Original Article

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# 5-Aminolevulinic acid combined with sodium ferrous ameliorated liver injury in a murine acute graft-versus-host disease model by reducing inflammation responses through PGC-1α activation

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**SUMMARY** Acute graft-versus-host disease (aGvHD) remains lethal as a life-threatening complication after allogeneic hematopoietic stem cell transplantation (HSCT). Inflammatory responses play an important role in aGvHD. 5-Aminolevulinic acid combined with sodium ferrous citrate (5-ALA/SFC) has been widely reported to have a major effect on the anti-inflammatory response; however, these effects in aGvHD models have never been reported. In this study, a murine aGvHD model was developed by transferring spleen cells from donor B6/N (H-2k$^{b}$) mice into recipient B6D2F1 (H-2k$^{b/d}$) mice. In addition to evaluating manifestations in aGvHD mice, we analyzed the serum ALT/AST levels, liver pathological changes, infiltrating cells and mRNA expression of inflammation-related cytokines and chemokines. 5-ALA/SFC treatment significantly ameliorated liver injury due to aGvHD and decreased the population of liver-infiltrating T cells, resulting in a reduced expression of pro-inflammatory cytokines and chemokines. Furthermore, the mRNA expression proliferator-activated receptor-γ coactivator (PGC-1α) was enhanced, which might explain why 5-ALA/SFC treatment downregulates inflammatory signaling pathways. Our results indicated that 5-ALA/SFC can ameliorate liver injury induced by aGvHD through the activation of PGC-1α and modulation of the liver mRNA expression of inflammatory-related cytokines and chemokines. This may be a novel strategy for treating this disease.

***Keywords*** 5-aminolevulinic acid, acute graft-versus-host disease, liver injury, inflammatory cytokines, PGC-1α.

## 1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is the most efficient treatment for many hematological malignancies and for primary immunodeficiencies (*1,2*). However, transplants also contain mature T cells, which can induce acute graft-versus-host disease (aGvHD), a life-threatening complication of allogeneic HSCT (*3*).

The immune response causes activated donor T cells to gain a cytolytic capacity and attack recipient tissue in order to eliminate foreign antigen-bearing cells. These donor T cells infiltrate target organs where the inflammatory response plays a very important role, including the skin, liver, lung and gastrointestinal tract, and ultimately induce end-organ tissue damage (*4,5*). Therefore, the major goals of HSCT are to modulate alloreactivity by donor allogeneic T cells without causing GvHD and to preserve the graft-versus-leukemia and graft-versus-infection effects.

Several strategies are being reported to treat aGVHD, and current therapies include the administration of extracellular mediators and receptors, regulation of intracellular signaling pathways, and regulation of translation and transcription (*6*). The successful reduction in inflammatory responses is a major strategy for dealing with aGvHD.

5-Aminolevulinic acid (5-ALA) is a precursor of heme found in plants, bacteria, fungi, and animals (*7*). It is an endogenous amino acid in animals and the first

compound produced by 5-ALA synthase in the heme biosynthetic pathway. Growing evidence supports the notion that 5-ALA combined with sodium ferrous citrate (5-ALA/SFC) exerts antioxidant, anti-inflammatory and anti-fibrotic properties (*8-11*). In addition, 5-ALA/SFC has been used to treat a variety of animal models, including inflammatory disease, transplantation, autoimmune disease and sclerodermatous issues induced by chronic GvHD, which may explain its anti-inflammatory, immunoregulation and cytoprotective properties exerted *via* the upregulation of heme oxygenase (HO)-1 expression and release of heme metabolites (*12,13*).

In the present study, we investigated the effects of 5-ALA/SFC treatment modulating inflammatory responses and liver injury in aGvHD mice. Evidence supporting the anti-inflammatory, immunoregulation and cytoprotective properties of 5-ALA/SFC was obtained, and possible mechanisms underlying these effects were confirmed. Such evidence included direct amelioration of liver injury, reduction in liver-infiltrating T lymphocytes and modulation of the liver mRNA expression of inflammatory-related cytokines and chemokines. Furthermore, 5-ALA/SFC treatment also enhanced the proliferator-activated receptor-γ coactivator (PGC-1α) expression in liver tissue, potentially explaining the downregulation of the inflammatory signaling pathways in the aGvHD model treated with 5-ALA/SFC.

## 2. Materials and Methods

### 2.1. Mice

Male 7- to 8-week-old C57BL/6NJcl × DBA/2NJcl (B6D2F1, H-2k$^{b/d}$) and 8- to 12-week-old C57BL/6NJcl (B6/J, H-2k$^{b}$) mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were maintained on a 12-h light-dark cycle and given free access to food and water except during the period of caloric restriction for the pair-feeding group. All animal manipulations were performed according to the recommendations of the Committee on the Care and Use of Laboratory Animals at the National Research Institute for Child Health and Development in Tokyo, Japan (Permission number: A2009-010-C11).

### 2.2. Experimental procedure for the aGvHD model and grouping

Spleens were aseptically harvested from B6/J mice separately, and the tissue was dissociated by rubbing to generate a single-cell suspension. Red blood cells were lysed using 10× phosphate-buffered saline (PBS) and pure water, viable cells were counted by trypan blue dye exclusion on a hemocytometer, and individual cells were resuspended in RPMI 1640 (Gibco BRL, Grand Island, NY). aGvHD was induced by the intravenous injection of 1 × $10^8$ B6/J donor splenocytes into unirradiated B6D2F1 recipients.

The mice were pair-fed, and body weight was monitored weekly. At the indicated time point on days 7 and 14, mice were sacrificed, and the serum, liver and spleen tissue were collected for the measurement of serum enzymes alanine transaminase (ALT) and aspartate transaminase (AST) levels, counting of liver-infiltrating cells and mRNA expression analyses.

Mice were randomly assigned to two groups: those receiving 5-ALA hydrochloride (100 mg/kg; neo ALA Co. Ltd, Tokyo, Japan) and SFC (157 mg/kg) (Komatsuya Corporation, Osaka, Japan) daily from days 0 to 7 or 14 as the 5-ALA/SFC-group, those receiving distilled water as the control group.

### 2.3. Histopathological analyses

For the histopathological examination, liver samples were collected after the animals were sacrificed by anesthesia and fixed in 10% formaldehyde, then embedded in paraffin. Sections of 4 μm were processed and stained with hematoxylin-eosin (HE; MUTO PURE CHEMICALS, Osaka, Japan) according to a previously described method (*14*). A light microscopic analysis was performed to assess the overall cellularity and liver damage using a digital camera (BX51, OLYMPUS, Tokyo, Japan). To facilitate a semiquantitative assessment of aGVHD, grading of aGVHD was confined to analysis of the liver. The histological assessment was performed according to the scoring system with slight modification (*15*). Inflammatory cell infiltrates in the portal tract and around the central veins were graded on a scale of 1 to 5 as follows: 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, ≥7 cells in thickness, involving > 50% of vessels. Scoring was performed in a blinded manner.

### 2.4. Liver enzyme measurements

Serum levels of ALT and AST are commonly used as biochemical indicators of liver injury. Blood samples were obtained to evaluate serum ALT and AST levels using commercially available kits (Fujifilm, Tokyo, Japan) measured by an automatic biochemical analyzer (DRI-CHEM 3500i; Fujifilm) according to the manufacturer's protocol.

### 2.5. Isolation of nonparenchymal cells (NPCs) from the liver

Mice were chosen randomly from each group on day 7 and 14 after the lymphocyte injection. The liver was

mashed and passed through a 70-μm nylon cell strainer on ice; then, the tissue liquid was centrifugated at 4°C for 1 min at 60 g, and the supernatant was collected for washing twice with PBS. NPCs were purified by centrifugation at room temperature for 25 mins over a 40% discontinuous Percoll gradient (Sigma-Aldrich, St. Louis, MO). The NPCs were then suspended in PBS for flow cytometry (FCM) and RLT buffer for a quantitative real-time polymerase chain reaction (PCR) analysis.

2.6. Flow Cytometer (FCM) analysis

NPCs and splenocytes were incubated with directly labeled antibodies for 30 mins, washed with PBS and fixed in 1% paraformaldehyde. Immune cell composition was determined *via* FCM. Liver NPCs and splenocytes were then treated with purified anti-mouse CD16/32 (BioLegend, San Diego, CA) and stained with a defined panel containing Live/Dead stain (L34957; Thermo Fisher Scientific, Waltham, MA) to mark the living and dead cells. In addition, liver NPCs in all panels were stained with anti-CD45 (30-F11; BioLegend) to gate the white blood cells (WBCs). NPCs and splenocytes then were stained with anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7), anti-NKp46 (29A1.4), anti-CD11b (M1-70), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-Ly6G (1A8), anti-Ly6C (HK1.4), anti-IA/IE (M5/114.15.2), anti-CD40 (3/23), anti-CD80 (16-10A1) and anti-CD86 (GL-1) mAbs conjugated with a particular fluorochrome, and corresponding isotype controls were used (BioLegend). The analysis of stained cells was performed with a BD FACS LSR Fortessa, Franklin Lakes, NJ), and analyzed by the FlowJo software program (Version 10.5.0; BD Biosciences).

2.7. RNA preparation and quantitative real-time PCR

To measure the inflammation and cytokine gene expression in liver tissue, total RNAs of liver were isolated with the Trizol reagent (Invitrogen, Carlsbad, CA). A total of 800 ng of each RNA sample was reverse-transcribed to cDNA using oligo (dT) primers and Super Script reverse transcriptase (Invitrogen). The target-specific primers and probes listed in Table 1 were designed based on the reported cDNA sequences and synthesized by Biosearch Technologies (Novato, CA). Four-step PCR was performed for 45 cycles. Quantitative real-time PCR was performed using the TaqMan and SYBR® Green-based system with an Applied Biosystem PRISM7700 (Thermo Fisher Scientific). 18s was used as the housekeeping gene. The final result was analyzed by the $2^{-\Delta\Delta}$Ct method.

2.8. Statistical analysis

The results were expressed as the mean ± standard deviation (SD). All data were analyzed using the GraphPad Prism software program (version 7.0, GraphPad Software, San Diego, CA). A one-tailed unpaired Student's *t*-test were used to compare two groups, and a one-tailed Wilcoxon's matched-pairs signed rank test was used to compare two groups. In cases with a normal distribution, a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was used to compare multiple groups, while in cases with a non-normal distribution, a one-way ANOVA with Dunn's multiple comparisons test was used to compare multiple groups. *P* values of $< 0.05$ were considered to indicate statistical significance.

**3. Results**

3.1. 5-ALA/SFC treatment ameliorated liver injury in aGvHD

To investigate the therapeutic effect of 5-ALA/SFC on liver injury, we used a mouse model of aGvHD, established by transferring $1 \times 10^8$ spleen cells from donor B6/J (H-$2k^b$) mice into recipient B6D2F1 (H-$2k^{b/d}$) mice, and recipient mice were treated with a combination of 5-ALA and SFC orally administered at 100 mg/kg and 157 mg/kg daily, respectively. In the aGvHD control group, the mouse weight loss was obvious. However, in the 5-ALA/SFC treatment group, the weight decreased significantly more slowly (Figure 1A). In addition, we also examined the serum levels of ALT and AST at the baseline before treatment as well as on days 7 and 14 in aGvHD mice. As shown as in Figure 1B, compared with the control group on day 14, the serum ALT and AST levels of the mice in the 5-ALA/SFC-treated group were significantly decreased. Furthermore, pathological changes in the liver were observed by light microscopy after HE staining. The histology of the representative liver specimen of aGvHD mice was observed after HE staining on days 7 and 14. The samples showed lymphoid cell infiltration, and the histology score was determined. 5-ALA/SFC treatment prevents pathological changes in the liver, and improvements in the liver samples of the 5-ALA/SFC treatment group were observed in all samples (Figure 1C).

3.2. 5-ALA/SFC treatment decreased the liver-infiltrating T cells on day 14 in aGvHD

In order to understand how 5-ALA/SFC treatment modulates the cellular response in aGvHD, we performed a multicolor FCM analysis on 5-ALA/SFC-treated mice on days 7 and 14 after the spleen cells had been transplanted. As shown as in Figure 2, we found that, compared with naïve mice, the populations of liver-infiltrating cells and spleen cells were increased on days 7 and 14 in the aGvHD model mice, indicating that liver and spleen inflammation was induced after donor cell transplantation. In addition, the total number of $CD3^+$ T

**Table 1. The sequences information of the primers and probes used in the study**

| Genes | Forward (5'- 3') primers | Reverse (5'- 3') primers | Probes |
|---|---|---|---|
| Taqman primers and probes | | | |
| PGC-1α | CATTTGATGCACTGACAGATGGA | CCGTCAGGCATGGAGGAA | CCGTGACCACTGACAACGAGGCC |
| HO-1 | CAGGGTGACAGAAGAGGCTAAGAC | TCTTTGTGTTCCTCTGTCAGCAGT | TCCTGCTCAACATTGAGCTGTTTTGAGGA |
| Nrf-2 | GCCCTCAGCATGATGGACTTG | TGCCTCCAAAGGATGTCAATCAA | AGTTGCCACCGCCAGGACTACAGTCC |
| Sirt-1 | CAGCATCTTGCCTGATTTGTAAATAC | CACCGAGGAACTACCTGATTAAAAA | TCTCCACGAACAGCTTCACAATCAACT |
| IFN-γ | AAGCGTCATTGAATCACACCTGA | ACCTGTGGGTTGTTGACCTCAA | ACTACCTTCTTCAGCAACAGCAAGGCGA |
| TNF-α | TGTCTACTGAACTTCGGGGTGAT | AACTGATGAGAGGGAGGCCAT | TCCCCAAAGGGATGAGAAGTTCCCAA |
| IL-1β | ACCCCAAAAGATGAAGGGCTG | GTGCTGCTGCGAGATTTGAAG | TCTCATCAGGACAGCCCAGGTCAAAGGT |
| CCL-2 | GTTGGCTCAGCCAGATGCAG | GTAGCTCTCCAGCCTACTCATTG | CCCACTCACCTGCTGCTACTCATTCACC |
| CCL-3 | TCCCAGCCAGGTGTCATTTTC | AGGCATTCAGTTCCAGGTCAG | ACCCAGGTCTCTTTGGAGTCAGCGCA |
| CCR-2 | CCTGTAAATGCCATGCAAGTTCA | CCGTGGATGAACTGAGGTAACATA | ACCAGAAGAGGGCATTGGATTCACCACAT |
| NF-κB | TGTCTGCCTCTCTCGTCTTCCT | CACCACTGTCACAGACGCTGT | CGAGGCAGCACATAGATGAACTCCGG |
| 18s | ATGAGTCCACTTTAAATCCTTTAACGA | CTTTAATATACGCTATTGGAGCTGGAA | ATCCATTGGAGGGCAAGTCTGGTGC |
| SYBR green primers and probes | | | |
| Sulf-2 | ATCCAGACCTTCTATTTCCAGGC | GTTGGGCCGGATGTTCCTG | |
| 18s | ATGAGTCCACTTTAAATCCTTTAACGA | CTTTAATATACGCTATTGGAGCTGGAA | |

cells was significantly increased on day 14, that of $CD4^+$ T cells was increased on day 7, and that of $CD8^+$ T cells was increased on day 14 (data not shown), indicating that $CD4^+$ and $CD8^+$ T cells play important roles in different phases of aGvHD.

Regarding the effect of 5-ALA/SFC treatment, we found that, compared with the control group, the T cell percentage among the liver-infiltrating $CD45^+$ cells was reduced in the 5-ALA/SFC treatment group. In addition, we detected other immune cells in the liver and spleen after splenocyte transplantation into recipient mice. As shown in Supplementary Figure S1A (*http://www.ddtjournal.com/action/getSupplementalData.php?ID=68*), compared with the data on day 7 the percentages of NK cells, NKT cells, neutrophils, monocytes, macrophages and dendritic cells among the liver-infiltrating cells were reduced on day 14, although there were no significant differences between the control and 5-ALA/SFC-treated groups, indicating that T cells are the main cells involved in aGvHD, and after 5-ALA/SFC treatment, the population of liver-infiltrating T cells is significantly decreased, with improvements in liver inflammation noted. Furthermore, we found that the populations of T cells, neutrophils, monocytes and macrophages were increased in the spleens of recipient mice, demonstrating spleen injury in aGvHD mice (Supplementary Figure S1B, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=68*).

### 3.3. 5-ALA/SFC treatment upregulated the PGC-1α expression in the liver tissue of aGvHD mice

Next, we wanted to determine the possible mechanism by which 5-ALA/SFC modulates the inflammatory response. PGC-1α regulates the molecular pathway linking oxidative stress and mitochondrial metabolism with the inflammatory response and metabolic syndrome (*16*). We therefore explored the expression of PGC-1α, which exerts a protective effect against inflammation in the liver tissue (*17-19*), as one potential cause of these phenomena.

As shown in Figure 3, we found that the mRNA expression of PGC-1α was significantly higher in 5-ALA/SFC-treated mice than in control mice. In addition, 5-ALA/SFC treatment enhanced the expression of mRNA from HO-1 and nuclear factor erythroid 2-related factor 2 (Nrf-2), which activate the transcription factor Sirtuin-1 (Sirt-1) to promote mitochondrial fusion and biogenesis, partly through an increase in the mRNA expression of PGC-1α. Thus, these data indicated that the activation of PGC-1α by 5-ALA/SFC through enhanced HO-1, Nrf-2, and Sirt-1 expression resulted in the improved modulation of the inflammatory response.

### 3.4. 5-ALA/SFC treatment decreased the mRNA expression of inflammation-related genes in the liver tissue of aGvHD mice

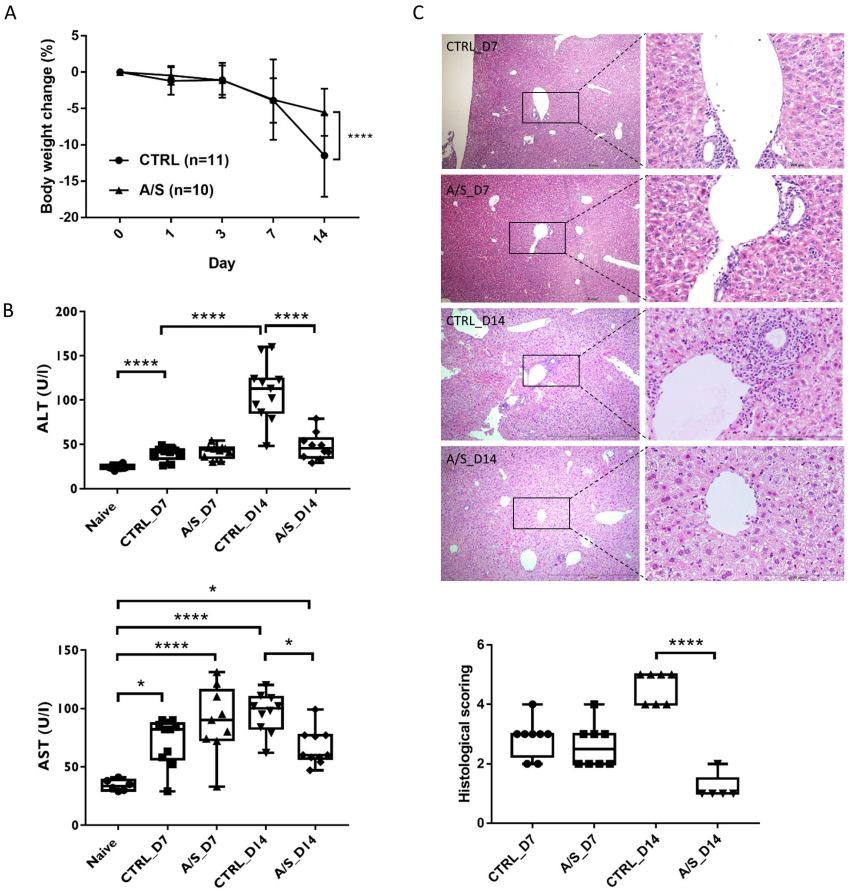


**Figure 1. 5-ALA/SFC treatment ameliorated liver injury in aGvHD mice. A.** The weight loss of the control group was markedly reduced during aGvHD, and in the 5-ALA/SFC-treated group, the weight reduced more slowly. Data were analyzed and presented as the mean ± SEM; $^{****}p < 0.0001$ compared with the control group. **B.** Serum ALT and AST levels were measured. Compared with the control group, the serum ALT and AST levels in the 5-ALA/SFC-treated group were significantly decreased. Data were analyzed and presented as the means ± SD; $^{*}p < 0.05$, $^{****}p < 0.0001$. **C.** The representative histological changes of the liver were observed on days 7 and 14 from aGvHD mice after HE staining, revealing inflammatory cell infiltration, which had improved in the liver samples from the 5-ALA/SFC treatment groups. Based on HE staining, the histology score was determined. 5-ALA/SFC treatment prevents pathological changes in the liver. Values are shown as the means ± SD; $^{****}p < 0.0001$.

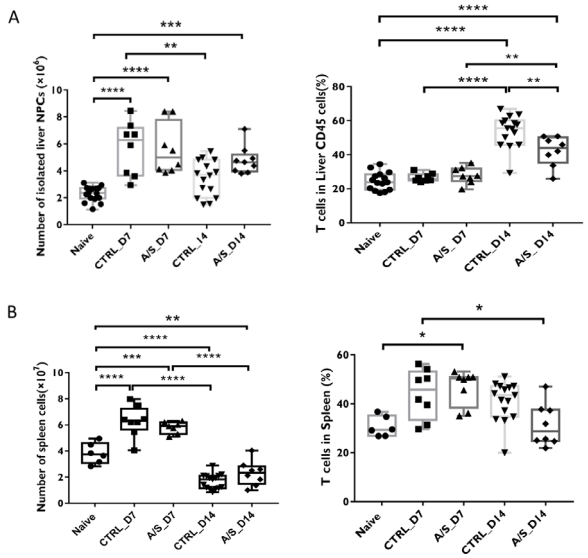


**Figure 2. 5-ALA/SFC treatment alleviated the activation of liver-infiltrating T cells in aGvHD mice.** Compared with the naïve group, the number of isolated liver NPCs on days 7 and 14 was increased in the aGvHD groups. Among the total lymphocytes, representative data showed that gated T cells were decreased in the 5-ALA/SFC-treated group. Percentages represent the means ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

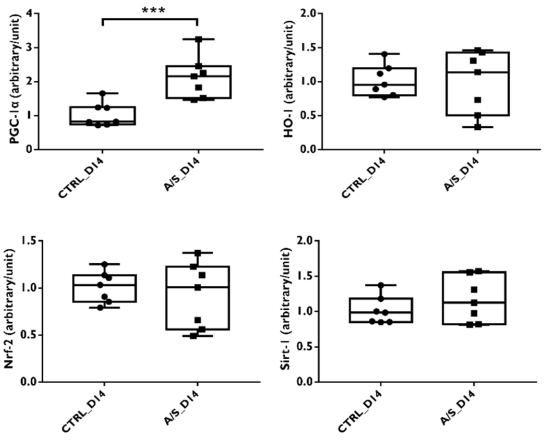


**Figure 3. 5-ALA/SFC treatment increased the mRNA expression of PGC-1α in aGvHD mice.** Liver tissue was collected on day 14, and the mRNA expression of PGC-1α, HO-1, Nrf-2 and Sirt-1 was analyzed by qRT-PCR, as described in the Materials and Methods. Compared with the control group, the mRNA levels were significantly different in the liver tissues of the 5-ALA/SFC group. Values are shown as the means ± SD; $^{***}p < 0.001$.

In order to analyze how 5-ALA/SFC ameliorated liver injury, we conducted quantitative real-time PCR for several kinds of inflammatory cytokines and chemokines in liver tissue that are known to be major contributors to the development of aGvHD and lead to liver injury. As shown as in Figure 4, the expression of TNF-α, IFN-γ, IL-1β, CC chemokine ligands 2 and 3 (CCL-2 and CCL-3, respectively) and NF-κB was increased in aGvHD mice, but 5-ALA/SFC treatment significantly reduced the expression of these genes. In addition, we found that 5-ALA/SFC treatment enhanced the mRNA expression of CC chemokine receptor 2 (CCR-2), which controls leukocyte migration during inflammatory processes and has dual pro- and anti-inflammatory actions. Furthermore, we found that, compared with control mice, the expression of heparan sulfate 6-O-endosulfatase (Sulf)-2, an extracellular sulfatase that acts on heparan sulfate proteoglycans and modulates multiple signaling pathways (*20*) was decreased after 5-ALA/SFC treatment.

## 4. Discussion

aGvHD was briefly induced by donor T cells attacking recipient tissue contained in the transplant. Based on previous experimental models, the development of aGvHD involves three stage: first, activation of antigen-presenting cells (APCs); then, donor T cell activation, proliferation, differentiation and migration; finally, target tissue destruction (*21,22*). Therefore, in addition to end-organ damage, aGvHD also results in the immune response of T-cell activation and expansion. Thus far, several strategies for combatting aGvHD have been proposed, include targeting mediators of cytokine storm, such as TNF-α and IL-1/IL-1β, with antagonists (*23*), as well as reducing the intensity of conditioning regimens to avoid causing excessive inflammation (*22*).

In the present study, we transferred B6/J splenocytes into recipient B6D2F1 mice to develop an aGvHD model and investigated the therapeutic effect of 5-ALA/SFC on this disease. During the development of this model, on days 7 and 14, the mice showed liver and spleen injury in addition to weight reduction. After treatment with a 5-ALA/SFC, however, an assessment of the serum and pathological features indicated that the liver damage had been ameliorated (Figure 1).

Many reports in addition to our own have shown that 5-ALA/SFC has an anti-inflammatory effect in different kinds of diseases (*24-33*). Liver injury is a major end-organ damage that occurs in aGvHD, wherein the inflammatory response dominates the disease phase (*21*). However, while there are already many strategies for dealing with this kind of disease, previous approaches have had a rather broad spectrum and often affect the overall immune system reconstruction (*34*). Few reports have described the inhibitory effect of 5-ALA/SFC on aGvHD, especially in liver injury. Therefore, in the present study, we administered 5-ALA/SFC to a murine aGvHD model to determine whether or not it had a protective effect against liver damage.

Regarding immune cells of the liver and spleen, recent studies have reported that donor T cells occupy a central role in mediating aGvHD. In our study, on day 14, although the total cells isolated from the liver were fewer than had been noted on day 7, the population of liver-infiltrating T cells, specially $CD8^+$ T cells, was increased, indicating that T cells play a central role in aGvHD. This population was significantly decreased following 5-ALA/SFC treatment, and a decrease was also noted in spleen cells compared with the control mice (Figure 2). This finding may explain the reduced weight, reduced serum level and pathological features of liver damage, although

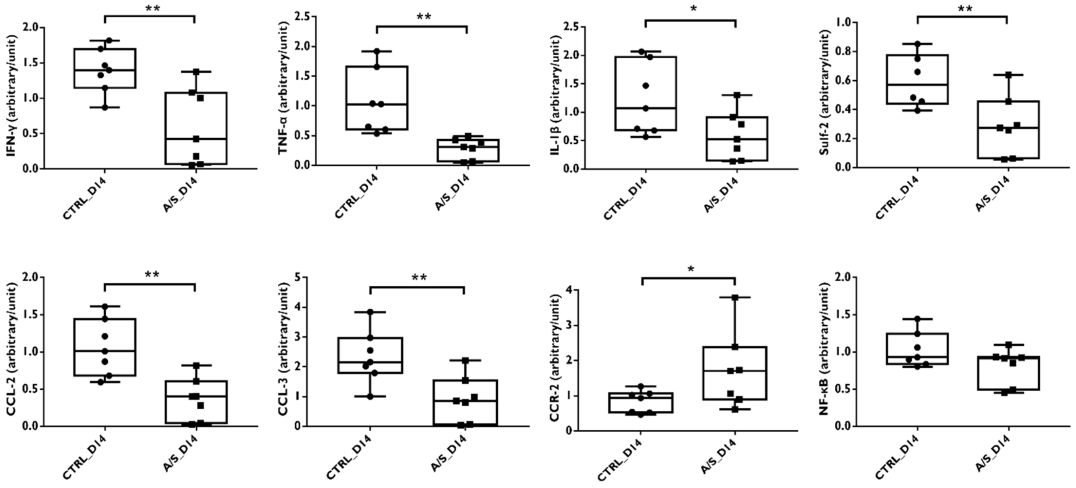


**Figure 4. 5-ALA/SFC treatment decreased the mRNA expression of inflammatory-related genes in aGvHD mice.** Liver were collected on day 14, and the mRNA expression of IFN-γ, TNF-α, IL-1β, CCL-2, CCL-3, CCR-2, Sulf-2 and NF-κB was analyzed by RT-PCR, as described in the Materials and Methods. Compared with the control group, the mRNA levels were significantly different in the liver tissues of the 5-ALA/SFC group. Values are shown as the means ± SD; $^{*}p < 0.05$, $^{**}p < 0.01$.

there were no significant differences in other immune cells, including NK cells, NKT cells, macrophages and dendritic cells, in the liver and spleen. However, the numbers of neutrophils and monocytes were significantly increased in the spleen following 5-ALA/SFC treatment (Supplementary Figure S1, *http://www.ddtjournal.com/action/getSupplementalData.php?ID=68*). These results indicate that 5-ALA/SFC treatment mainly targets T cells, which are primarily involved in aGvHD, resulted in the amelioration of liver inflammation and improvement in its function.

PGC-1α plays a beneficial role in the regulation of hepatic steatosis and insulin resistance by enhancing the IL-10-mediated anti-inflammatory response (*35,36*). In addition, inflammation can be regulated through changes in cellular reactive oxygen species induced by PGC-1α (*37*). Future studies should endeavor to identify the molecular mechanisms involved in the PGC-1α-mediated downregulation of inflammatory signaling pathways. Regarding the possible protective mechanism of 5-ALA/SFC against liver injury in the aGvHD model, we hypothesized that the activation of PGC-1α signaling might be intimately involved. We demonstrated that the mRNA expression of PGC-1α was significantly increased in the 5-ALA/SFC treatment group compared with the control group, which might be sufficient to enhance the mitochondrial function and ameliorate hepatic inflammatory cell infiltration (Figure 3). Therefore, we proposed that PGC-1α might played a vital role in our aGvHD mouse model by mediating the inflammatory response and reducing oxidative damage. In addition to an increased expression of PGC-1α, 5-ALA/SFC treatment also resulted in an increased mRNA expression of HO-1, Nrf-2 and Sirt-1, suggesting that the upregulation of PGC-1α might be achieved *via* the Nrf-2, HO-1 and Sirt-1 pathway, which enhances the mitochondrial function *in vitro* (*38,39*). Of note, Sirt-1 can deacetylate downstream targets, such as NF-κB, which is a key transcriptional factor in pro-inflammation responses (Figure 3) (*40*). Our data showed that with the expression of Sirt-1 and PGC-1α was increased while that of NF-κB was reduced (Figure 4). HO-1's oxidative stress-protective effects are strongly associated with Sirt-1 upregulation. The increases in both Sirt-1 and HO-1 found in this study point to the existence of a common mechanism mediating the anti-inflammatory, anti-proliferative and cytoprotective effects, all of which result from oxidative stress induced by both Sirt-1 and HO-1 (*41*). Taken together, our data show that 5-ALA/SFC treatment significantly increased the PGC-1α expression in liver tissue, highlighting a possible mechanism underlying the protective effect of 5-ALA/SFC.

Because inflammatory cytokines and chemokines are well-known inducers of leukocyte trafficking and activation and contribute to the pathogenesis of aGvHD (*2*), we detected the mRNA expression of related cytokines and chemokines in the liver. We found that 5-ALA/SFC treatment significantly reduced the expression of IFN-γ, TNF-α, IL-1β, CCL-2 and CCL-3 while increasing the CCR-2 expression (Figure 4). CCR-2 and its main ligand, CCL-2, are a chemokine receptor-ligand pair that controls leukocyte migration during inflammatory processes (*42*). It is important to note that CCR-2 has both pro- and anti-inflammatory actions. The proinflammatory role of CCR-2 is dependent on APCs and T cells, whereas the anti-inflammatory role is dependent on its expression in regulatory T cells (*43*). In our study, the enhancement of CCR-2 was attributed to an anti-inflammatory function, resulting in the inhibition of the release of cytokines, such as TNF-α, INF-γ and IL-1β (Figure 4). However, in the present study, we found that, compared with control mice, the expression of Sulf-2 was reduced after 5-ALA/SFC treatment (Figure 4). This result suggested that Sulf-2 would offer considerable advantages as a therapeutic target, playing a protective role against epithelial injury and inflammation of the liver in aGvHD (*44*). Taken together, these results were consistent with the findings of an increased expression of PGC-1α and the liver pathologic features, indicating that 5-ALA/SFC can modulate the inflammatory response *via* the HO-1, Nrf-2, Sirt-1 and PGC-1α pathways.

In conclusion, our data showed that 5-ALA/SFC was effective for treating liver injury by modulating the inflammatory response in aGvHD mice. The mechanistically therapeutic effect of 5-ALA/SFC may rely on PGC-1α activation and reducing the liver mRNA expression of inflammatory-related cytokines and chemokines. Our research may offer a novel therapeutic option for aGvHD, and these data may encourage future studies of this promising therapeutic agent for the treatment of aGvHD.

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*Conflict of Interest*: HI, KT, MN and TT are employees of SBI Pharmaceuticals Co., Ltd.

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