Bifidobacterium mitigates autoimmune hepatitis by regulating IL-33-induced Treg/Th17 imbalance via the TLR2/4 signaling pathway

Authors: Jianguo Song, Juan Dai, Xueping Chen, Fei Ding, Yanbo Ding, Liang Ma and Liwen Zhang

DOI: 10.14670/HH-18-669
Article type: ORIGINAL ARTICLE
Accepted: 2023-10-18
Epub ahead of print: 2023-10-18

This article has been peer reviewed and published immediately upon acceptance.
Articles in “Histology and Histopathology” are listed in Pubmed.
Pre-print author’s version
**Bifidobacterium mitigates autoimmune hepatitis by regulating IL-33-induced Treg/Th17 imbalance via the TLR2/4 signaling pathway**

Jianguo Song¹²#, Juan Dai²#, Xueping Chen⁴#, Fei Ding², Yanbo Ding², Liang Ma¹²*, Liwen Zhang³*

¹Department of Gastroenterology, The Fifth People’s Hospital of Xinjiang Uygur Autonomous Region, Xin Jiang, China.
²Department of Gastroenterology, The First People’s Hospital of Changzhou, The Third Affiliated Hospital of Soochow University, Changzhou, Jiangsu, China.
³Department of Pediatrics, the Second People’s Hospital of Changzhou, Affiliate Hospital of Nanjing Medical University, Changzhou, Jiangsu, China.
⁴Department of Gastroenterology, The People’s Hospital of Wuqia, Xinjiang, China.

These authors made equal contributions to this study.
*Corresponding authors:
Liang Ma, Department of Gastroenterology, The First People’s Hospital of Changzhou, The Third Affiliated Hospital of Soochow University, Changzhou, Jiangsu, 213000, People’s Republic of China. Tel: 86-150061610635, Fax: +86 0519 88112137. E-mail: mmaliang212@126.com.
Liwen Zhang, Department of Pediatrics, the Second People’s Hospital of Changzhou, Affiliate Hospital of Nanjing Medical University, Changzhou, Jiangsu, China, 213000, People’s Republic of China. Tel: 86-15006121810, Fax: +86 0519 88065524, E-mail: zhangliwenlove827@126.com.

**Abstract**

The present work aims to evaluate the efficacy of Live Combined Bifidobacterium, Lactobacillus and Enterococcus Capsules (LCBLECs), a probiotic drug containing *Bifidobacterium*, in the treatment of autoimmune hepatitis (AIH). In this study, a mouse model of experimental autoimmune hepatitis (EAH) was established to investigate the effects of LCBLECs on AIH. The results showed that LCBLECs improved dysbiosis of gut microbiota, reduced liver injury, restored liver function, and maintained Treg/Th17 balance in EAH mice. In addition, LCBLECs restored Treg/Th17 balance in EAH mice by downregulating IL-33 production. Besides, LCBLECs also suppress IL-33 upregulation in EAH mice by inhibiting the TLR2/4 signaling pathway. Furthermore, LCBLECs also mitigated dysbiosis of gut microbiota and enhanced the efficacy of conventional treatment for AIH patients. To sum up, our findings revealed that LCBLECs exerted therapeutic effects on EAH mice by improving Treg/Th17 imbalance in an IL-33-dependent manner via the TLR2/4 signaling pathway and relieved the clinical symptoms of AIH patients, indicating *Bifidobacterium* supplementation with LCBLECs might be a potential adjuvant therapy for AIH treatment.

**Keywords:** Bifidobacterium, autoimmune hepatitis, IL-33, Treg/Th17 imbalance, TLR2/4
Introduction

Autoimmune hepatitis (AIH) is a chronic inflammatory liver disease characterized by interface hepatitis, circulating autoantibodies, and hyper-gammaglobulinemia (Yuksel et al., 2015). If left untreated, AIH may develop rapidly into liver cirrhosis and failure (Durazzo et al., 2019). Although AIH etiology and pathogenesis remain largely unknown, it is generally believed that AIH usually occurs in genetically susceptible hosts as a consequence of excessive immune responses towards liver tissue (van Gerven et al., 2016). Regulatory T cells (Tregs) play a crucial role in the maintenance of immune balance to prevent autoimmune diseases (Scheinecker et al., 2020). As a member of the T-helper effector cell family, interleukin IL-17-producing Th cells (Th17s) can induce inflammation and autoimmune tissue injury by promoting production of proinflammatory cytokines (Singh et al., 2013). Studies have reported that Treg/Th17 imbalance contributes to liver injury in the pathological process of liver diseases (Zhang et al., 2019; Lu et al., 2021; Zhao et al., 2022). Recently, it has been demonstrated that Treg/Th17 imbalance is associated with poor prognosis of AIH patients (Vuerich et al., 2021). However, the underlying mechanism of Treg/Th17 imbalance in AIH remains to be further understood.

As key organs in nutrient absorption and metabolism, the gut and the liver are closely connected through the biliary tract, portal vein and systemic circulation (Zeuzem, 2000; Nie et al., 2021). This bidirectional interaction between gut and the liver is called the gut–liver axis (Song and Zhang, 2022). As evidenced by previous studies, the alteration of gut microbiota composition plays a crucial role in the cross-talk of the gut–liver axis and is closely associated with inflammation, oxidative stress, and lipid deposition in the liver (Meng et al., 2018). Studies have also shown that the gut–liver axis exists and plays a crucial role in the development of liver diseases, including AIH (Wang et al., 2022b). It is widely recognized that probiotics are beneficial regulators of gut microbiota, among which Bifidobacterium is a probiotic that has been widely studied and utilized to inhibit the growth of pathogenic bacteria in the intestinal tract, maintain intestinal health, and protect the intestinal barrier (Wang et al., 2022a). In addition, prophylactic administration of Bifidobacterium also has a favorable impact on AIH (Zhang et al., 2020). Although it has been reported that Bifidobacterium can improve immune function by regulating Th17/Treg balance (Dong et al., 2022; Sapra et al., 2022), it is still unclear whether Bifidobacterium can protect against AIH by improving Th17/Treg imbalance.

The present study aimed to investigate the efficacy and the specific mechanisms of Bifidobacterium in AIH treatment using Live Combined Bifidobacterium, Lactobacillus and Enterococcus Capsules (LCBLECs), a probiotic mix containing Bifidobacterium. Based on the results of LCBLEC administration for experimental autoimmune hepatitis (EAH) mice and AIH patients, it was found that LCBLECs alleviated the symptoms of EAH mice and promoted the efficacy of conventional treatment for AIH patients, suggesting that oral supplementation of Bifidobacterium might exhibit potential therapeutic effects on AIH.
**Materials and Methods**

**Patients**

20 AIH patients at active phase were selected from AIH patients diagnosed in The Third Affiliated Hospital of Soochow University. AIH diagnosis was based on Simplified Criteria for the Diagnosis of Autoimmune Hepatitis promulgated by International Autoimmune Hepatitis Group (IAIHG) in 2008 and Practice Guidelines for Diagnosis and Management of Autoimmune Hepatitis promulgated by American Association for the Study of Liver Disease (AASLD) in 2010. Criteria for AIH patients at active phase: 1) fatigue, arthralgia, jaundice; 2) serum alanine aminotransferase (ALT) level > 10-fold of upper limit of normal (ULN); 3) serum aspartate aminotransferase (AST) level ≥ 5-fold of ULN, and γ-globulin level ≥ 2-fold of ULN; 4) histological findings of bridging necrosis or multinuclear necrosis. Each patient enrolled provided written informed consent. The study was approved by the Ethics Committee of The Third Affiliated Hospital of Soochow University.

The enrolled patients were randomly assigned to Group I (conventional treatment; n=10) and Group II (conventional treatment + LCBLECs; n=10). All AIH patients in Group II were given LCBLECs (0.66g × 3/d t.i.d.). 2 weeks later, serum samples and fecal samples were collected from AIH patients in Group I and Group II for further analysis.

**Animal study**

Wildtype (WT) and IL-33 knockout (IL-33−/−) female C57BL/6 mice (6-8 weeks old; 18-20g) were purchased from Model Animal Research Center of Nanjing University and kept in specific pathogen-free conditions. Mice were randomly divided into six groups (n=6): Control (WT), EAH (WT), EAH (WT)+LCBLECs, Control (IL-33−/−), EAH (IL-33−/−), and EAH (IL-33−/−)+LCBLECs. To induce EAH, mice received intraperitoneal injection of S-100 liver antigen emulsified in complete Freund’s adjuvant (CFA) on Day 0 and Day 7, as previously described (Liang et al., 2021). From day 15 to day 28, LCBLECs were administered by gavage (0.345 g/kg/day) (Zhao et al., 2013). Finally, all mice were sacrificed to collect serum samples, liver tissues and feces for further analysis. All animal procedures were permitted by Ethics Committee of The Third Affiliated Hospital of Soochow University (Approval No.: [2019]KY048-01).

**Hematoxylin and eosin (H&E) staining**

H&E staining was applied for histopathological evaluation of liver tissues. In brief, liver tissues were fixed in 4% paraformaldehyde for 48 h, embedded in paraffin, prepared into sections (4 µm), deparaffinized in xylene, and rehydrated. Then, the sections were examined under light microscopy for tissue injury, necrosis, and infiltrating leukocytes.
**Biochemical analysis**
ALT, aspartate aminotransferase (AST), Immunoglobulin G (IgG), Immunoglobulin M (IgM), and Immunoglobulin A (IgA) levels in serum samples from mice and AIH patients were determined using the automated chemistry analyzer (BioMajesty, Japan).

**RT-qPCR**
To detect abundance of gut microbiota in fecal samples, bacterial genomic DNA was extracted from fecal samples and purified for RT-qPCR analysis. The abundances of *Lactobacillus*, *Bifidobacterium*, *Clostridium leptum*, and *Bacteroides fragilis*, *Escherichia coli* were quantified by RT-qPCR in StepOne™ Real-Time PCR System using SYBR® Premix Ex Taq™ reagents. To detect gene expression, total RNA was extracted from liver tissues of mice using TRIzol (Invitrogen, CA) and reversely transcribed into cDNA using Quantitect Reverse Transcription Kit (Qiagen, USA). Then, RT-qPCR was performed in StepOne™ Real-Time PCR System using SYBR® Premix Ex Taq™ reagents. Relative gene expression was normalized to β-actin and quantified by $2^{-\Delta\Delta Ct}$ method. The primers used for RT-qPCR were as follows in Table 1.

**ELISA**
The concentrations of IL-33 and its soluble receptor ST2 (sST2) in serum samples from AIH patients and liver tissue supernatant from mice were determined using IL-33/sST2 ELISA kit (R&D Systems, USA) as per manufacturer’s instructions.

**Gas chromatography-mass spectrometry (GC-MS)**
The concentrations of short-chain fatty acids (SCFAs) in fecal samples from mice and AIH patients were determined by GC-MS as previously described (Jiang et al., 2020).

**Flow cytometry**
Flow cytometry was applied to detect Th17s and Tregs in liver tissues. Briefly, liver tissues were aseptically removed from mice and prepared into monocyte suspensions. After intracellular staining with specific anti-cytokine antibodies, the number of Th17s (CD3⁺CD4⁺IL-17a⁺) and Tregs (CD3⁺CD4⁺CD25⁺FOXP3⁺) was determined by FACS flow cytometry. The antibodies used for flow cytometry analysis were as follows: anti-CD3 (ab11089; Abcam), anti-CD4 (ab133616; Abcam), anti-IL-17a (ab302922; Abcam), anti-CD25 (Cat. No.: 561780; R&D Systems), and anti-FOXP3 (ab215206; Abcam).
**Western blotting**

Total protein was isolated from liver tissue by RIPA Buffer (Beyotime), separated by 10% SDS/PAGE and electroblotted onto PVDF membranes. Next, PVDF membranes were blocked with 5% skimmed milk. Then, the membranes were incubated with primary antibody at 4°C overnight and treated with secondary antibody at 37 °C for 45 min. Finally, the protein bands were visualized using BeyoECL Plus (Beyotime). The protein level of sST2 was detected by using an anti-ST2 antibody. Primary antibodies used for western blotting assay were: anti-IL-33 antibody (ab187060; Abcam), anti-ST2 antibody (ab228543), anti-TLR2 antibody (ab213676; Abcam), anti-TLR4 antibody (Cat. No.: 48-2300; Invitrogen), anti-MyD88 antibody (ab219413; Abcam), anti-IRAK1 antibody (ab238; Abcam), anti-TRAF6 antibody (ab33915; Abcam), anti-NF-κB p-p65 antibody (ab76302; Abcam), anti-NF-κB p65 antibody (ab32536; Abcam), and anti-GAPDH antibody (ab8245; Abcam). Secondary antibodies used were: Anti-Rabbit/Mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (ab97085, ab98799; Abcam).

**Statistical analysis**

All data were expressed as mean±SD. Comparisons were performed by one-way Analysis of Variance (ANOVA) using SPSS 19.0 (SPSS Inc., USA). Graphs were plotted using GraphPad Prism 6.0. Values with p < 0.05 were deemed significant in statistics.

**Results**

**LCBLECs ameliorate dysbiosis of gut microbiota in EAH mice**

EAH mice exhibited a significant decrease in *Lactobacillus*, *Bifidobacterium*, *Clostridium leptum*, and *Bacteroides fragilis* levels and a significant increase in *Escherichia coli* level compared with mice in the control group, which was substantially abated by LCBLEC administration (Fig. 1A). As important metabolites of gut microbiota, SCFAs, including acetic acid, butyric acid, and propionic acid, are deeply involved in immune regulation and autoimmunity by regulating the generation of Th17s and Tregs (Haase et al., 2018). Therefore, acetic acid, butyric acid, and propionic acid levels in feces of mice from each group were measured. As indicated in Fig. 1B-D, LCBLECs significantly eliminate the reduction of acetic acid, butyric acid, and propionic acid levels in EAH mice. Therefore, LCBLECs effectively mitigated EAH-induced dysbiosis of gut microbiota.

**LCBLECs alleviate EAH severity in mice**

To explore the effect of LCBLECs on AIH progression, we established an EAH model based on C57BL/6 mice. H&E staining results showed that LCBLECs significantly attenuated EAH-induced liver injury, including destruction of liver structure, large areas of necrosis, and infiltration of inflammatory cells, in mice (Fig. 2A). In addition,
serum levels of liver enzymes (ALT and AST) and autoantibodies (IgG, IgA, and IgM) were also determined. As shown in Fig. 2B-F, LCBLECs partially reversed the elevation of ALT, AST, IgG, IgA, and IgM levels in serum samples of EAH mice. The above results demonstrated that LCBLECs exerted liver-protective effects on EAH mice.

**LCBLECs relieve Treg/Th17 imbalance in EAH mice**

It has been demonstrated that *Bifidobacterium* could restore Treg/Th17 imbalance in autoimmune diseases (Lopez *et al.*, 2016). To explore the specific effects of LCBLECs on Treg/Th17 balance in EAH mice, flow cytometry was applied to detect the frequencies of Tregs and Th17s in liver tissues of mice. The results showed that LC BLEC treatment markedly increased the percentages of Tregs and reduced the number of Th17s in liver tissues from EAH mice (Fig. 3A and B). These results indicated that LC BLECs might help restore Treg/Th17 balance in liver tissues from EAH mice.

**LCBLECs regulate Treg/Th17 imbalance in an IL-33-dependent manner**

As a new member of the IL-1 family, IL-33 plays a critical role in AIH pathogenesis and severity (Abe *et al.*, 2019). In addition, IL-33 has also been identified as a key factor for maintenance of Treg/Th17 balance in numerous diseases (Cheng *et al.*, 2022). Hence, it was hypothesized that LC BLECs might regulate Treg/Th17 balance in EAH mice via modulating IL-33 secretion. It was shown that LC BLECs inhibited IL-33 and sST2 levels in liver tissues (Fig. 4A and B) and serum samples (Fig. 4C) from EAH mice. Next, we used IL-33−/− mice to further investigate the role of IL-33 in the restorative effects of LC BLECs on EAH-induced Treg/Th17 imbalance. Treatment with LC BLECs reversed EAH-induced reduction of Tregs and increase of Th17s in liver tissues from WT mice, while IL-33−/− mice showed few changes in the frequencies of Tregs and Th17s (Fig. 4D and E). Therefore, LC BLECs relieved Treg/Th17 imbalance EAH mice via IL-33.

**LCBLECs suppress EAH-induced IL-33 upregulation by deactivating the TLR2/4 signaling pathway**

Toll-like receptors (TLRs), a group of pattern-recognition receptors (PRRs), are essential regulators of both innate and adaptive immune responses (Yu *et al.*, 2017). Among them, TLR2 and TLR4 have been identified as key regulators of signaling pathways in AIH (Cai *et al.*, 2022; Zhou *et al.*, 2022). *Bifidobacterium* has been identified as an inhibitor of both TLR2 and TLR4 signaling pathway (Al-Sadi *et al.*, 2021; Chen *et al.*, 2021). Therefore, the expression of related genes and proteins in TLR2/TLR4 signaling pathway were detected. As shown in Fig. 5A, TLR2 and TLR4, MyD88, IRAK1 and TRAF6 mRNA expressions significantly increased in liver tissues from EAH mice, which was effectively
abrogated by treatment with LCBLECs. Furthermore, Western blotting results indicated that LCBLECs exerted inhibitory effects on TLR2, TLR4, MyD88, IRAK1, TRAF6, and NF-κB p-p65 protein levels in EAH mice (Fig. 5B). Taken together, LCBLECs might repress IL-33 upregulation in EAH mice by inhibiting the hyperactivation of the TLR2/4 signaling pathway.

**LCBLECs improve clinical symptoms of AIH patients**

To determine the clinical effect of LCBLECs on AIH, AIH patients were randomly assigned to Group I (conventional treatment; n=10) and Group II (conventional treatment + LCBLECs; n=10). AIH patients in Group II were orally administrated with LCBLECs (0.66g×3/d t.i.d.). As shown in Fig. 6A, AIH patients in Group II exhibited higher *Lactobacillus*, *Bifidobacterium*, *Clostridium leptum*, and *Bacteroides fragilis* levels and lower *Escherichia coli* level than AIH patients in Group I. Similarly, LCBLECs further increased acetic acid, butyric acid, and propionic acid levels in feces from AIH patients (Fig. 6B). Biochemical results revealed that LCBLEC administration further promoted the reduction of ALT, AST, IgG, IgA, and IgM levels in AIH patients induced by conventional treatment (Fig. 6C-G). Moreover, LCBLEC treatment further decreased serum IL-33 and sST2 levels of AIH patients, compared with conventional treatment (Fig. 6H and I). Taken together, cotreatment with LCBLECs exhibited better effects on clinical symptoms of AIH patients than conventional treatment alone.

**Discussion**

AIH incidence has been rising in recent years, which is a significant threat to human health globally (Feld and Heathcote, 2003). Therefore, it is necessary to find novel and effective therapeutic agents for AIH treatment. Probiotics exert beneficial effects in the prevention and treatment of autoimmune disorders by restoring altered gut microbiota, maintaining intestinal barrier, and regulating immune homeostasis (Shahbazi et al., 2020). As a major genus of intestinal probiotics, *Bifidobacterium* has been studied for its immunoregulatory role in autoimmune diseases (Rinaldi et al., 2019). However, there were only few studies on the application of *Bifidobacterium* in AIH treatment. In this work, LCBLECs substantially improved EAH-induced dysbiosis of gut microbiota in mice, as evidenced by the increase in *Lactobacillus*, *Bifidobacterium*, *Clostridium leptum*, and *Bacteroides fragilis* population and the decrease in *Escherichia coli* population. In addition, LCBLECs reduced ALT, AST, IgG, IgA, and IgM levels in serum samples from EAH mice, indicating LCBLECs might alleviate EAH severity by reducing liver injury and restoring liver function. Furthermore, LCBLECs also restored the altered gut microbiota in AIH patients and significantly improved the efficacy of conventional treatment for AIH patients. The above results suggested that LCBLECs altered the composition of gut microbiota, thereby exerting liver-protective effects in AIH. Previous studies have reported the crucial role of the Treg/Th17 balance in
autoimmune diseases (Lee, 2018). Our team has also previously reported the importance of the Treg/Th17 balance in AIH pathogenesis (Liang et al., 2018). In addition, *Bifidobacterium* has been proven as an important regulator of Treg/Th17 balance in autoimmune diseases (Shi et al., 2018). Therefore, we explored how *Bifidobacterium* affect Treg/Th17 balance in AIH progression. The results showed that LCBLECs reversed the reduction of Tregs and the increase of Th17s in liver tissues from EAH mice. These results indicated that LCBLECs may exert therapeutic effects on AIH by maintaining the Treg/Th17 balance. As documented in previous studies, IL-33 plays an important role in AIH pathogenesis and severity (Abe et al., 2019). In addition, IL-33 also serves as an inducer for Treg/Th17 imbalance in LPS-stimulated acute respiratory distress syndrome (Cheng et al., 2022). Hence, we assumed that LCBLECs might restore Treg/Th17 balance in EAH mice via inhibiting IL-33 production. Our results confirmed that administration with LCBLECs effectively abated EAH-induced increase of IL-33 and sST2 levels. Besides, LCBLEC treatment further promoted the decrease in serum IL-33 and sST2 levels of AIH patients caused by conventional treatment. Furthermore, LCBLEC treatment significantly increased the frequencies of Tregs and reduced the frequencies of Th17s in IL-33−/− mice; however, the frequencies of Tregs and Th17s in IL-33−/− mice had barely changed, implying LCBLECs regulated the balance between Tregs and Th17s in EAH mice via IL-33.

TLRs play critical roles in the innate recognition of pathogens besides bridging both innate and adaptive immune responses (Kawai and Akira, 2011). Among them, TLR2 and TLR4 have been identified as key regulators of autoimmune response in AIH (Chi et al., 2018). Therefore, we wondered whether TLR2 or TLR4 signaling was involved in LCBLEC-mediated downregulation of IL-33 in EAH mice. Herein, LCBLECs decreased TLR2, TLR4, MyD88, IRAK1 and TRAF6 mRNA expression in liver tissues from EAH mice. In addition, TLR2, TLR4, MyD88, IRAK1, TRAF6, and NF-κB p-p65 protein levels also declined after treatment with LCBLECs. These results indicated that LCBLECs might reinstate the Treg/Th17 balance in EAH mice by regulating IL-33 production via the TLR2/4 signaling pathway.

The EAH model used in this study is a chronic EAH model which has been widely applied in the exploration of the potential mechanisms of AIH (Ma et al., 2007). As an acute EAH model, Concanavalin A (ConA)-induced EAH model has also been frequently used in the investigation of T-cell function in AIH (Zheng et al., 2018). In this study, we have demonstrated the protective role of LCBLECs in a chronic EAH model. Therefore, we can speculate that LCBLECs may also exert therapeutic effects in ConA-induced EAH, which will be further verified in future studies.

**Conclusion**

To sum up, our findings revealed that LCBLECs exerted therapeutic effects on EAH mice by improving dysbiosis of gut microbiota and Treg/Th17 balance. In addition, it
was also demonstrated that LCBLECs restored Treg/Th17 balance in EAH mice in an IL-33-dependent manner via the TLR2/4 signaling pathway. Furthermore, LCBLECs also mitigated dysbiosis of gut microbiota and enhanced the efficacy of conventional treatment for AIH patients. Hence, *Bifidobacterium* supplementation with LCBLECs might be a potential adjuvant therapy for AIH treatment.

**Funding**

This study was sponsored by grants from the Top Talent of Changzhou “The 14th Five-Year Plan” High-Level Health Talents Training Project (2022260), the Changzhou Sci&Tech Program (Grant No. CZQM2020011; CZQM2020079), Natural Science Foundation of Xinjiang Uygur Autonomous Region (2021D01F64), the Applied Basic Research Programs of Science, Technology Department of Changzhou city (CJ20210109; CJ20190095; CJ20160031), Natural Science Foundation of Xinjiang Uygur Autonomous Region (2022D01A307), Xinjiang Kirgiz Autonomous Prefecture Medical and Health Technology Project (Grant No. 43; No.44); the National Natural Science Foundation of China (No. 81700500).

**References**


Song Q. and Zhang X. (2022). The role of gut-liver axis in gut microbiome dysbiosis associated NAFLD and NAFLD-HCC. Biomedicines 10, 524.


Yuksel M., Wang Y., Tai N., Peng J., Guo J., Beland K., Lapierre P., David C., Alvarez...
Zhang H., Jiang Z. and Zhang L. (2019). Dual effect of t helper cell 17 (Th17) and regulatory T cell (Treg) in liver pathological process: From occurrence to end stage of disease. Int. Immunopharmacol. 69, 50-59.

Figure Legends
Figure 1. LCBLECs ameliorate dysbiosis of gut microbiota in EAH mice. (A) Lactobacillus, Bifidobacterium, Clostridium leptum, Bacteroides fragilis, and Escherichia coli levels in feces of mice from Control (WT) (n=5), EAH (WT) (n=5), and EAH (WT)+LCBLECs (n=5) groups by RT-qPCR. (B) acetic acid, butyric acid, and propionic acid levels in feces of mice by GC-MS. *P < 0.05; **P < 0.01.

Figure 2. LCBLECs alleviate EAH severity in mice. (A) HE staining of liver tissues of mice from Control (WT) (n=5), EAH (WT) (n=5), and EAH (WT)+LCBLECs (n=5) groups. (B-F) serum levels of liver enzymes (ALT and AST) and autoantibodies (IgG, IgA, and IgM) by biochemical analysis. *P < 0.05; **P < 0.01.

Figure 3. LCBLECs relieve Treg/Th17 imbalance in EAH mice. (A and B) Detection of the frequency of Treg (CD3+CD4+CD25+FOXP3+) and Th17 (CD3+CD4+IL-17a+) cells by flow cytometry. *P < 0.05; **P < 0.01.
Figure 4. LCBLECs regulate Treg/Th17 imbalance in an IL-33-dependent manner. (A and B) IL-33 and sST2 mRNA and protein levels in liver tissues of mice from Control (WT) (n=5), EAH (WT) (n=5), and EAH (WT)+LCBLECs groups (n=5) by RT-qPCR and Western blotting. (C) Serum IL-33 and sST2 levels by ELISA. (D and E) Detection of the frequency Treg (CD3+CD4+CD25+FOXP3+) cells and Th17 (CD3+CD4+IL-17a+) cells in liver tissues of mice from Control (WT) (n=5), EAH (WT) (n=5), EAH (WT)+LCBLECs (n=5), Control (IL-33−/−) (n=5), EAH (IL-33−/−) (n=5), and EAH (IL-33−/−)+LCBLECs (n=5) groups by flow cytometry. *P<0.05; **P<0.01.

Figure 5. LCBLECs suppress EAH-induced IL-33 upregulation by deactivating the TLR2/4 signaling pathway. (A) TLR2, TLR4, MyD88, IRAK1 and TRAF6 mRNA expressions in liver tissues of mice from Control (WT) (n=5), EAH (WT) (n=5), and EAH (WT)+LCBLECs (n=5) groups by RT-qPCR. (B) TLR2, TLR4, MyD88, IRAK1, TRAF6, NF-κB p65, and NF-κB p-p65 protein levels in liver tissues by Western blotting. *P<0.05; **P<0.01.

Figure 6. LCBLECs improve clinical symptoms of AIH patients. (A) Lactobacillus, Bifidobacterium, Clostridium leptum, Bacteroides fragilis, and Escherichia coli levels in feces of AIH patients from Group I (n=10) and Group II (n=10). (B) acetic acid, butyric acid, and propionic acid levels in feces from AIH patients. (C-G) ALT, AST, IgG, IgA, and IgM levels in serum samples from AIH patients. (H and I) IL-33 and sST2 levels in serum samples from AIH patients. *P<0.05; **P<0.01.

Table 1. Primers used for RT-qPCR analysis of gene expression.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence 5′-3′</th>
</tr>
</thead>
</table>
| IL-33 | Forward: TTCCTGTCTGTATTTGAGAAACCT  
   Reverse: TTTGCCGGGGAAATCTTGGG     |
| sST2  | Forward: CCAGCCCTTCATCTGGGTATC  
   Reverse: ATGGGTGTGTTCTAGGGCGG   |
| TLR2  | Forward: ATCCCCCTTCATCTGGGTATC  
   Reverse: GCCCGGAGCTAGGAGGTA   |
| TLR4  | Forward: TCCCTGATAGAGGTAGCCCTAGAG  
   Reverse: TCAAGGGGTTGAAGCTCAGA |
| MyD88 | Forward: GATGACCCCCTAGGACAAACG  
   Reverse: ACTCGATATCGTTGGGGCAG |
| IRAK1 | Forward: CCTCAGATCCCCAGAACAGGTAT  
   Reverse: AGTTTCTCTGTGGGGCAAGGG |
| TRAF6 | Forward: GGAGTTTGGACCACCTCTTG  
   Reverse: CTTGTGCCCTGCACTCCCTTA |
## A

<table>
<thead>
<tr>
<th>Gut microbiota</th>
<th>Control (WT)</th>
<th>EAH (WT)</th>
<th>EAH+LCBLECs (WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>1±0.125</td>
<td>0.313±0.103</td>
<td>0.642±0.105</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>1±0.161</td>
<td>0.269±0.213</td>
<td>0.651±0.113</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td>1±0.201</td>
<td>0.356±0.113</td>
<td>0.613±0.121</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>1±0.134</td>
<td>0.312±0.104</td>
<td>0.689±0.211</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1±0.113</td>
<td>2.131±0.211</td>
<td>1.377±0.131</td>
</tr>
</tbody>
</table>

## B

- Acetic acid (mmol/g): EAH (WT) > Control (WT)
- Butyric acid (mmol/g): EAH (WT) > Control (WT)
- Propionic acid (mmol/g): EAH+LCBLECs (WT) > Control (WT)

- EAH (WT) vs Control (WT): **p < 0.01
- EAH+LCBLECs (WT) vs Control (WT): **p < 0.001
- EAH+LCBLECs (WT) vs Control (WT): *p < 0.05
- EAH (WT) vs Control (WT): *p < 0.05
**Gut microbiota**

<table>
<thead>
<tr>
<th>Gut microbiota</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>1±0.113</td>
<td>3.135±0.312</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>1±0.124</td>
<td>2.556±0.224</td>
</tr>
<tr>
<td>Clostridium leptum</td>
<td>1±0.136</td>
<td>2.846±0.288</td>
</tr>
<tr>
<td>Bacteroides fragilis</td>
<td>1±0.152</td>
<td>2.935±0.301</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1±0.115</td>
<td>0.216±0.019</td>
</tr>
</tbody>
</table>

**Analyte**

- **Acetic acid (mmol/g)**
  - Group I: [Data]
  - Group II: [Data]

- **Butyric acid (mmol/g)**
  - Group I: [Data]
  - Group II: [Data]

- **Propionic acid (mmol/g)**
  - Group I: [Data]
  - Group II: [Data]