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Glutamine protects mice from acute graft-versus-host disease (aGVHD)

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ABSTRACT

Despite current immunosuppressive therapies, acute graft-versus-host disease (aGVHD) is a major cause of morbidity and mortality in allogeneic hematopoietic stem cell transplantation (HSCT). In the present study, therapeutic effects of intraperitoneal glutamine (Gln) administration (1 g/kg/day) in a mouse aGVHD model were evaluated. Gln administration significantly inhibited the GVHD-induced inflammation and tissue injury in the intestine, liver, skin and spleen. Gln therapy improved the score of clinical evidence of aGVHD and prolonged the median survival of aGVHD mice. Gln administration in aGVHD mice increased the fraction of Foxp3+/CD4+/CD25+ cells in the blood measured on day 7, and decreased the serum levels of tumor necrosis factor-α measured on days 7, 14 and 21 after aGVHD induction. These results demonstrated that Gln administration may be useful in protecting the host from aGVHD.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an important treatment modality in the management of a number of malignant and nonmalignant hematopoietic disorders [1]. Unfortunately, the utility of HSCT is limited by transplant-related complications, including graft-versus-host disease (GVHD) [1]. Currently available posttransplantation immunosuppressive therapies are insufficient and GVHD remains a major cause of morbidity and mortality in allogeneic HSCT [1].

The pathophysiology of aGVHD is believed to be a multistep process. The initial step involves the development of an inflammatory milieu resulting from damages to host tissues induced by preparative chemotherapy or radiotherapy. Damaged tissues secrete inflammatory cytokines, including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin (IL)-1 [2–4]. In the second step, antigen-presenting cells from both the recipient and the donor, in concert with inflammatory cytokines, trigger the activation of donor-derived T cells, which expand and differentiate into effector T cells [4]. In the third step, activated donor T cells mediate cytotoxicity targeted towards host tissues through Fas–Fas ligand interactions [5–7], perforin–granzyme B [5], and the production of cytokines, such as TNF-α [8]. aGVHD predominantly affects the skin, upper and lower gastrointestinal tracts, liver, and occasionally the eyes and oral mucosa [1].

The treatment and prevention of aGVHD is based on disruptions of this multistep pathophysiologic phenomenon. aGVHD has been usually treated with corticosteroid, cyclosporine, tacrolimus, mycophenolate mofetil, antithymocyte globulin and modulating agents of TNF-α or IL-2 [1]. However, many patients with aGVHD remain refractory to these agents and are associated with increased mortality [1]. Thus, the exploration of alternative approaches for aGVHD treatment is needed.

Glutamine (Gln) is the most abundant free amino acid in the body [9]. Although it is a non-essential amino acid, Gln is a preferred energy fuel for cells with rapid turnover such as lymphocytes and enterocytes [10,11]. Gln plays an important role in nitrogen- and carbon-skeleton exchanges among different tissues,

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where this amino acid fulfills many different physiological functions [12]. Gln has immune modulating activities including the inhibition of proinflammatory cytokine release, and the attenuation of depletion of glutathione synthesis [13,14]. Gln has been shown to improve survival, and to enhance immune and gut barrier functions in animal models of endotoxin shock [15]. Gln was also found to improve allergic bronchial inflammation through the suppression of Th2-dependent phenotypes [16]. Clinical trials in human subjects have also demonstrated that Gln treatment is safely used in a number of patient populations [17].

The immune modulating function and the safety in clinical use of Gln led us to investigate the effect of Gln on aGVHD using a mouse model.

2. Materials and methods

2.1. Animals

Specific pathogen-free C57BL/6J (H-2^b) and BALB/c (H-2^d) mice were purchased from Damul Animal Center (Daejon, Korea) and housed at the Chonbuk National University Hospital Animal Care Facility (Chonbuk, Korea). (C57BL/6J × BALB/c) F1 (H-$2^{b/d}$) mice were bred in the facility, and were used for experiments between the age of 6 and 8 weeks. Mice were maintained under guidelines established by the Chonbuk National University Hospital Animal Care Committee, which also approved experimental protocols. The investigation conformed to EU Directive 2010/63/EU for animal experiments. Mice were age and sex matched at the onset of each experiment. All experiments described in this work were performed at least three times with highly concordant results.

2.2. Acute GVHD (aGVHD) model

Splenocytes were obtained from of BALB/c (H-2^d) mice as previously described [18] and suspended at 1×10^8 cells/0.5 mL in phosphate-buffered saline (PBS). aGVHD was induced by transferring the splenocytes (1×10^8 cells/mouse) of BALB/c mice (H-2^d) into the tail vein of (C57BL/6J × BALB/c) F1 (H-$2^{b/d}$) mice. Recipient mice were treated with 900 cGy total body irradiation (TBI) within 4–8 h before the splenocyte injection. TBI was administered in a single dose using a Shepherd Mark I cesium irradiator (Shepherd, San Fernando, CA). The dose rate was 83.3 cGy/min.

2.3. Administration of glutamine and measurement of survival

Glutamine (Fluka, Steinhein, Germany) dissolved in distilled water (0.2 ml/mouse/injection) was administered by intraperitoneal injection once a day from day 0 to day 21 after the splenocyte infusion (aGVHD induction). Survival of the mice was measured by observing the cage daily beginning from the day of aGVHD induction. Clinical evidence of aGVHD was evaluated and scored as previously described [19].

2.4. Histologic evaluation

Mice were euthanized with CO_2 gas, and the small intestine, liver, abdominal skin and spleen were isolated on day 21 after the donor splenocyte infusion. The isolated tissues were fixed by immersion in 4% paraformaldehyde and blocked in paraffin for histologic study. Tissue sections (5 μm in thickness) were deparaffinized with xylene and rehydrated with graded ethanol. The sections were stained with hematoxylin and eosin. Tissue sections were screened by an examiner blinded to the treatment received by each animal. To facilitate a semiquantitative assessment of aGVHD, grading of aGVHD was confined to analysis of the liver. Inflammatory cell infiltrates in the portal tract and around the central veins were graded on a scale of 1 to 5 reflecting Grade 1: normal or minimal perivascular cuffing, Grade 2: perivascular cuffing, 1–2 cells in thickness, involving up to 10% of vessels, Grade 3: perivascular cuffing, 1–4 cells in thickness, involving 10–30% of vessels, Grade 4: perivascular cuffing, 3–6 cells in thickness, involving 30–50% of vessels, Grade 5: perivascular cuffing, greater than seven cells in thickness, involving greater than 50% of vessels.

2.5. FACS analysis

The following monoclonal antibodies were used for flow cytometric analysis: anti-CD4 FITC (BD Biosciences, San Diego, CA, USA), anti-CD8 FITC (eBioscience, San Diego, CA, USA), anti-B220 PE (eBioscience), anti-CD25 PE (BD Biosciences), anti-Foxp3 PE (BD Biosciences), and anti-H-2^b allophycocyanin (BD Biosciences).

Blood samples were harvested by retroorbital puncture on days 7, 14 and 21 after aGVHD induction, and briefly exposed to Tris-buffered 0.16 M ammonium chloride to remove red blood cells. Remained cells were used for FACS analysis as previously described [18].

2.6. Measurement of cytokines

Blood samples (0.5 ml) were harvested by retroorbital puncture on days 7, 14 and 21 after the splenocyte infusion. Samples were centrifuged at 500 *g* for 10 min in order to obtain the serum, and frozen at −70 °C until experimental uses. The levels of TNF-α and IFN-γ were measured as previously described [18].

2.7. Statistical analysis

Data were expressed as mean ± SD. Statistical comparisons between groups were conducted with the unpaired *t* test using GraphPad Prism version 5 (GraphPad Software, USA) unless otherwise specified. Survival distributions were estimated by the Kaplan–Meier method, and compared using the log-rank test. Experimental outcomes of six separate experiments were similar and experimental results were therefore pooled. A *p* value of 0.05 or less was considered statistically significant for the treatment comparisons.

3. Results

3.1. Effect of Gln therapy on histologic severity of GVHD

We first investigated to find out the effective therapeutic dose of Gln in aGVHD mice. Three different doses of Gln (0.5, 1, and 2 g/kg body weight of mouse) were administered to the corresponding groups of mice (ten mice/group) through once-daily intraperitoneal injection beginning from the day of aGVHD induction. Normal (non-aGVHD) mice and untreated aGVHD mice served as controls. Following 21 days of Gln treatment, aGVHD-induced perivascular infiltrations of inflammatory cells (aGVHD-induced pathologic changes) were analyzed in hematoxylin and eosin-stained liver tissue sections using a semiquantitative scoring system (pathologic grade). To facilitate to find out the appropriate therapeutic dose of Gln, the analysis of pathologic grades of aGVHD was confined to the liver in this experiment. Treatment with 1 g/kg Gln appeared to improve the pathologic grades of aGVHD in the liver better than treatment with 0.5 g/kg Gln, whereas treatment with 2 g/kg resulted in a similar improvement of aGVHD pathologic grades as compared with treatment with 1 g/kg Gln (Fig. 1). We therefore chose 1 g/kg Gln as the optimal therapeutic dose, and used only three groups [normal control (non-aGVHD) group,

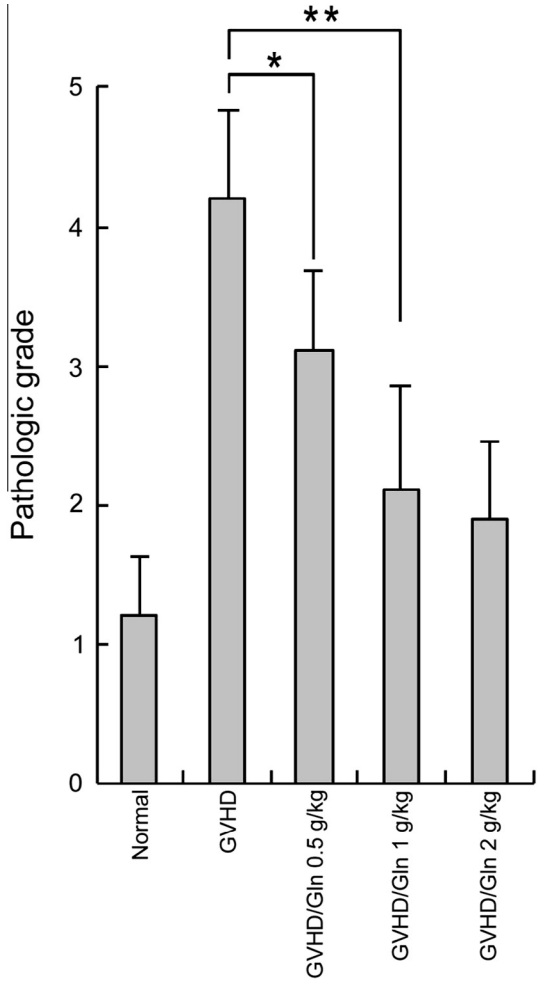

Fig. 1. Effect of glutamine on aGVHD in the liver. aGVHD was induced by injecting BALB/c splenocytes (1×10^8 cells/mouse) (H-2^d) into the tail vein of irradiated (900 cGy) (C57BL/6J × BALB/c) F1 mice (H-$2^{b/d}$). Three different doses of Gln (0.5, 1, and 2 g/kg body weight of mouse) were administered to the corresponding groups of mice (ten mice/group) through once-daily intraperitoneal injection beginning from the day of aGVHD induction. On day 21 after aGVHD induction, the liver was harvested and aGVHD-induced perivascular infiltrations of inflammatory cells were analyzed in hematoxylin and eosin-stained tissue sections using a semiquantitative scoring system (pathologic grade). Normal non-aGVHD mice and untreated aGVHD mice served as controls. Results were presented as mean ± SD. $^*p < 0.05$,$^{**}p < 0.01$.

untreated aGVHD group, and Gln (1 g/kg)-treated aGVHD group] in the following experiments.

Histologic changes of the small intestine, liver, skin and spleen were analyzed on day 21 after aGVHD induction in the presence or absence of Gln treatment. Induction of aGVHD caused inflammatory cell infiltrations and target tissue destructions in all the analyzed organs. Granulomatous changes were additionally observed in the spleen. In contrast, Gln treatment inhibited the aGVHD-induced inflammatory cell infiltrations and tissue destructions (Fig. 2).

3.2. Effect of Gln therapy on aGVHD-related morbidity and mortality

In order to evaluate the effect of Gln administration on the severity of aGVHD and survival of aGVHD mice, six consecutive experiments were performed using two animal groups [untreated aGVHD, and Gln (1 g/kg/day)-treated aGVHD] (five mice/group in each experiment). Because the results were similar in each experiment, they were pooled for analysis. The aGVHD score was significantly reduced in mice receiving Gln compared with untreated aGVHD mice (Fig. 3A). Gln therapy also prolonged the median survival of aGVHD mice from 13.0 days to 34.0 days ($p < 0.001$) (Fig. 3B).

3.3. Effect of Gln therapy on serum levels of TNF-α and IFN-γ in aGVHD mice

Serum levels of TNF-α and IFN-γ in aGVHD mice were measured in the presence or absence of Gln therapy on days 7, 14 and 21 after aGVHD induction. Serum levels of TNF-α were increased and maintained at similar levels throughout the test periods. As shown in Fig. 4A, serum levels of TNF-α were significantly decreased in Gln-treated (1 g/kg/day) aGVHD mice compared to untreated aGVHD mice on days 7, 14 and 21. In contrast, serum levels of IFN-γ were significantly increased only on day 7 in aGVHD mice compared to normal mice, but markedly decreased on days 14 and 21. There were no significant differences in serum levels of IFN-γ between untreated and Gln-treated aGVHD mice.

3.4. Effect of Gln therapy on immunophenotypic changes of blood lymphocytes in aGVHD mice

We examined the fractional difference between donor and host-derived blood leukocytes on days 7, 14 and 21 after aGVHD induction in the presence or absence of Gln therapy. Since most of blood leukocytes were observed to be donor-derived (>95%, data not shown) at those time points, we therefore analyzed immunophenotypes without determination of donor vs. host origin in the following experiments. As shown in Fig. 4B, CD4+/CD25+ cells and Foxp3+/CD4+/CD25+ cells were significantly increased in Gln-treated aGVHD mice compared with untreated aGVHD mice on day 7, but no significant differences were observed in their fractions between untreated and Gln-untreated aGVHD mice on days 14 and 21. There were no differences in the fractions of CD4+, CD8+, and B220+ cells between untreated and Gln-treated aGVHD mice on days 7, 14 and 21. Compared to the fractions on day 7, fractions of CD4+/CD25+, Foxp3+/CD4+/CD25+, CD4+ and CD8+ cells significantly decreased on days 14 and 21. In contrast, the fraction of B220+ cells significantly increased on days 14 and 21 compared to their fractions on day 7.

In parallel experiments, the total blood leukocyte count was measured. Gln treatment did not affect the total leukocyte count of aGVHD mice, although wide range was observed (data not shown), indicating that the fractional changes of the immunophenotypic subsets were not due simply to the fractional changes of other subsets.

4. Discussion

The principal target organs of aGVHD are the intestine, liver, skin and immune system [20,21]. We found that Gln therapy significantly reduced aGVHD in the small intestine, liver, skin and spleen. The spleen is one of the largest extramedullary hematopoietic organ and an important peripheral lymphoid organ playing a role as a source of peripheral T lymphocytes [22]. In aGVHD mice, the normal architecture was severely destructed and granulomatous changes were apparent in the spleen. Treatment with Gln resulted in significant improvements of these inflammatory and granulomatous changes, suggesting that Gln might protect the immunologic injury of the spleen from aGVHD. We examined the effect of Gln on aGVHD at 21 days after the injection of donor splenocytes. The timing of this protocol was chosen on the basis of previous studies demonstrating that a peak of donor cell engraftment was observed between 2 and 3 weeks after donor splenocyte injection in the GVHD model [23,24].

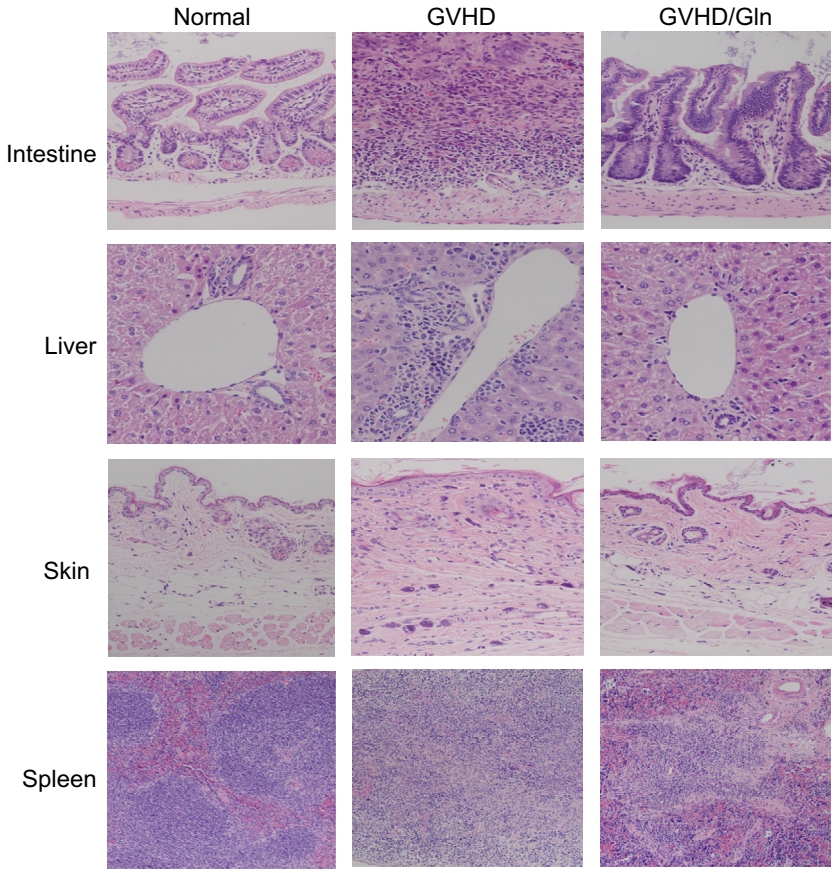

Fig. 2. Effect of glutamine on aGVHD pathology. aGVHD was induced by injecting BALB/c splenocytes (1×10^8 cells/mouse) (H-2^d) into the tail vein of irradiated (900 cGy) (C57BL/6J × BALB/c) F1 mice (H-$2^{b/d}$). Glutamine (1 g/kg/day) was administered intraperitoneally on days 0–21 after aGVHD induction. Target tissues were harvested and evaluated with hematoxylin-eosin staining on day 21 after aGVHD induction. Normal non-aGVHD mice and untreated aGVHD mice served as controls. Representative results of three separate experiments were presented (five mice/group in each experiment). Original magnification, ×400.

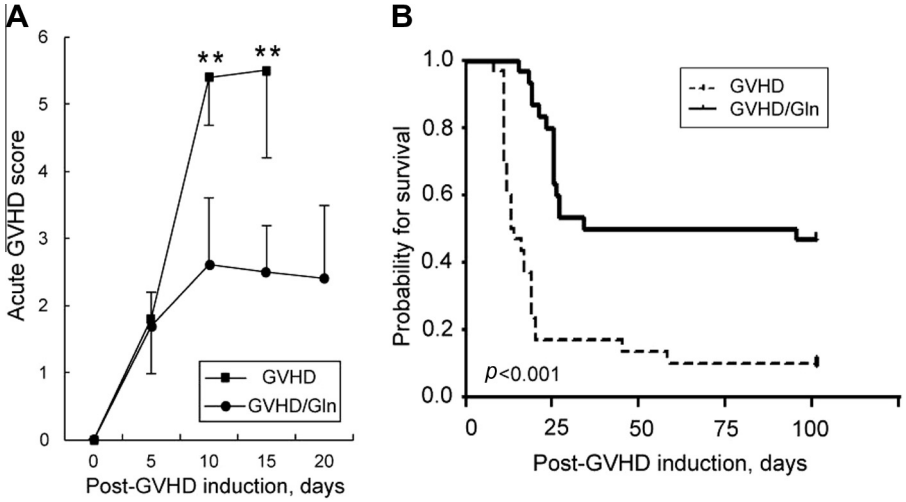

Fig. 3. Effect of glutamine on acute GVHD score and survival of aGVHD mice. aGVHD was induced by injecting BALB/c splenocytes (1×10^8 cells/mouse) (H-2^d) into the tail vein of irradiated (900 cGy) (C57BL/6J × BALB/c) F1 mice (H-$2^{b/d}$). Glutamine (1 g/kg/day) was administered intraperitoneally on days 0–21 after aGVHD induction. Acute GVHD score was evaluated on days 5, 10, 15 and 20 (A). Untreated aGVHD mice served as controls. Thirty mice per group pooled from 6 separate experiments were analyzed. Results are presented as mean ± SD. $^{**}p < 0.01$. In parallel experiments, survival was measured by the Kaplan-Meier method (B).

Previous studies using HSCT animal models have showed critical roles of inflammatory cytokines in the development of aGVHD [2–4]. Induction of aGVHD has been known to cause synthesis of pro-inflammatory cytokines including TNF-α and IFN-γ [25]. In the present study, serum levels of TNF-α were increased and maintained at similar levels throughout the test periods. Gln treatment resulted in a significant decrease in serum levels of TNF-α in aGVHD mice during the periods. These results suggested that Gln might inhibit inflammatory changes, and improve aGVHD by at least partly decreasing the production of TNF-α. In contrast, serum levels of IFN-γ were significantly increased on day 7 after the induction of aGVHD, but markedly decreased on days 14 and 21.

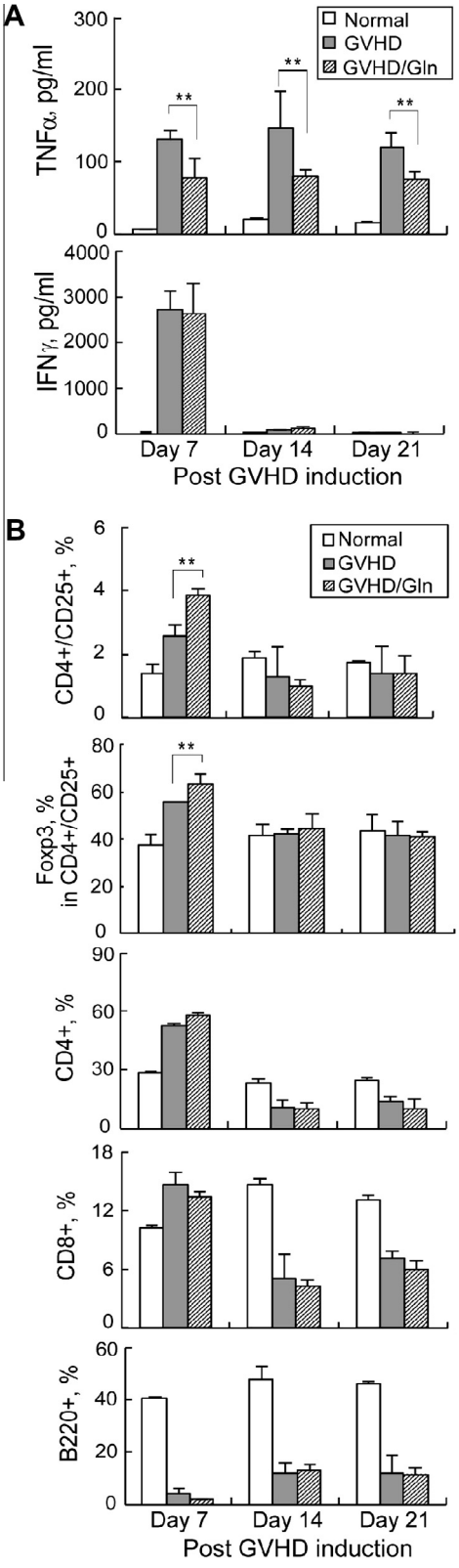

Fig. 4. Effect of glutamine on serum levels of cytokines and blood leukocyte immunophenotypic changes in aGVHD mice. aGVHD was induced by injecting BALB/c splenocytes (1×10^8 cells/mouse) (H-2^d) into the tail vein of irradiated (900 cGy) (C57BL/6J × BALB/c) F1 mice (H-$2^{b/d}$). Glutamine (1 g/kg/day) was administered intraperitoneally on days 0–21 after aGVHD induction. Serum levels of cytokines and blood leukocyte immunophenotypes were measured on days 7, 14 and 21 after aGVHD induction. Normal non-aGVHD mice and untreated aGVHD mice served as controls. Representative results of three separate experiments were presented (five mice/group in each experiment). Data are expressed as mean ± SD. $^{**}p < 0.01$.

Gln treatment had no effects on IFN-γ levels throughout the test periods. The significance of these differential effects of Gln between TNF-α and IFN-γ levels remains to be further studied. The Gln-induced inhibition of TNF-α production is consistent with the previous findings that Gln supplementation decreases the production of some pro-inflammatory cytokines which may be associated with the inhibition of nuclear factor-κB and/or the inhibition of p38 mitogen-activated protein kinase (MAPK) [17]. Gln-induced immune modulations were also suggested to be related with the increased induction of heat shock protein expression, and the inhibition of cytosolic phospholipase A2 through the inactivation of p38 and JNK via the induction of MAPK phosphorylase-1 [16,17,26].

Donor-derived T cells have been known to be the most important immune effector cells during the induction and propagation of aGVHD [1]. Regulatory T cells which are positive for CD4/CD25 or Foxp3/CD4/CD25 can modulate GVHD by regulating donor-derived T cells [1]. CD4+/CD25+ cells and Foxp3+/CD4+/CD25+ cells significantly increased in Gln-treated aGVHD mice on day 7 after aGVHD induction. This result suggests that Gln may inhibit inflammatory changes, and improve aGVHD by increasing the numbers of these cells. However, no significant differences were observed in their fractions between untreated and Gln-treated mice on days 14 and 21 after aGVHD induction, suggesting that the effect of Gln on these cells is restricted to the earlier phase of aGVHD induction. Compared to immunophenotypic changes on day 7, the fractions of CD4+/CD25+, Foxp3+/CD4+/CD25+, CD4+ and CD8+ cells significantly decreased in contrast with the increased fractions of B220+ cells on days 14 and 21 after aGVHD induction. Although the significance of these time-dependent changes remains unclear, the results suggested that immunologic reactions involving these cells may be more important during the earlier phase of aGVHD. Gln treatment had no effect on the fractional changes of CD4+, CD8+ and B220+ cells on days 7, 14 and 21 after aGVHD induction. Further evaluation is needed to elucidate the significance of the results.

The present study indicated that Gln administration improved aGVHD. The protective effect of Gln is suggested to be associated with the fractional increase in blood CD4+/CD25+ and Foxp3+/CD4+/CD25+ cells and the reduction of serum TNF-α. These findings suggest that the administration of Gln in aGVHD may become a novel strategy to reduce the severity and to improve the outcome of aGVHD. Since Gln has cytoprotective effects via various mechanisms including the enhanced synthesis of endogenous antioxidant glutathione [17], this function may also contribute to the improvement of aGVHD-induced tissue injuries.

A number of studies have shown that parenteral administration of Gln as a component of supportive nutritional therapy is safe and improves clinical outcomes in critically ill patients [27–29]. Although Gln can be produced in sufficient quantities under stable conditions, it becomes a limiting amino acid during metabolic stresses, which are associated with higher susceptibility to infections and impairment of the immune response [30–32]. Therefore, it is likely that a decrease in the intracellular concentration of Gln occurs during aGVHD. Supplementation with Gln may correct such abnormalities. In this regard, the clinical use of Gln in patients with aGVHD may be beneficial.

Further works are required to elucidate mechanisms by which Gln administration improves aGVHD, and to clarify the exact role of Gln-induced changes of CD4+/CD25+ and Foxp3+/CD4+/CD25+ cell fractions and serum TNF-α levels in controlling aGVHD.

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