelial cells. Compared to the DC group, the expression levels of ICAM-1, P-selectin, and E-selectin mRNA were suppressed in the DG group (Figure 8).

3.6. T Lymphocyte Subsets in the Colon Mucosa. CD3 is the cell surface marker of T cells. CD3 and CD4 double-positive cells are considered Th cells, whereas Tc cells coexpress CD3.
and CD8. As shown in Figure 9, the immunoreactive intensity of Th cells was higher in the DC group than the DG group. However, intensities of Tc cell populations did not differ between the DC and DG groups (Figure 10).

4. Discussion

DSS is a heparin-like polysaccharide which results in acute chemical toxicity that disrupts the intestinal epithelial cell
Figure 6: Percentage of adhesion molecule- and chemokine receptor-expressing cytotoxic T (Tc) cells in blood. CD8-positive blood lymphocytes were gated to analyze expressions of PSGL-1, α4β7 integrins, CD11a, and CCR9 by flow cytometry ((a)–(d)). (e) Expression of CCR9 by CD8 and α4β7 integrin double-positive blood lymphocytes. Values are shown as the mean ± SEM. *Significantly different from the C group (P < 0.05). †Significantly different from the DC group (P < 0.05).

Recent studies indicated that DSS-induced colitis is characterized by extensive crypt and epithelial cell damage with ulceration, tissue edema, and infiltration of immune cells predominantly in the distal colon that mimics the histological features of UC [22]. Recent studies indicated that DSS-induced morphological and biochemical damage also extends to the small intestines [23]. Susceptibilities to DSS-induced colitis differ in various inbred mouse strains [24]. A single cycle of DSS exposure to the C57BL/6 strain was found to develop acute colitis which later proceeds to chronic inflammation, and T cell
migration to the colon plays an important role in the progression to chronicity [25]. Regarding the high sensitivity to DSS, C57BL/6 mice were used in this study to analyze the consequences of DSS exposure on adhesion molecules and chemokine receptors involved in T cell trafficking to the intestines.

IBD is associated with a massive influx of immune cells into the gut. Previous studies indicated that increased local...
secretion of proinflammatory cytokines by the inflamed colon leads to upregulated expressions of vascular adhesion molecules, resulting in a sustained influx of inflammatory cells, and the data were presented as the fold change in gene expression relative to the calibrator. Values are shown as the mean ± SEM. *Significantly different from the C group (P < 0.05). †Significantly different from the DC group (P < 0.05).

**Figure 8:** Gene expression of endothelial adhesion molecules in colon tissues. mRNA levels were analyzed by a real-time PCR. The C group was used as a calibrator, and the data were presented as the fold change in gene expression relative to the calibrator. Values are shown as the mean ± SEM. *Significantly different from the C group (P < 0.05). †Significantly different from the DC group (P < 0.05).
competes as a Gln source with Tc cells. Further studies are needed to investigate the molecular mechanisms involved in gene expressions of adhesion molecules and CCR9 regulated by Gln.

In conclusion, this study showed for the first time that pretreatment with oral Gln reduced adhesion molecule- and CCR9-expressing T cells induced by DSS exposure. The inhibitory abilities against adhesion molecule and CCR9 expressions were more obvious in Th cells than Tc cells. Also, Gln supplementation reduced gene expressions of endothelial adhesion molecules in colons, prevented BW loss, and attenuated colon edema in colitic mice. Our results imply that dietary Gln prevented Th cell trafficking into colon tissues and provide a new mechanism of Gln supplementation that has beneficial effects on ameliorating the severity of acute DSS-induced colitis.

Conflict of Interests

The authors declare that they have no conflict of interests.
**Figure 10:** Representative immunofluorescence images of cytotoxic T cells. In the left column, cell surface antigens were stained for CD3 (green, FITC), and the staining of CD8 cells (red, rhodamine) is illustrated in the middle column. The nucleus is stained with DAPI (blue), and the last column presents a colocalized fluorescence image of CD3⁺CD8⁺ T cells.

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