Synergistic effects of *Lactobacillus rhamnosus* culture supernatant and bone marrow mesenchymal stem cells on the development of alcoholic steatohepatitis in mice

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**Abstract:** The gut microbiota has been shown to play an important role in chronic liver disease. It has been found that both *Lactobacillus rhamnosus* and its culture supernatant have the potential to mitigate alcoholic steatohepatitis. However, the exact mechanism is still not fully understood. Bone marrow mesenchymal stem cells have immunosuppressive effects with few side effects. The synergistic effect between *Lactobacillus rhamnosus* culture supernatant and bone marrow mesenchymal stem cells (BMMSCs) deserves further observation. In this study, a mouse model of chronic alcoholic hepatitis was established by eight weeks of Lieber-DeCarli liquid diet feeding; and LGG-s, BMMSCs or a combination of the two were used to explore a new therapeutic method for alcoholic liver disease and to study the mechanism. The results showed that the combined LGG-s and BMMSC treatment might have a synergistic effect and could improve the symptoms of alcoholic hepatitis by regulating inflammation, autophagy and lymphocyte subsets through the PI3k/NF-κB and PI3K/mTOR pathways. With the treatment, the autophagy rate accelerated, and alcohol-induced natural killer B (NKB) cell and follicular helper T (TFH) cell numbers decreased. These findings suggest that the development of alcoholic hepatitis may occur via PI3K/NF-κB and PI3K/mTOR pathway overactivation as well as through NKB and TFH cell imbalances. Moreover, LGG-s and BMMSCs can regulate these factors and alleviate the disease.

**Keywords:** Gut microbiota, mTOR, bone marrow mesenchymal stem cells, alcoholic liver disease, natural killer-like B cells

**Introduction**

With a gradual and steady decrease in viral hepatitis B infections due to the use of the hepatitis B vaccine as well as the improvement of living conditions, fatty liver diseases, especially steatohepatitis, have steadily increased in recent decades and become a primary issue to the health of the liver [1]. In both alcoholic and nonalcoholic fatty liver disease, fatty liver progresses from steatosis, steatohepatitis, cirrhosis to hepatocellular carcinoma [2]. Steatohepatitis is an inflammatory condition in the liver, and investigation of steatohepatitis has revea-

led that lipid peroxidation and local inflammatory cytokines released by the accumulation of fat in liver cells are the major causes of liver fibrosis and cirrhosis [3].

Inflammation is a complex biological response that involves cytokines, immune cells and blood vessels. Although several cytokines are important in inflammation, NF-κB is one of the most inflammation-inducible factors in nonalcoholic fatty liver disease [4, 5], and its expression is elevated in alcoholic liver disease [6]. However, NF-κB overexpression alone cannot explain the autophagy and Th17-Treg percentage imbal-
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Changes in the proportion of lymphocyte subsets play a crucial role in immune regulation [21, 22]. We previously found that changes in the proportion of Th17 and Treg cells could affect liver damage in mice with alcoholic hepatitis. By adjusting this ratio, the alcohol-induced damage to the liver could be reduced to some extent [11]. Recently, both natural killer B cells (NKB) and follicular helper T cells (TFH) have been found to be immune adjuvant cells that have immunomodulatory effects [23-27]. Therefore, their role in the regulation of liver inflammation needs to be investigated.

Recently, gut microbiota has been found to be involved in several diseases in humans, including alcoholic fatty liver diseases [28, 29]. Dysbiosis of gut microbiota has been demonstrated to increase liver fat accumulation [30]. Moreover, fecal transplantation from patients with nonalcoholic fatty liver disease to mice treated with a high-fat diet can exacerbate liver damage [31]. Furthermore, treatment of gut microbiota to correct the dysbiosis improves clinical outcomes in a nonalcoholic disease animal model [11, 32]. Treatment of chronic-binge alcoholic model mice with LGG culture supernatant improves intestinal barrier function and the balance of Treg and TH17 cells [11]. We have previously shown that bone marrow mesenchymal stem cells and LGG-s can regulate Th17 and Treg cells, affecting the damage of alcoholic liver disease to the liver. The similar mechanisms of BMMSCs and LGG-s led us to consider whether there are direct or indirect synergistic effects between them and whether the use of both interventions can enhance the protection against liver damage caused by alcohol. According to the hypothesis above, this study aimed to explore the possible synergistic effects and the underlying mechanism.

Material and methods

Preparation of LGG-s

LGG was purchased from (ATCC, Rockville, MD) and cultured in MRS broth according to the manufacturer’s guidelines. When the LGG density reached $10^9$ colony-forming units/ml (CFU/ml), the LGG supernatant (LGG-s) was collected by filtering through 0.22 mm filters. LGG-s was stored at 0-4°C for use.

Animal experiments and sample collection

Wild-type (WT) male C57BL/6 mice weighing 18–20 g at six weeks of age were purchased from the Shanghai Laboratory Animal Center (Shanghai, China). Mice were fed a Lieber-DeCarli control diet for 2 weeks for adaptation to a liquid diet and then fed a different proportion of Lieber-DeCarli ethanol diet for one week starting at a ratio of the Lieber-DeCarli control diet to the Lieber-DeCarli ethanol diet (5% ethanol) of 2:1 to 1:1 and increasing to 1:2 on days 2, 4 and 6, with the exception of mice in the normal control group. At the end of the adaptation period, mice were fed a complete Lieber-DeCarli ethanol diet. There were 5 experimental groups with 6 mice in each group: group 1: normal control group (N) with Lieber-DeCarli control diet only; group 2: alcohol model group (M) with Lieber-DeCarli ethanol diet for 4 weeks; group 3: LGG-s treatment group (L) with Lieber-DeCarli ethanol diet plus LGG-s in the diet (at a dose equivalent to $10^9$ CFU/ml per mouse per day) [11, 33]; group 4: BMMSC treatment group (B) with a Lieber-DeCarli ethanol diet plus injection of BMMSCs via the tail vein once per week for a total of 3 times at the beginning of the second week, third week and fourth week; and group 5: LGG-s and BMSC treatment group (LB) with Lieber-DeCarli ethanol diet plus the combination of LGG-s and BMSC groups as described for groups 3 and 4. At the end of the 7th week, all mice were euthanized by collecting blood from the tail vein and then by cervical dislocation as outlined in Figure 1. Mice were treated according to the protocols reviewed and approved by the Institutional Ethics Committee of Wenzhou Medical University, which were in accordance with the Guiding Principles for the Care and Use of Laboratory Animals in China.

Liver enzymes assay

The collected blood was centrifuged at 1500 rpm at room temperature for 10 minutes to
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obtain serum. Serum was then stored at -80°C until measurement. Serum ALT and AST levels were measured by an automatic biochemistry analyzer (AU5800, Beckman Coulter, USA) at the clinical biochemical laboratory of the First Affiliated Hospital of Wenzhou Medical University.

Histochemical staining of the liver by HE and oil red O

Paraformaldehyde (4%) was used to fix liver tissue. After fixation in paraformaldehyde and embedding in paraffin, tissue was prepared into 4-μm-thick slices and stained with hematoxylin and eosin (HE). Another part of the liver was quickly frozen after removal from mice. Frozen tissue was cut into 5-μm-thick slices and stained with oil red O in a standard protocol. Three randomly selected fields in each slide were measured with a digital image analyzer (KS400, Carl Zeiss Vision, Germany) by a technician blinded to the tissue grouping. Histological assessment was conducted by two clinical pathologists who were blind to the experiment.

Real-time reverse transcription-polymerase chain reaction (RT-PCR) assay

Total RNA was isolated from mouse liver tissue using an RNA isolation kit (RNAiso, Aidlab Biotechnologies Co., Beijing, China). Reverse transcriptase was conducted by a PrimeScript RT reagent kit (Takara, Otsu, Shiga, Japan). Real-time PCR was performed with a QuantStudio™ 5 Real-Time PCR System (Thermo Fisher, Waltham, MA USA) to test the expression of FOS and microRNA-29c. Expression values were compared using the delta-delta-Ct method, FOS was normalized to GAPDH and microRNA-29c was normalized to U6. RT-PCR was performed by a 7500 real-time PCR system (Applied Biosystems, Life Technologies, USA) following a PCR cycle of denaturation at 94°C for 30 seconds, annealing at 60°C for all genes for 60 seconds, and extension at 72°C for 2 minutes for 40 cycles. Primers were as follows: GAPDH: forward primer (5'->3') AAGAAGGTTGTAAGCAGG, reverse primer (5'->3') GAAGGTGGAAGAGTGGGAGT with Tm = 60°C, raptor: forward primer (5'->3') CACGTCTTCCTTGTCTTAC, reverse primer (5'->3') GCCTTGCTTCTCTGACTGCTC with Tm = 60°C, rictor: forward primer (5'->3') CTCGCCTCGCAGCACATATACT, and reverse primer (5'->3') AGCCTTCAGAATTGCGCTGTC with Tm = 60°C. Each mRNA expression value was calculated based on the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. The final data were analyzed by the 2-ΔΔCt method.

Western blot analysis

Cytoplasmic protein in the liver tissue was extracted by using ice-cold RIPA solution (Biotime, Beijing, China). Twenty micrograms of the protein sample from each mouse was denatured for five minutes with 4 x gel loading buffer. After separation by 10% SDS-PAGE, proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-p, Darmstadt, Germany). Membranes were blocked with 5% BSA in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) at 22°C for one hour and incubated with specific antibodies at 4°C for 12 hours. The membranes were then washed and incubated with a horseradish peroxidase-conjugated secondary antibody (1:5,000, Biosharp, Hefei, China) for one hour at 22°C. Protein levels were detected by chemiluminescence using an ECL kit (Thermo, Massachu-
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Flow cytometry analysis

Mouse spleens from different groups were removed and washed with PBS. The splenic lymphocytes were isolated by a Spleen Lymphocyte Separation kit from Multiscience (Multiscience, Hangzhou, China). After isolation of lymphocytes from the spleen, 500 µl of cell suspension solution was used to detect NKB cells and TFH cells. NKB cells were defined as CD19+NK1.1+CD3- B cells, and TFH cells were defined as CD4+CXCR5+ T cells. Splenic lymphocytes were incubated with fluorescently labeled CD3-FITC (eBioscience, CA, USA), CD19-APC (eBioscience) and NK1.1-PE (eBioscience), CD4-PerCP5.5 (eBioscience), and CXCR5-PE (eBioscience) monoclonal antibodies at room temperature for 30 minutes. Mouse IgG2a kappa labeled with PE (eBioscience) was used as an isotype control to avoid nonspecific binding. CD3 CD19+NK1.1+ NKB cells and CD4+ CXCR5+ FTH T cells were analyzed using the BD FACSCalibur platform (BD Bioscience, CA, USA), and the percentages of NKB cells to total B cells and TFH cells to total lymphocytes were analyzed by FlowJo software.

Statistics

Comparisons between multiple sets of data were performed using two-way analysis of variance (ANOVA), LSD and Dunnett’s T3 tests in SPSS 20.0 software. For the cell experiments, three independent experiments were performed for each parameter. Data are presented as the mean ± SEM. A P value of less than 0.05 was considered to be statistically significant.

Results

LGG-s treatment, BMMSC treatment and combination treatment all have a positive effect on alcohol-induced abnormal liver function

Body weights of mice in different groups were measured once every week. As shown in Figure 2A, the body weight of mice from all groups gradually increased. Although there was a gap between the body weight of control mice and body weight of mice in other groups, the difference was not statistically significant. Moreover, the body weight of mice in different groups was very close at the end of the experiment. Liver injury was assessed by aminotransferases, as indicated in Figure 2B and 2C. Both serum ALT and AST levels were significantly elevated in mice fed the Lieber-DeCarli ethanol diet compared with mice fed the Lieber-DeCarli control diet. In addition, compared with the control treatment, treatment with either LGG-s, BMMSCs or a combination of LGG-s and BMMSCs significantly reduced both serum ALT and AST levels compared.

LGG-s BMMSC combination treatment decreases liver tissue damage and liver lipid accumulation

Liver tissue injury and lipid deposition were also examined by liver histology, as shown in Figure 3. There was a significant liver injury in mice fed the Lieber-DeCarli ethanol diet com-
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Figure 3. Histological examination of the livers from different groups. (A) Shows the HE staining of the livers from different groups (original magnification: ×200), and (B) displays the oil red O staining of the liver (original magnification: ×200).
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pared to mice fed the Lieber-DeCarli control diet (Figure 3A). However, LGG-s, BMMSCs, and the combination of LGG-s and BMMSCs significantly reduced liver injury but did not completely reverse the liver injury. The lipid deposition results are shown in Figure 3B. Compared to mice fed the Lieber-DeCarli control diet, mice fed the Lieber-DeCarli ethanol diet showed significant lipid deposition. Moreover, all three treatment options reduced lipid deposition but did not return lipid deposition in the liver back to the level in livers of normal mice. These improvements were increased in the mice that received a combination of LGG-s and BMMSCs. In addition, there was no significant evidence of liver fibrosis as assessed by Masson’s trichome staining due to the short course of ethanol intake (data not shown).

The therapeutic effect of the combined treatment is mediated by NF-kB, PI3K, and mTOR

The mechanism of liver injury was further investigated by examining the expression and phosphorylation of the inflammatory signaling molecules NF-kB and mTOR, as shown in Figure 4. There was a significant decrease in phosphorylated forms of NF-kB (Figure 4A), mTOR (Figure 4B) and PI3K (Figure 4C).

The mechanism of the combined therapy may involve autophagy

Since the PI3K/mTOR signaling pathway is also involved in autophagy, the expression of autophagy proteins (raptor, rictor and LC3II/I) was further examined in the liver, as shown in Figure 5. The mRNA levels of raptor and rictor showed that raptor mRNA levels were significantly decreased in mice were treated with all three options compared to mice fed the Lieber-DeCarli ethanol diet. Moreover, a significant reduction in raptor mRNA levels was observed in mice treated with a combination of LGG-s and BMMSCs. In addition, rictor mRNA levels were the opposite of raptor mRNA levels. There was a significant increase in rictor mRNA in mice treated with a combination of LGG-s and BMMSCs compared to mice only fed the Lieber-DeCarli ethanol diet (Figure 5A). The protein levels of raptor and rictor showed the same trends as observed at the mRNA levels (Figure 5B). mTOR is a complex with two forms: mTORC1 and mTORC2. The Raptor and Rictor proteins represent the distinctive components of these two forms, respectively (28). Furthermore, LC3II protein is a marker of autophagosome. The ratio of LC3II to LC3I can reflect the relative activity of autophagy. Their expression level was examined, as shown in Figure 5B. Although there was an increase in the LC3II/LC3I ratio in mice fed the Lieber-DeCarli ethanol diet compared to mice fed the normal control diet, there was no statistically significant difference among the treated groups except for the group of mice treated with the combination of LGG-s and BMMSCs, indicating an effect of the combination of LGG-s and BMMSC treatment on autophagy.

Combined therapy regulates immune cells

Since our previous investigation revealed that BMMSCs play an important role in immune reg-
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ulation and the treatment of liver disease, the presence of NKB and TFH cells was investigated, as shown in Figure 6. The flow cytometry profile of NKB cells shown in Figure 6A reveals a significant increase in NKB cells in the spleens of mice fed the Lieber-DeCarli ethanol diet alone. However, treatment with all three options reduced the number of NKB cells in the spleen, with the most significant reduction observed in mice treated with the combination of LGG-s and BMMSCs. The flow cytometry profile of TFH cells is shown in Figure 6B, with similar changes in TFH T cell numbers in the different groups.

Discussion

In our previous publications, we investigated the role of bone marrow mesenchymal stem cells in the alleviation of inflammation in mice with acute liver injury and autoimmune hepatitis [33, 34]. Moreover, we investigated the therapeutic activity of LGG-s in acute alcoholic liver disease in mice [11]. However, whether the combination of LGG-s and BMMSCs affects alcoholic liver disease remains unclear. Therefore, we designed an experiment to examine the therapeutic effects of a combination of LGG-s and BMMSCs on the attenuation of chronic-binge alcohol-induced liver injury. We examined inflammation, autophagy and immunomodulatory cell balance in mice.

The pathogenesis of alcoholic liver disease has been extensively investigated, but there is still no effective treatment for this disease. Since alcoholic liver disease, especially alcoholic steatohepatitis (ASH), involves several aspects of cellular and molecular biology, a single treatment will not completely heal alcohol-induced liver damage. In the liver, ASH shows both inflammation and autophagy effects, which are mediated by either the PI3K/NF-κB or PI3K/mTOR signaling pathways. It has been shown that the NF-κB signaling pathway is a major intracellular signaling pathway that induces the release of inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) [5, 36]. The Lieber-DeCarli model of alcoholic liver injury is
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A

NK1.1 PE

Group N

0.682%

Group M

7.73%

CD3<sup>+</sup> Cells

Group L

3.51%

Group B

2.21%

Group LB

1.14%

CD19 APE

CD3<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>+</sup> B cells

B

CXCR5 PE

Group N

0.096%

Group M

1.95%

Group L

0.637%

Group B

1.05%

Group LB

0.450%

CD4 Perp-cy5.5
Figure 6. Flow cytometry analyses of NKB cells and TFH cells in the spleens from different groups. (A) Shows the NKB cell profile in the five experimental groups, and (B) displays the TFH cell profile in different groups. NKB cells in total B cells and TFH cells in total lymphocytes are shown as dot graphs in (A and B), respectively. Data are presented as the mean ± SEM from six mice in each group. *Represents P<0.05, **represents P<0.01 vs group M. & and && represents P<0.05 and P<0.01 between any 2 groups below the line.
a good model to investigate inflammation of the liver. In this model, normal alcohol intake is simulated while providing sufficient daily calorie intake for the mice [35]. The results of body weight, serum ALT, AST, HE and oil red O staining analyses showed that the model was successfully established in our study. In addition, NF-kB signaling was activated in the model mice. With three treatment options, we observed that with reduced NF-kB signaling, there was a decrease in liver injury. The activation of NF-kB signaling occurs through its phosphorylation, and the phosphorylation level reflects the degree of inflammation in the tissue [5, 36]. In our experiments, the activity of NF-kB in alcoholic mice was increased, whereas it was decreased in mice treated with the combination of LGG-s and BM-MSCs. This result indicates that the combination of LGG-s and BM-MSCs could inhibit NF-kB phosphorylation and reduce inflammation.

Autophagy is another pathological event in this model, as there was an increased level of LC3II/LC3I and activation of the mTOR signaling pathway in alcoholic mice. The signaling pathway of mTOR is associated with autophagy and innate immune regulation [24]. In this experiment, we found that the autophagy marker protein LC3II was elevated in alcoholic mice, which is consistent with the activation of autophagy. mTOR signaling is involved in both mTORC1 and mTORC2, which mediate either increased or decreased autophagy in cells [37, 38]. Although we observed that mTOR phosphorylation was reduced in mice treated with both LGG-s and BM-MSCs and that LC3II level was also reduced, there was a low level of mTOR activity and low LC3II level in control mice. The differences between control mice and treated mice could be due to the ratio of mTORC1 to mTORC2 in the cells. The mTORC1 and mTORC2 signaling pathways involve raptor and rictor, respectively [39]. Although the phosphorylation of mTOR was decreased in mice treated with both LGG-s and BM-MSCs, the level of mTORC2-associated rictor was elevated, which is different from that in the control mice. Therefore, although mTORC1 to mTORC2 in the cells. The mTORC1 and mTORC2 signaling pathways involve raptor and rictor, respectively [39]. Although the phosphorylation of mTOR was decreased in mice treated with both LGG-s and BM-MSCs, the level of mTORC2-associated rictor was elevated, which is different from that in the control mice. Therefore, although mTORC1 accounts for most of the mTOR complex, the role of mTORC2 is not negligible. It is the change in the proportion of the mTOR complex that regulates autophagy. We believe that the appropriate activation of autophagy alleviates liver damage in mice treated with both LGG-s and BM-MSCs.

Our previous experiments showed that the treatment of LGG-s could affect the ratio of TH17 to Treg cells in mice, and the change in the proportion of these lymphocyte populations could improve the immune status of mice and thus protect the liver of mice with alcoholic hepatitis [11]. TFH cells are a subpopulation of T regulatory cells and are regulated by Treg cells and the mTOR signaling pathway. The activation of the mTOR pathway can promote TFH cell maturation and differentiation, and different components of mTOR complexes can regulate TFH cells differently. For example, raptor/mTORC1 mainly promotes CD4 T cell proliferation, which is necessary for TFH differentiation, while rictor/mTORC2 promotes Akt activation through TCF1 expression, which regulates TFH differentiation without severely affecting T cell proliferation [24-27]. Mature TFH cells are involved in innate immunity and humoral immunity [24-27]. However, few studies have been conducted on TFH cells in mice with alcoholic hepatitis, and there have been no investigations about the effect of interventions with probiotics and bone marrow mesenchymal stem cells on TFH cells in alcoholic liver disease. In this experiment, the proportion of TFH cells increased in mice with alcoholic hepatitis and decreased after treatment with LGG-s, BM-MSCs or the combination of LGG-s and BM-MSCs. Our findings reveal that inflammation in alcoholic hepatitis is closely associated with lymphocyte subsets, and the mTOR signaling pathway also plays a key role. Another type of lymphocyte, NKB cells, are a type of B cell with functions similar to those of helper T cells. NKB cells play an important role in infectious disease through the excretion of several cytokines. Our findings also found increases in the NKB cell population in alcoholic mice, and these populations decreased in response to treatment with LGG-s, BM-MSCs and the combination of LGG-s and BM-MSCs. These findings are consistent with the role of NKB cells in inflammation. In general, our findings reveal that in addition to playing a role in bacterial infections, TFH and NKB cells also play an influential role in diseases such as alcoholic hepatitis. These findings suggest that in alcoholic liver disease, the balance of the overall lymphocyte ratio is critical and therapeutic effect of BM-MSCs and LGG-s is mediated by this ratio. Moreover, these findings also suggest that mTOR signaling may also be involved in the reg-
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ulation of the function and quantity of NKB cells through mTORC1 and mTORC2 mechanisms.

There are limitations to the current study. First, we did not directly verify that the BMMSC and LGG-s combination therapy regulates NF-kB and mTOR by acting on PI3K. Second, we cannot confirm whether our combination therapy acts directly on NKB cells or ultimately through the mTOR signaling pathway or both. Third, we did not directly analyze the effect of the changes in the ratio of TFH cells to NKB cells on the liver.

In summary, our study demonstrates that the LGG-s and BMMSC combination therapy can more effectively alleviate liver injury in alcoholic hepatitis than the individual therapies. The enhanced effect of the combination therapy may be related to its regulation of inflammation and autophagy through the NF-kB and mTOR signaling pathways. Moreover, the combination of LGG-s and BMMSCs in the treatment of alcoholic liver injury may also be related to the regulation of the balance between NKB and TFH cells.

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Disclosure of conflict of interest

None.

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