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Mesenchymal stem/stromal cells armored by FGF21 ameliorate alcohol-induced liver injury through modulating polarization of macrophages

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Abstract

Background: Alcohol-associated liver disease (ALD) is a major health care challenge worldwide with limited therapeutic options. Although mesenchymal stem/stromal cells (MSCs) represent a newly emerging therapeutic approach to treat ALD, thus far, there have been extensive efforts to try and enhance their efficacy, including genetically engineering MSCs. FGF21, an endocrine stress-responsive hormone, has been shown to regulate energy balance, glucose, and lipid metabolism and to enhance the homing of MSCs toward injured sites. Therefore, the purpose of this study was to investigate whether MSCs that overexpress FGF21 (FGF21-MSCs) improve the therapeutic effect of MSCs in treating ALD.

Methods: Human umbilical cord-derived MSCs served as the gene delivery vehicle for the FGF21 gene. Human umbilical cord-derived MSCs were transduced with the FGF21 gene using lentiviral vectors to mediate FGF21 overexpression. We utilized both chronic Lieber-DeCarli and Gao-binge models of ethanol-induced liver injury to observe the therapeutic effect of FGF21-MSCs. Liver injury was phenotypically evaluated by performing biochemical methods, histology, and inflammatory cytokine levels.

Results: Compared with MSCs alone, administration of MSCs over-expressing FGF21(FGF21-MSCs) treatment significantly enhanced the therapeutic effect of ALD in mice, as indicated by the alleviation of liver injury with reduced steatosis, inflammatory infiltration, oxidative stress, and hepatic apoptosis, and the promotion of liver regeneration. Mechanistically, FGF21 could facilitate the immunomodulatory function of MSCs on macrophages by

Abbreviations: AF, EtOH-fed; AH, alcohol-associated hepatitis; ALD, alcohol-associated liver disease; BMDMs, bone marrow-derived macrophages; CCL2, C-C motif chemokine ligand 2; FGF21-MSCs, MSCs overexpressing FGF21; LPS, lipopolysaccharide; MSCs, mesenchymal stem/stromal cells.

Qian Huai and Cheng Zhu contributed equally to this work.

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setting metabolic commitment for oxidative phosphorylation, which enables macrophages to exhibit anti-inflammatory inclination.

Conclusions: Our data elucidate that MSC modification by FGF21 could enhance their therapeutic effect in ALD and may help in the exploration of effective MSCs-based cell therapies for the treatment of ALD.

INTRODUCTION

Alcohol use disorder is a major cause of advanced liver disease worldwide and substantially contributes to a social and economic burden.[1] Alcohol-associated liver disease (ALD) encompasses a spectrum of pathological features, progressing from steatosis, to steatohepatitis, fibrosis, and ultimately cirrhosis, all of which may result in an acute hepatic inflammatory condition termed alcohol-associated hepatitis (AH). Once developed, AH can progress to cirrhosis and liver cancer.[2] Severe AH represents an acute and often devastating form of all ALD pathologies and is also characterized by a sudden onset of jaundice and clinical signs of hepatic decompensation, along with an intense systemic inflammatory response and high short-term mortality.[3] Despite our growing biological understanding of ALD, the effective treatment of AH remains challenging.

Chronic inflammation plays a pivotal role in the pathogenesis of AH. Alcohol exposure causes multiple inflammatory processes accompanied by signals that coordinate the recruitment of immune cells and the activation of inflammasomes, as well as changes in the expression of cytokines and chemokines.[4] Of these, the accumulation of immune cells is a histological hallmark of liver inflammation and is correlated with disease progression. Noticeably, multiple types of immune cells are involved in the pathogenesis of AH, including neutrophils, resident and infiltrating macrophages, and other cell types in innate and adaptive immune systems.[4] In mild and chronic AH, the number of hepatic macrophages increases, and infiltrating monocyte-derived macrophages are believed to contribute to this expansion and the pathogenesis of AH.[5] A potential mechanism of alcohol-induced immunomodulatory function is through alterations in the gut microbiome, disruption of gut barrier integrity, and translocation of bacterial products and metabolites, including lipopolysaccharide (LPS), into the systemic circulation, which can contribute to inflammation and organ damage, particularly in the liver.[6] For example, binge alcohol consumption causes leakage of gut-derived LPS, leading to Toll-like receptor 4-induced signaling in liver macrophages, including both resident and infiltrating macrophages.[7] In ALD, macrophages are hypersensitive to LPS, leading to increased inflammatory responses, which are commonly classified as M1 macrophages, as opposed to M2 macrophages, which usually arise in Th2 responses in wound healing. Activated M1 macrophages produce high amounts of pro-inflammatory cytokines such as TNF, IL-1β, IL-12, IL-18 and IL-23, which help to induce Th1 and Th17 cell inflammatory responses, thereby promoting inflammation. In contrast, activated M2 macrophages secrete large amounts of IL-10, IL-1R antagonist, and TGF-β, subsequently suppressing inflammation and promoting tissue repair.[4] Therefore, the regulation of cell fate determination of M1 and M2 macrophages in the evolution and progression of ALD is an obvious area of research interest. Of note, alcohol-induced hepatocyte injury and pro-inflammatory activation of hepatic macrophages initiate a cytokine storm, which in turn propagates the recruitment of leukocytes, especially neutrophils that perpetuate hepatic inflammation.[8] Thus, modulating the inflammatory response is a promising therapeutic strategy for the improvement of ALD. While patients with severe AH are treated with corticosteroids as first-line anti-inflammatory medications, the use of corticosteroids in AH is linked to significant side effects, including fungal infections, which limit its potential. Given that many immune cells and inflammatory factors play a dual role in liver injury and regeneration, an all-encompassing treatment approach is required, as opposed to merely inhibiting or stimulating inflammatory responses. Although new therapies are undergoing evaluation, relatively few effective and safe treatments are available for ALD.

Mesenchymal stem/stromal cell (MSC)-based cellular therapy has progressed from a skeptical idea to a viable clinical option. The safety and efficacy of MSCs have been shown in a range of clinical settings, including autoimmune diseases, organ failure, GvHD and COVID-19, while also being reported to ameliorate liver injury in the context of both acute and chronic liver diseases.[9–11] Tellingly, several clinical data have shown that MSCs can safely improve clinical outcomes of patients with alcohol-associated cirrhosis.[12] The pleiotropic effects of MSCs represent a potential advantage over pharmacological therapies and principally not only focus on their limited differentiation potentials but, more importantly, on their role in immunomodulation, resulting in a favorable immune microenvironment and releasing growth factors to activate endogenous tissue repair.[13] Of note, MSCs can modulate injurious immune responses and reduce the occurrence of cytokine storm, which were the major causes of liver injury that led to the progression of

severe AH. While MSCs alone have been the mainstay of therapeutic studies, thus far, there have been extensive efforts to try and enhance their efficacy, including enrichment and/or priming of MSCs along with genetic engineering of cells.[11] In humanized mice, preconditioning MSCs with cytokines can dramatically reduce acute liver injury caused by alcohol consumption, leading to a notable improvement in both survival and hepatic function.[14] In addition, engineered MSCs can be armed as "Trojan horses" to safely deliver cytokines, including TRAIL, type I IFNs, and IL-2.[15–17] Thus, the versatile development of engineering strategies in MSC-based clinical applications contributes to strengthening innate functions (eg, immunomodulatory and regenerative properties) and expanding the therapeutic scope of MSCs, thereby maximizing clinical potency in treating diseases.

FGF21 is an atypical member of FGF family that can enter the circulation and act in an endocrine manner.[18] Once secreted, FGF21 elicits its biological effects by binding and activating a receptor complex composed of the co-receptor KLB and a conventional FGF receptor tyrosine kinase (FGFR1c).[19] The metabolic action of FGF21 is pleiotropic, including lipid and glucose metabolism and energy expenditure.[20] Starvation, protein deficiency, simple sugars, and ethanol, all induce circulating FGF21 levels in humans.[21] FGF21 signaling to the central nervous system is important for its effects on regulating energy balance and macronutrient preference.[22] Moreover, FGF21 signaling has also been shown to regulate alcohol consumption. Endogenous FGF21 and pharmacologic administration of FGF21 suppress alcohol consumption in rodents and nonhuman primates through direct FGF21 signaling to the central nervous system.[23,24] However, FGF21 has a very short half-life; FGF21 analogs were designed to have a longer half-life than native FGF21 while recapitulating the receptor activity profile of the native hormone. Accordingly, clinical trials with FGF21 analogs have consistently demonstrated improvements in lipids in both healthy volunteers and patients with NASH or diabetes.[25,26] Engineered MSCs that consistently overexpress FGF21 maintain high and stable levels of FGF21, improve migration to injured sites, and decrease MSC apoptosis.[27,28] Thus, MSCs overexpressing FGF21 (FGF21-MSCs) may confer a promising genetic modification to maximize MSCs-based cellular therapy.

The therapeutic potential of FGF21 and MSCs in ALD has already been investigated; however, a paucity of literature has addressed the therapeutic effects of engineered MSCs that overexpress FGF21 in the treatment of ALD. In the current study, we tested the hypothesis that FGF21-MSCs are a superior tool for improving the therapeutic effect of MSCs on ALD. We found that FGF21-MSCs administration greatly ameliorated alcohol-induced liver injury in both Gao-binge and chronic ethanol-feeding models of ALD, as demonstrated by reduced steatosis, reduced hepatocyte death, reduced hepatic inflammation and oxidative stress, and increased liver regeneration. Mechanistically, we demonstrated that FGF21 could facilitate the immunomodulatory function of MSCs on macrophages by setting metabolic commitment for oxidative phosphorylation, which enables macrophages to exhibit anti-inflammatory inclination. Our novel observations suggest that MSC modification by FGF21 could enhance their therapeutic effect in ALD and may help in the exploration of effective MSCs-based cell therapies for the treatment of ALD.

METHODS

Mice

Male C57BL/6J wild-type mice were purchased from the Experimental Animal Center of Anhui Medical University. All animal experiments were approved by the Animal Ethics Committee of Anhui Medical University (No. LLSC20231222). The mice were housed in a pathogen-free and temperature-regulated facility with a 12-hour light-dark cycle. Both the National Institute on Alcohol Abuse and Alcoholism model of ALD and the chronic ethanol-feeding model were used as described.[29,30]

Isolation and culture of BMDM

As described,[31,32] to obtain bone marrow-derived macrophages (BMDMs), bone marrow cells were isolated from the femur and tibia of C57BL/6 mice. After washed, the cell suspension was passed a 70 μm cell strainer and plated in a 6-well plate at a density of 2×10^6 with macrophage colony-stimulating factor (40 ng/mL) in high-glucose Dulbecco's modified Eagle's medium (DMEM)/F12 with additive of 10% fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Supernatants from indicated groups were added at a ratio of 2:1 with the culture medium at 24 hours following cell seeding. After culturing for 3–6 days, freshly prepared medium with supernatant derived from MSCs (medium: supernatant = 2:1) was replenished. To evaluate the effect of supernatants from each group in macrophages polarization, these cells were left untreated (control) or were treated with lipopolysaccharide (LPS, 100 ng/mL, Sigma-Aldrich) + IFN-γ or IL-4 (20 ng/mL, ProteinTech) + IL-13 (20 ng/mL, ProteinTech) for 24 hours on day 6.

Flow cytometry

MSCs were harvested and incubated with the appreciated antibodies for 30 minutes in the dark at room temperature and then analyzed by flow cytometry. Intrahepatic leukocytes were isolated as described.[31] The following antibodies were used for flow cytometry: CD45, CD11b, Ly6G, F4/80, CD163 (BioLegend). Data

were acquired from a FACS Caliber system (BD) and analyzed using FlowJo (Tree Star, USA).

Statistical analysis

The results were presented as mean ± SEM, and all statistical analyses were analyzed using GraphPad Prism software version 9.0 (GraphPad, San Diego, CA, USA). Data were compared by the application of an unpaired two-tailed Student *t* test and one-way ANOVA or two-way ANOVA. Differences with *p* values less than 0.05 were considered statistically significant among groups.

Additional methods

Detail methods for animal models, MSCs and the culture supernatants preparation, serum biochemistry, ELISA, histopathological and immunohistochemistry staining, immunofluorescence staining, and TUNEL Assay, western blot analysis, real-time PCR, RNA-seq analysis, and lentivirus-mediated gene overexpression are available in Supplemental materials and methods, http://links.lww.com/HC9/A836.

RESULTS

Characterization of MSCs

The cultured adherent MSCs showed a spindle-shaped fibroblast-like morphology. MSCs were transfected with lentiviral vectors carrying the FGF21-3xflag-ZsGreen-PURO gene. In fact, the morphology of FGF21-MSCs remained unchanged compared with unmodified MSCs (Figure 1A). Subsequently, we investigated whether genetic modification affected the biological characteristics of MSCs. Flow cytometry results showed that the phenotype of MSCs surface markers (CD90, CD105) were positively expressed, and hematopoietic stem cell markers (CD19, CD34, CD45) were absent in FGF21-MSCs, which was consistent with typical MSCs (Figure 1B). The transduction efficiency was assessed using fully automated live cell fluorescence microscopy imaging (Figure 1C). Three days after infection, we detected remarkedly upregulated expression of FGF21 at the mRNA and protein levels in FGF21-MSCs compared with Vector-MSCs through RT-qPCR and western blot (Figure 1D), respectively. To assess the homing of transplanted MSCs, we injected Flag-labeled FGF21-MSCs into liver-injured mice through the tail vein. Flag tag-positive FGF21-MSCs were detected in the recipient mice after transplantation (Supplemental Fig. S1A, http://links.lww.com/HC9/A836). Bioluminescence images represented fluorescent signals in different organs after 12 hours of tail vein FGF21-MSCs infusion. Signals are stronger in the lungs and liver than in other organs after administration (Supplemental Fig. S1B, http://links.lww.com/HC9/A836). The MSCs overexpressing FGF21 at passages 5-8 were used for in vivo cell therapy in the subsequent alcohol-induced liver injury models. In summary, these results indicated that Human umbilical cord-derived MSCs were successfully modified with FGF21 without altering their intrinsic characteristics.

MSCs overexpressing FGF21 ameliorate alcohol-induced liver injury in mice

The protocols for establishing the chronic and Gao-binge models of chronic plus binge ethanol feeding are shown in Figure 2A and Supplemental Figure S2A, http://links.lww.com/HC9/A836. Mice were divided into 4 groups according to different treatments: pair-fed plus PBS, EtOH-fed (AF) plus PBS, AF plus MSCs, and AF plus FGF21-MSC groups. After ethanol feeding, serum levels of alanine aminotransferase and aspartate aminotransferase, 2 common markers of hepatocellular damage, increased significantly compared to the pair-fed group (Figure 2B). Compared with the AF control mice, serum alanine aminotransferase and aspartate aminotransferase levels were significantly decreased in both the MSC group and the FGF21-MSC group, and more significantly decreased levels were found in the FGF21-MSC group. Similarly, H&E and Oil Red O staining of liver tissue sections showed that histopathological injury was more efficiently improved in FGF21-MSC-treated mice, which has remarkably ameliorated steatosis (Figure 2C). Similar results were also observed in the Gao-binge model (Supplemental Fig. S2B, http://links.lww.com/HC9/A836, S2C, http://links.lww.com/HC9/A836). Taken together, these results indicated that FGF21-MSCs could have enhanced potency and more effectively alleviated alcohol-induced liver injury in mice.

Administration of FGF21-MSCs reduces hepatic lipid accumulation in mice with alcohol-induced liver injury

H&E and Oil Red O staining were performed to evaluate the degree of liver fatty accumulation. MSC-treated and FGF21-MSC-treated animals had remarkably lessened liver vacuoles and lipid droplet accumulation. Most importantly, FGF21-MSC treatment had much fewer vacuoles and lipid droplet accumulation compared with MSC treatment. These results revealed that FGF21-MSC treatment had a protective effect on the damage of liver function in AF mice. Ethanol intake has been reported to cause hepatic steatosis through increasing lipid synthesis.[33] Interestingly, hepatic mRNA levels of lipogenesis-related genes, including *Gpat1, Dgat1, Dgat2, Srebp1c, Fasn, Scd1, Elovl6, and Acc* deceased in alcohol-fed mice compared with the pair-fed mice (Figure 2D and Supplemental Fig. S2D, http://links.lww.

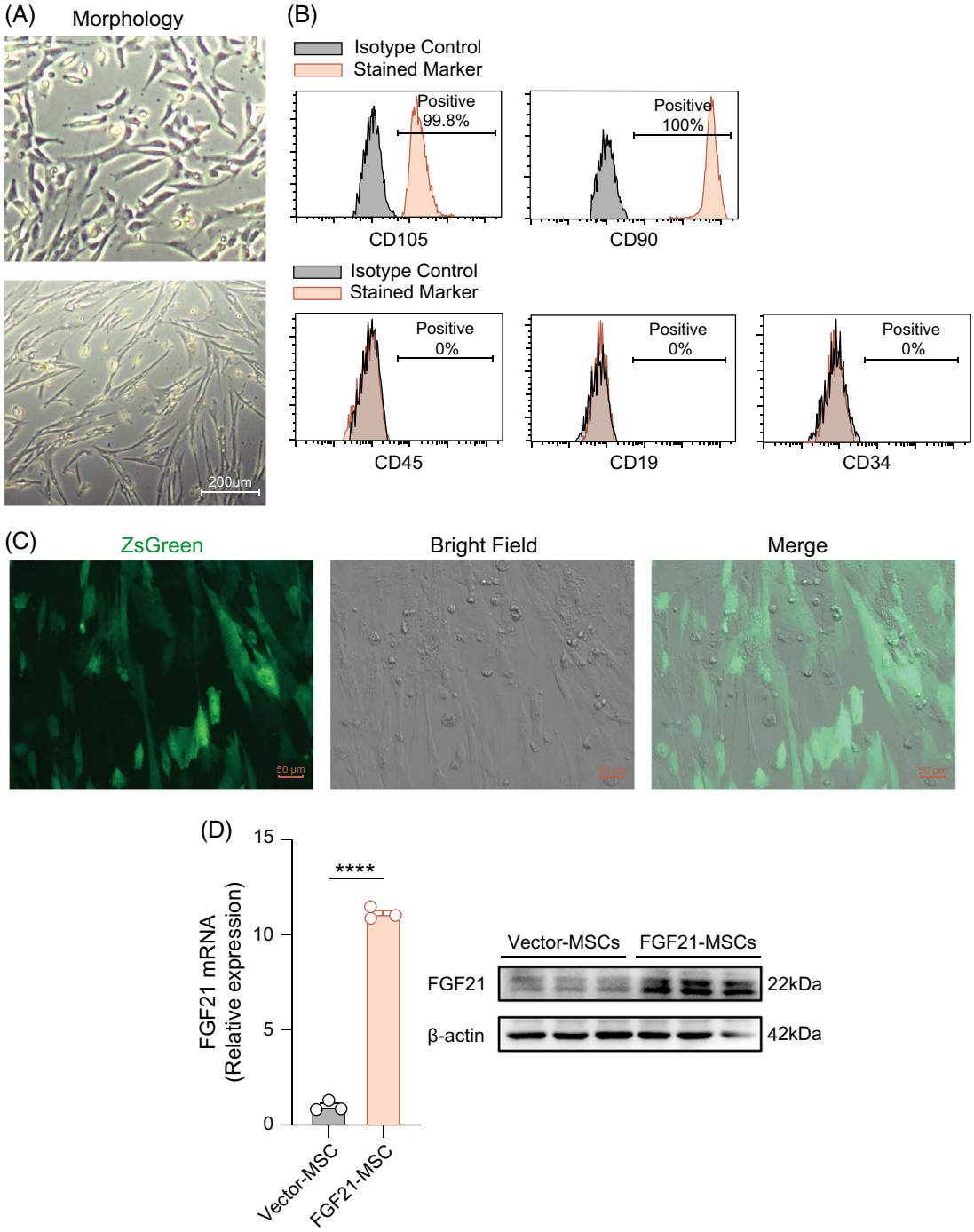

FIGURE 1 Characterization of human umbilical-derived MSCs. (A) The spindle-shaped, fibroblast-like morphology of MSCs (top) and FGF21-MSCs (bottom). Scale bar: 20 μm. (B) Flow cytometry analysis of FGF21-MSCs phenotypes. (C) The green fluorescence of FGF21-MSCs was observed using fluorescence microscopy. Scale bar: 50 μm. (D) RT-qPCR and western blot confirmed overexpression of FGF21 in MSCs by transferring lentivirus. Data are shown as mean ± SEM, n = 3, ****$p < 0.0001$. Statistical significance was assessed by Student *t* test. Abbreviation: MSCs, mesenchymal stem/stromal cells.

com/HC9/A836), suggesting hepatic de novo lipogenesis may not be critical in alcohol-induced steatosis in mice by preclinical chronic and Gao-binge ethanol-feeding models.

In addition, alcohol intake has been shown to reduce hepatic fatty acid oxidation, which also contributes to the accumulation of free fatty acids in the liver.[34,35] In fact, we validated the expression levels of mRNA for peroxisome proliferator-activated receptor α (*PPARα*) and carnitine palmitoyltransferase 1a (*Cpt1a*), 2 key genes implicated in hepatic fatty acid oxidation. They were down-regulated by ethanol-feeding (Figure 2E). Importantly, we found that hepatic mRNA levels of 2 fatty acid oxidative-related genes were significantly

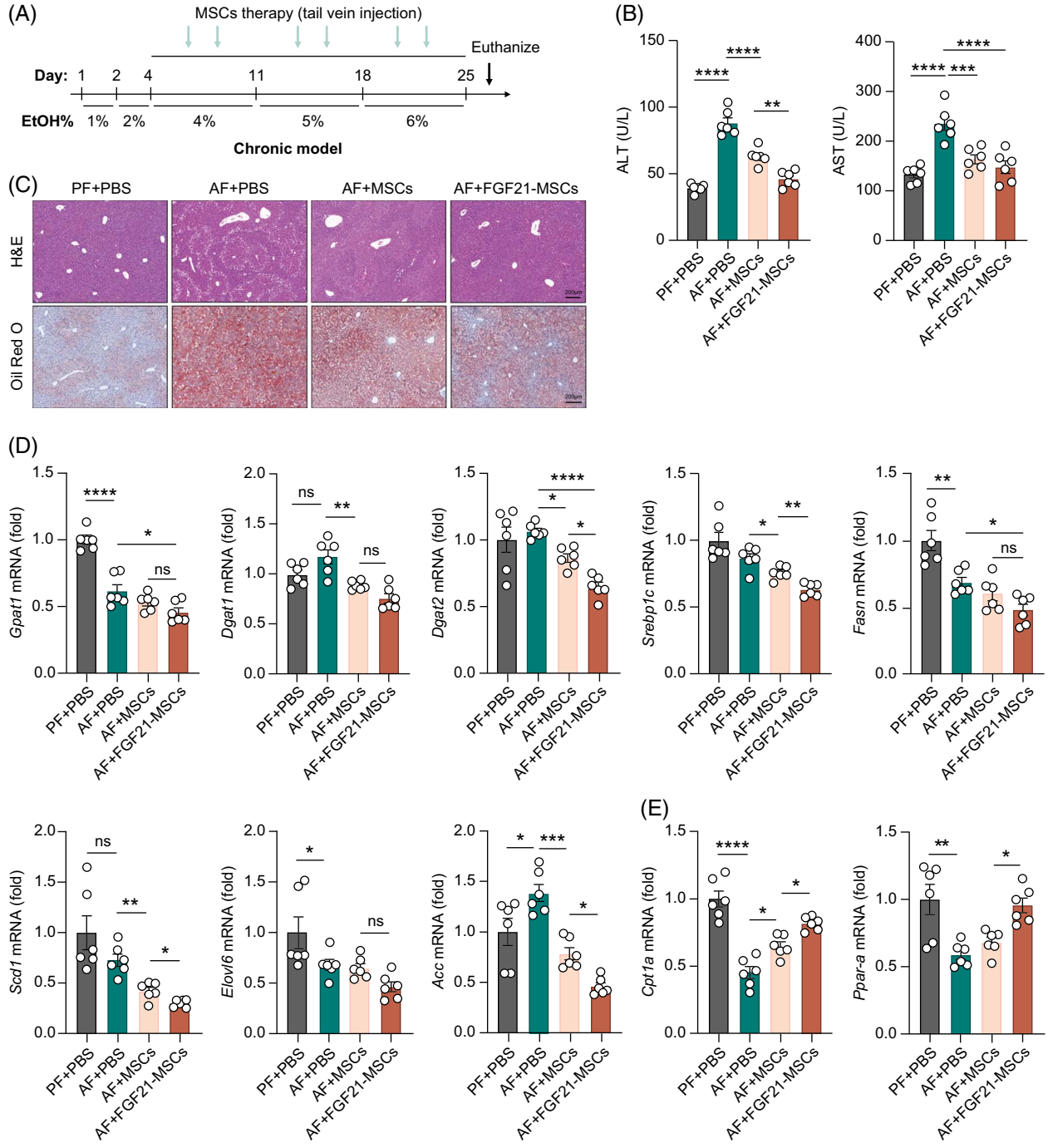

FIGURE 2 Administration of FGF21-MSCs potently reduces hepatic lipid accumulation in mice with chronic alcohol-induced liver injury. (A) Pattern diagram of chronic alcohol-induced liver injury in mice prevented by tail vein injection of MSCs. (B) Serum levels of ALT and AST in each group (n = 6). (C) Representative H&E and Oil red O staining of the liver sections from each group (n = 6). Scale bar: 200 μm. (D) Hepatic mRNA levels of lipogenesis genes (*Gpat1, Dgat1, Dgat2, Srebp1c, Fasn, Scd1, Elovl6, and Acc*) were measured by RT-qPCR in mice. (E) Hepatic mRNA levels of fatty acid catabolic genes (*Cpt1a, Ppar-a*) were measured by RT-qPCR in mice. Data are shown as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$; ns, no significance. Statistical significance was assessed by one-way ANOVA. Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; H&E, hematoxylin and eosin; MSCs, mesenchymal stem/stromal cells.

increased in FGF21-MSCs treatment relative to AF group after either chronic or acute ethanol feeding (Supplemental Fig. S2E, http://links.lww.com/HC9/A836). These observations suggest that fatty acid oxidation may be critical in the process of reducing hepatic lipid accumulation after FGF21-MSC treatment.

FGF21-MSCs transplantation efficiently attenuates alcohol-induced hepatic inflammation

Inflammation is one of the well-established characteristics of alcohol-induced liver injury[36,37]; therefore, we

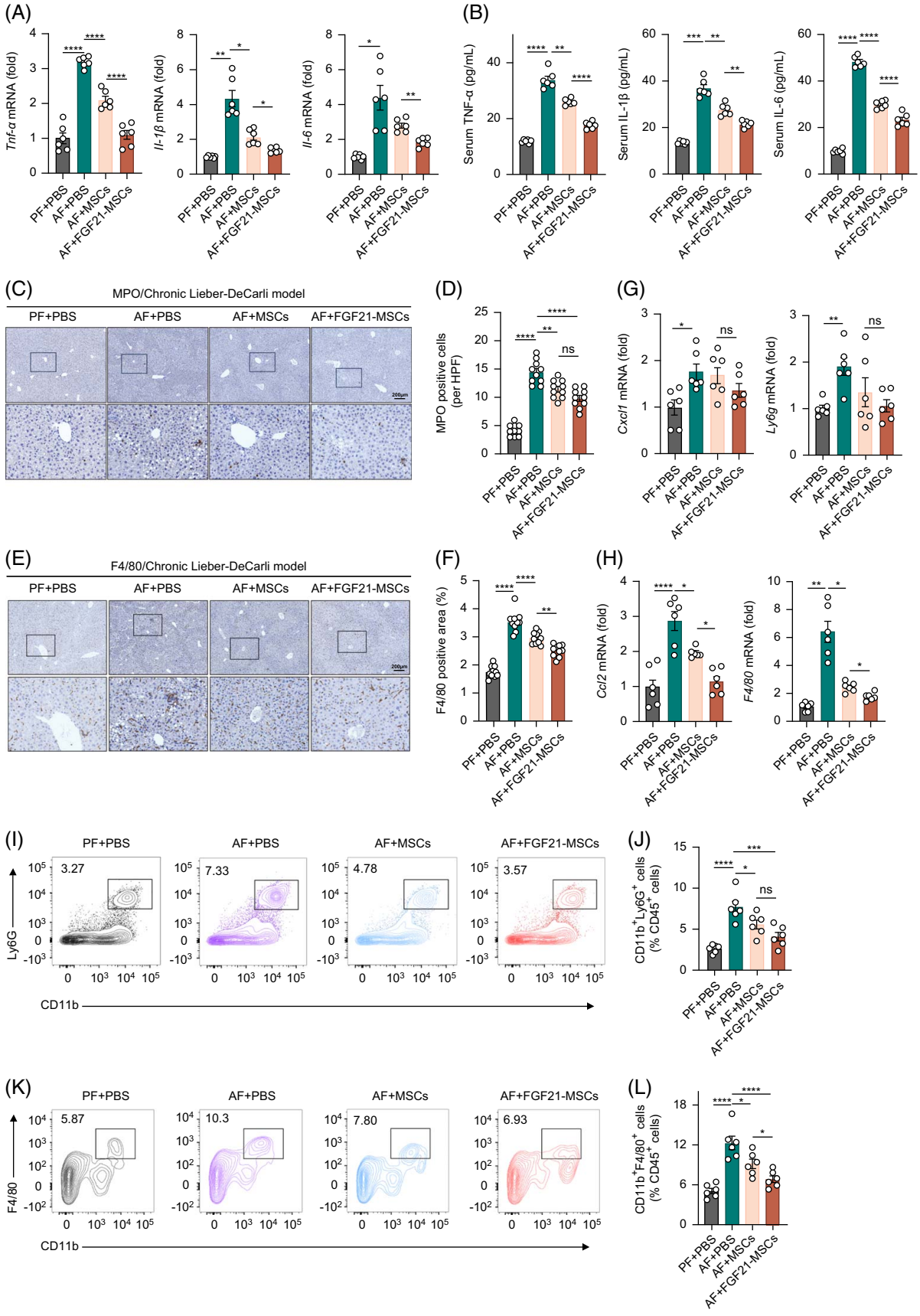

FIGURE 3 FGF21-MSCs administration reduces production of inflammatory mediators and macrophage infiltration in chronic alcohol-fed mice. (A) Hepatic mRNA levels of *Tnf-α, Il-1β, and Il-6* were measured by RT-qPCR in mice. (B) Serum TNF-α, IL-1β, and IL-6 were measured by ELISA. (C) Representative images of immunostaining of MPO staining of infiltrated neutrophils in livers of each group of mice. Scale bar: 200 μm.

(D) Quantifications of MPO-positive cells from (C). (E) Representative images of immunostaining of F4/80 positive macrophages/KCs in livers of each group of mice. Scale bar: 200 μm. (F) Quantifications of F4/80 positive area from (E). (G) Hepatic mRNA levels of *Cxcl1* and *Ly6g* were measured by RT-qPCR in mice. (H) Hepatic mRNA levels of *Ccl2* and *F4/80* were measured by RT-qPCR in mice. (I) Representative images of flow cytometric analysis of hepatic neutrophils ($CD11b^+$ and $Ly6G^+$) from each group of mice. (J) Percentage of $CD11b^+$ $Ly6G^+$ cells in liver tissues from each group. (K) Representative images of flow cytometric analysis of hepatic macrophages ($CD11b^+$ and $F4/80^+$) from each group of mice. (L) Percentage of $CD11b^+$ $F4/80^+$ cells in liver tissues from each group. Data are shown as mean ± SEM (n = 4–6). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; ns, no significance. Statistical significance was assessed by one-way ANOVA. Abbreviations: CXCL1, chemokine (C-X-C motif) ligand 1; MPO, myeloperoxidase; MSCs, mesenchymal stem/stromal cells.

examined the inflammatory response in the liver after MSCs or FGF21-MSCs infusion. Notably, mRNA levels of inflammatory cytokines and chemokines, including *Tnf-α, Il-1β, Il-6, Cxcl1*, and *Ccl2*, were significantly inhibited in the MSC group, and in the FGF21-MSC group, the levels of these cytokines decreased more obviously (Supplemental Fig. S3A, http://links.lww.com/HC9/A836). Similarly, MSC or FGF21-MSC infusion could also decrease hepatic inflammatory response after chronic ethanol feeding. Compared with pair-fed mice, chronic ethanol-fed mice had increased mRNA expression of pro-inflammatory cytokines in the liver, namely *Tnf-α, Il-1β*, and *Il-6* (Figure 3A), but this induction was ameliorated in the FGF21-MSCs group. Consistently, serum levels of TNF-α, IL-1β, and IL-6 were higher in ethanol-fed mice than their counterparts (Figure 3B).

Hepatic macrophages and neutrophil infiltration play an important role in contributing to the progression of liver injury.[4,38] Therefore, we examined the infiltration levels of macrophages and neutrophils in the liver of each group by anti-F4/80 and anti-myeloperoxidase immunohistochemical staining, respectively. We found that hepatic macrophages and neutrophils were accumulated in PBS-treated mice after chronic ethanol feeding, but this accumulation was diminished with MSC treatment (Figure 3C-F). Meanwhile, more significantly decreased levels of hepatic macrophages and neutrophils were found in the FGF21-MSC group than in the MSC group following Gao-binge ethanol feeding in mice (Supplemental Fig. S3B-E, http://links.lww.com/HC9/A836). This was consistent with F4/80 and Ly6g mRNA expression from the whole liver mRNA extracts (Figure 3G, H and Supplemental Fig. S3A, http://links.lww.com/HC9/A836). Consistent with the histological evidence for decreased inflammatory cells after chronic ethanol feeding to mice treated with MSCs, expression of mRNA of the chemokines *Cxcl1* and *Ccl2* in the liver was also decreased in MSCs treatment mice compared to PBS treatment following ethanol feeding. Although MSC treatment could significantly decrease the infiltration of neutrophils in mice after either chronic or acute ethanol-feeding model, compared to MSCs alone, FGF21-MSC treatment only significantly decreases the infiltration of neutrophils in mice after acute ethanol-feeding, rather than chronic ethanol-feeding (Figure 3C, D, Supplemental S3B, http://links.lww.com/HC9/A836 and S3C, http://links.lww.com/HC9/A836). In addition, we isolated immune cells from liver tissue and used flow cytometric analysis to examine the state of neutrophils and macrophages in the liver of chronic ethanol-feeding mice. Consistent with the immunohistochemistry results, hepatic neutrophils ($CD11b^+Ly6G^+$) and macrophages ($CD11b^+F4/80^+$) were increased in ethanol-feeding mice compared with pair-fed mice (Figure 3I-L). MSC treatment decreased neutrophil and macrophage infiltration in the liver of ethanol-feeding mice as determined by flow cytometry analysis. Compared with MSCs, FGF21-MSC treatment significantly reduced $CD11b^+F4/80^+$macrophage infiltration in the liver of alcohol-fed mice (Figure 3K, L). However, no difference in the infiltration of neutrophils was observed between MSC and FGF21-MSC treatment in the chronic ethanol-feeding model (Figure 3J). Together, these data suggest that FGF21-MSCs transplantation efficiently attenuates alcohol-induced hepatic inflammation.

FGF21-MSCs infusion alleviates hepatic apoptosis and promotes liver regeneration in the liver of ALD mice

In addition, we evaluated the levels of apoptosis and proliferation in livers from each group by TUNEL staining and immunohistochemical staining of Ki-67, respectively. TUNEL staining confirmed that hepatocyte apoptosis was decreased when infused with MSCs, especially in the FGF21-MSC group (Figure 4A, B). We also found that proliferating cells, indicated by a Ki67-positive signal, were substantially increased in the livers of both MSC and FGF21-MSC groups compared to the other 2 groups, especially in the FGF21-MSC group (Figure 4C, D). Interestingly, positive signals were mostly observed in hepatocytes. Therefore, we hypothesized that Ki67-positive proliferating cells are hepatocytes, and FGF21-MSC treatment promotes liver regeneration in the liver of ALD mice. Comparable findings were likewise observed in the Gao-binge model (Supplemental Fig. S4A-D, http://links.lww.com/HC9/A836). Taken together, these results indicate that FGF21-MSCs infusion alleviates hepatic apoptosis and promotes liver regeneration.

Protective effects of MSCs overexpressing FGF21 against oxidative stress in ALD mice

Oxidative stress is one of the major pathophysiological mechanisms contributing to the pathogenesis of ALD.[37]

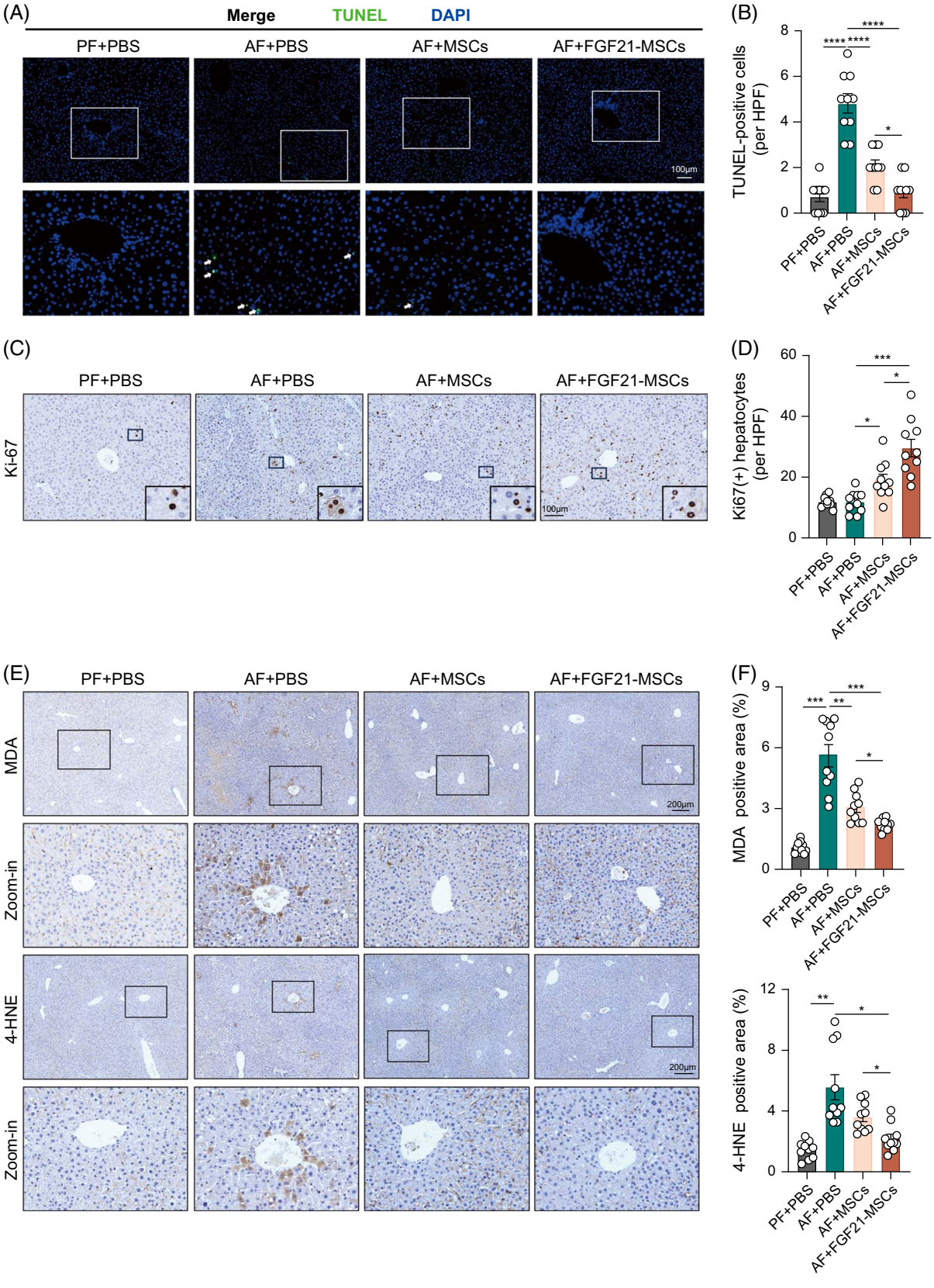

FIGURE 4 FGF21-MSCs infusion attenuates hepatic apoptosis and oxidative stress and promotes liver regeneration in chronic alcohol-induced liver injury mice. (A) Apoptosis level was detected using TUNEL (green) staining of liver tissue from each group. The nuclei were counterstained with DAPI (blue). White arrows pointed out positive apoptosis nuclei. Scale bar: 100 μm. (B) Quantifications of TUNEL positive cells per HPF from (A). (C) Hepatocyte proliferation was assessed by anti-Ki-67 immunohistochemical staining of liver tissue sections from each group. Scale bar: 100 μm. (D) Quantifications of Ki67(+) hepatocytes per high power field (HPF) from (C). (E) Representative images of

immunostaining of MDA and 4-HNE in livers of each group of mice. Scale bar: 200 μm. (F) Quantifications of MDA positive area and 4-HNE positive area from (E). Data are shown as mean ± SEM (n = 4–6). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Statistical significance was assessed by one-way ANOVA. Abbreviations: HPF, high power field; MDA, malondialdehyde; MSCs, mesenchymal stem/stromal cells; 4-HNE, 4-hydroxynonenal.

Oxidative stress triggers cell damage by altering proteins, lipids, and DNA content, thereby affecting protein expression, gene transcription, cell apoptosis, and immune cell activation, which is mediated through the generation of reactive oxygen species.[36] Therefore, immunohistochemistry analysis was performed for detecting oxidative stress levels, especially 4-hydroxynonenal and malondialdehyde, which are markers of the end product of lipid peroxidation. Mice treated with FGF21-MSCs displayed significantly decreased malondialdehyde and 4-hydroxynonenal contents compared with mice in the MSCs group or PBS group (Figure 4E, F). The Gao-binge model showed similar results as well (Supplemental Fig. S4E, http://links.lww.com/HC9/A836, S4F, http://links.lww.com/HC9/A836). In fact, oxidative stress and excessive cell death induced by alcohol and its metabolites can also exacerbate the progression of inflammation in the liver. These results suggest that the protective effects of MSCs overexpressing FGF21 against oxidative stress in the pathogenesis of alcohol-induced liver injury.

FGF21-MSCs treatment enables macrophages to acquire oxidative phosphorylation-dependent anti-inflammatory properties

The transcriptome sequencing of 3 MSCs transfected with lentivirus-FGF21 and 3 matched MSCs transfected with lentivirus vector was performed (Figure 5A-C). The data revealed 465 differentially expressed genes between the FGF21-MSC group and Vector-MSC group, including 131 down-regulated genes and 334 upregulated genes (Figure 5B). Among the upregulated genes, we found that some were immune mediators involved in the transformation of the monocyte/macrophage phenotype, such as CSF1, TGF-β, TSG-6, PGE2, and CCL2 (Figure 5C). This result was also confirmed by RT-qPCR (Figure 5D). In fact, several studies confirmed the role of MSCs on macrophage polarization in different models.[39,40] Thus, we tended to explore whether MSCs could induce M2 macrophages in ALD to promote inflammation resolution. To assess the impact of MSCs or FGF21-MSCs on macrophages, BMDMs and supernatants from MSCs or FGF21-MSCs were co-cultured together, and the transcriptional changes of specific M1/M2 marker genes evaluated by RT-qPCR. This revealed that M2 polarization-related marker levels (*Arg1, Ym1,* and *CD206*) were significantly increased when co-cultured with supernatant from FGF21-MSCs (Figure 5E). However, M1 polarization-related marker levels (*TNF-α, IL-1β,* and *Nos2*) were significantly decreased when co-cultured. In vitro studies confirmed the MSC-derived FGF21 on macrophage polarization, as the expression of M2 markers of BMDMs treated with MSC-conditioned medium (MSCs-sup) or FGF21-MSC-conditioned medium (FGF21-MSCs-sup) were increased. Taken together, our results demonstrated that MSCs induced M2 macrophages to resolute inflammation in ALD through FGF21. Consistent with our in vitro findings, we utilized double immunofluorescent staining of CD 68 and CD163 (markers of mouse M2 macrophages) to evaluate macrophage polarization in the liver. The results showed that more $CD68^+CD163^+$ macrophages were observed in the FGF21-MSCs group than in other ethanol-feeding groups (Figure 5F). We also used flow cytometry to examine the $CD163^+$ macrophage population, and we found that $CD163^+$ macrophages were increased in the livers of mice treated with FGF21-MSCs in comparison with MSCs treatment mice following chronic ethanol feeding (Figure 5G, H). These data together suggest that FGF21-MSCs treatment promotes macrophage activation with an M2-like phenotype in the presence of IL-4/IL-13 or alcohol.

To further explore the molecular mechanism involved in FGF21-MSCs–induced M2 polarization in macrophages, transcriptome analysis was performed and revealed the effects of different treatment groups on macrophages. Principal component analysis showed a clear segregation of the transcriptional programs among 3 groups (Figure 6A). A volcano plot was constructed showing differential expression analysis results between FGF21-MSCs-sup versus MSCs-sup, and MSCs-sup versus CM, with red representing up-regulation and blue indicating down-regulation (Figure 6B and Supplemental Fig. S5A, http://links.lww.com/HC9/A836). To elucidate the possible biological functions of these differentially expressed genes after FGF21-MSCs treatment, Kyoto Encyclopedia of Genes and Genomes pathway enrichment and gene ontology functional annotation analysis were performed (Figure 6C, D and Supplemental Fig. S5B, http://links.lww.com/HC9/A836, S5C, http://links.lww.com/HC9/A836). This analysis revealed a number of pathways and processes associated with the chemokine signaling pathway, regulation of cytokine production, and inflammatory response that were significantly affected after FGF21-MSCs-sup treatment. We then created a gene expression heatmap and found a different expression pattern in the 2 groups (Figure 6E). Consistent with our transcriptomics studies, the expression of genes involved in oxidative phosphorylation in BMDMs was higher in the FGF21-MSCs-sup group (Figure 6F), compared with other groups, while the expression

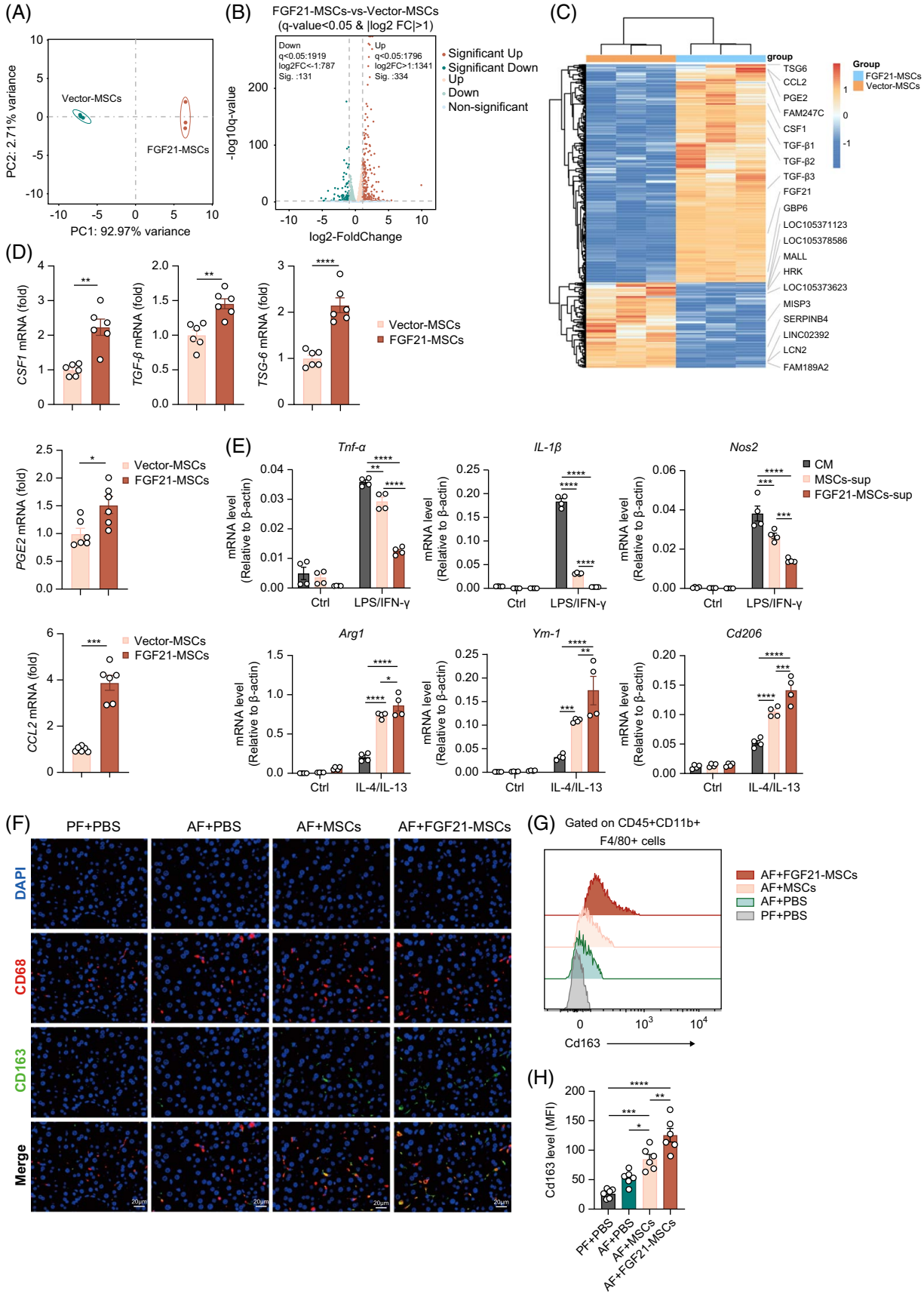

FIGURE 5 FGF21-MSCs treatment promotes macrophage activation with an M2-like phenotype in vitro and in vivo. (A) (PCA of Vector-MSCs and FGF21-MSCs. (B) Volcano plot showing DE analysis results between FGF21-MSCs versus Vector-MSCs. (C) Cluster heatmap of FGF21-MSCs and Vector-MSCs. (D)The mRNA levels of *CSF1, TGF-β, TSG-6, PGE2,* and *CCL2* in Vector-MSCs and FGF21-MSCs. (E) The mRNA

levels of M1 pro-inflammatory markers (*Tnf-α, Il-1β, and Nos2*) and M2 anti-inflammatory markers (*Arg1, Ym1, and Cd206*) in BMDMs following indicated condition (n = 4 per group). (F) Representative images of liver immunofluorescence straining of M2 markers, CD68 (red), and CD163 (green). The nuclei were counterstained with DAPI (blue); n = 4-6 per group. (G) Representative images of flow cytometric analysis of M2 macrophage markers (Cd163) in hepatic macrophages from each group. (H) Quantification of Cd163 level (MFI). Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Statistical significance was assessed by Student *t* test, one-way ANOVA, or two-way ANOVA. Abbreviations: BMDMs, bone marrow-derived macrophages; DE, differential expression; MFI, mean fluorescence intensity; MSCs, mesenchymal stem/stromal cells; PCA, principal component analysis.

levels of glycolysis-related genes were decreased after FGF21-MSCs treatment (Figure 6G). These data supported the immunomodulatory effects of FGF21-MSCs on macrophages in shaping the anti-inflammatory preference of maturing macrophages. Taken together, these results potentially provided new insights on FGF21-MSCs may be beneficial in inducing the macrophage phenotypic switch toward M2.

DISCUSSION

The therapeutic potential of FGF21 and MSCs in ALD has already been investigated; however, the paucity of literature has addressed the effects of engineered MSCs that overexpress FGF21 in the treatment of ALD. To our knowledge, this study is the first attempt to evaluate the potential therapeutic benefits of FGF21 gene-modified MSCs in mice by preclinical chronic and Gao-binge ethanol-feeding models. Ethanol-induced liver injury, hepatic inflammation, and hepatocyte death were greatly reduced in mice after FGF21-MSC treatment compared with MSCs alone (Figure 7). These findings underline the significance of FGF21 signaling as a potential therapeutic target in ALD, and it provides a possible strategy of novel MSC-based cell therapy for the clinical treatment of AH.

Corticosteroids alone and in combination with pentoxifylline can reduce short-term mortality in patients with severe AH.[41] However, the use of corticosteroids in AH is correlated with considerable adverse effects, including fungal infections limiting its potential.[42] Other treatments need to be developed for patients who do not have remission or cannot tolerate corticosteroid treatments, and stem cell therapy is a potentially important treatment. MSCs can regulate the differentiation and activation of almost all immune cells,[9] and are easy to culture, expand and preserve ex vivo. Due to their excellent immunomodulatory effects, MSCs have been widely used in the treatment of T-cell–mediated hepatitis.[43] ALD is the most prevalent type of chronic liver disease worldwide, with limited therapeutic options, resulting in high morbidity and mortality. Chronic and binge alcohol consumption over a sustained period of time leads to an increase in the risk of ALD and other related diseases that causes detrimental health and social consequences for individuals.[36] Globally, an estimated 741,300 or 4.1% of all new cases of cancer in 2020 were attributable to alcohol consumption.[1] However, unlike T-cell–mediated hepatitis, basic and clinical reports for stem cell-based ALD therapy are scarce. A phase 2 trial using autologous bone marrow-derived mesenchymal stem cells to treat alcohol-associated cirrhosis found that stem cell transplantation safely and effectively improved histologic fibrosis and liver functions.[12] However, the curative effect of MSCs alone is unsatisfactory, so it is necessary to find ways to enhance the immune regulatory function of MSCs.

MSC-based cell therapies have gradually become a promising option for promoting liver regeneration and repairing liver injury in various liver diseases, including ALD. Extensive research has demonstrated that various trophic factors secreted by MSCs play essential roles in liver regeneration and repair through alleviating inflammation, apoptosis, and oxidative stress as well as promoting tissue regeneration.[11] However, in addition to possible tumorigenesis, another major problem for MSC therapy is poor therapeutic efficacy, which was largely due to low retention and poor survival of infused MSCs, as demonstrated by clinical studies.[44] Gene modification is considered as a strategy for augmenting the therapeutic benefits of transplanted MSCs. MSCs have been genetically modified to increase the expression of CCR2, thereby enhancing their homing to the injured liver and improving their therapeutic effect.[45] Additionally, MSCs overexpressing the Smad7 gene could be a feasible approach to mitigate liver cirrhosis, providing new insights for stem cell-based gene therapy targeting TGF-β1 signaling.[46] Genetically modified MSCs could produce trophic cytokines or other beneficial gene products to enhance their therapeutic effects on various liver diseases by engineering MSCs with viral or nonviral vectors in preclinical models. However, there are still some challenges in clinical administration, like the genetic modification of stem cells through the manipulation of target genes, which can enhance the rate of stem cell survival and engraftment in damaged liver tissue and improve their therapeutic potential. First, genetically modified MSCs, while altering their biological functions in a beneficial way, also pose new safety risks, such as chromosomal instability, gene off-targeting, and insertional mutagenesis. Second, the sufficient dosage of transplanted cells, optimal timing, and injection frequency are still being determined. Finally, the transplantation route is unclear, and the risk of unanticipated differentiation and tumorigenicity causes safety concerns. Unlike other viral vectors and nonviral

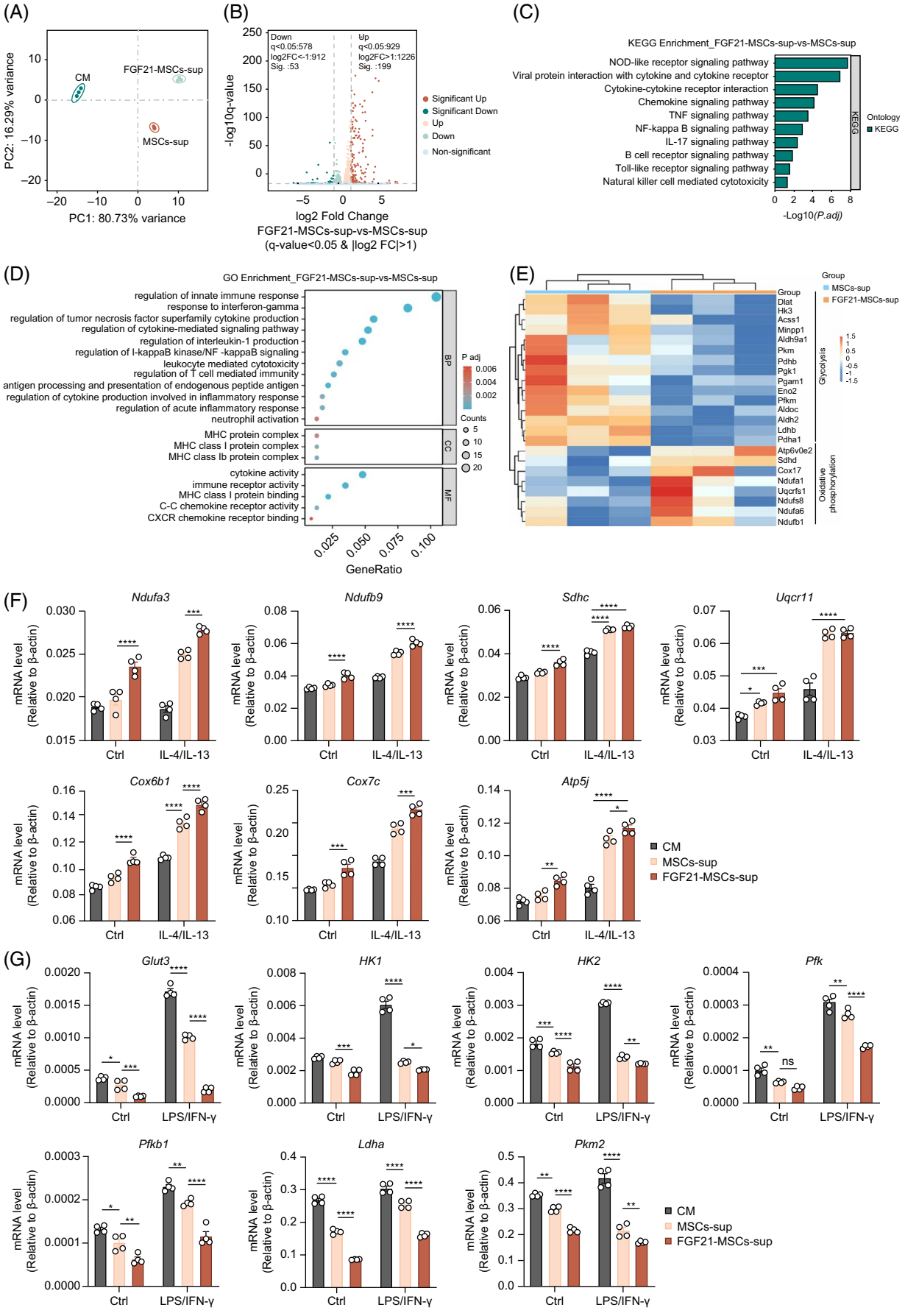
(A)
PC2: 16.29% variance
PC1: 80.73% variance
CM
FGF21-MSCs-sup
MSCs-sup
(B)
-log10q-value
log2 Fold Change
FGF21-MSCs-sup-vs-MSCs-sup
(q-value<0.05 & |log2 FC|>1)
Down
q<0.05:578
log2FC<-1:912
Sig. :53
Up
q<0.05:929
log2FC>1:1226
Sig. :199
Significant Up
Significant Down
Up
Down
Non-significant
(C)
KEGG Enrichment_FGF21-MSCs-sup-vs-MSCs-sup
NOD-like receptor signaling pathway
Viral protein interaction with cytokine and cytokine receptor
Cytokine-cytokine receptor interaction
Chemokine signaling pathway
TNF signaling pathway
NF-kappa B signaling pathway
IL-17 signaling pathway
B cell receptor signaling pathway
Toll-like receptor signaling pathway
Natural killer cell mediated cytotoxicity
KEGG
Ontology
KEGG
-Log10(P.adj)
(D)
GO Enrichment_FGF21-MSCs-sup-vs-MSCs-sup
regulation of innate immune response
response to interferon-gamma
regulation of tumor necrosis factor superfamily cytokine production
regulation of cytokine-mediated signaling pathway
regulation of interleukin-1 production
regulation of I-kappaB kinase/NF -kappaB signaling
leukocyte mediated cytotoxicity
regulation of T cell mediated immunity
antigen processing and presentation of endogenous peptide antigen
regulation of cytokine production involved in inflammatory response
regulation of acute inflammatory response
neutrophil activation
MHC protein complex
MHC class I protein complex
MHC class Ib protein complex
cytokine activity
immune receptor activity
MHC class I protein binding
C-C chemokine receptor activity
CXCR chemokine receptor binding
BP
CC
MF
P adj
Counts
GeneRatio
(E)
Group
MSCs-sup
FGF21-MSCs-sup
Dlat
Hk3
Acss1
Minpp1
Aldh9a1
Pkm
Pdhb
Pgk1
Pgam1
Eno2
Pfkm
Aldoc
Aldh2
Ldhb
Pdha1
Atp6v0e2
Sdhd
Cox17
Ndufa1
Uqcrfs1
Ndufs8
Ndufa6
Ndufb1
Glycolysis
Oxidative phosphorylation
(F)
Ndufa3
Ndufb9
Sdhc
Uqcr11
Cox6b1
Cox7c
Atp5j
mRNA level (Relative to β-actin)
Ctrl
IL-4/IL-13
CM
MSCs-sup
FGF21-MSCs-sup
(G)
Glut3
HK1
HK2
Pfk
Pfkb1
Ldha
Pkm2
Ctrl
LPS/IFN-γ
ns

FIGURE 6 FGF21-MSCs enables macrophages to commit OXPHOS that shapes an anti-inflammatory phenotype. (A) Principal component analysis (PCA) of genes from BMDMs pretreated with CM, MSCs-sup and FGF21-MSCs-sup. (B) Volcano plot showing DE analysis results between FGF21-MSCs-sup versus MSCs-sup. (C) KEGG pathway enrichment of gene set. (D) GO enrichment of gene set. (E) Gene expression heatmap of genes involved in oxidative phosphorylation and glycolysis metabolism in BMDMs. (F) The mRNA levels of OXPHOS-related genes (*Ndufa3, Ndufb9, Sdhc, Uqcr11, Cox6b1, Cox7c and Atp5j*) in BMDMs following indicated condition. BMDMs were either untreated (control) or stimulated with IL-4+IL-13. (G) The mRNA levels of glycolysis-related genes (*Glut3, Hk1, Hk2, Pfk, Pfkb1, Ldha and Pkm2*) in BMDMs following indicated condition. BMDMs were either untreated (control) or stimulated with LPS+IFN-γ. Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$; ns, no significance. Statistical significance was assessed by two-way ANOVA. Abbreviations: BMDMs, bone marrow-derived macrophages; DE, differential expression; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; LPS, lipopolysaccharide; MSCs, mesenchymal stem/stromal cells; OXPHOS, oxidative phosphorylation; PCA, principal component analysis.

vectors, lentivirus vectors have the ability to efficiently transduce stem cells, adapt to long exogenous gene fragments, integrate exogenous genes into the host cell genome to achieve sustained expression of exogenous gene functions, and exhibit low and high immunogenicity on biosafety—a feature that has been used in several human gene therapy clinical trials.[47] To date, no adverse reactions related to treatment-related toxicity or systemic effects of lentivirus vectors have been reported, likely due to the high biological safety of these vectors.[48] Thus, we chose a lentivirus vector for genetic engineering in the current study. Our results show that lentivirus-mediated FGF21-engineered MSCs can be safely administered to ALD mouse models without causing systemic involvement or treatment-related toxicity.

FGF21, an endocrine stress-responsive hormone, has been shown to regulate energy balance, glucose, and lipid metabolism. Previous studies have shown that overexpression of FGF21 in MSCs exhibited enhanced homing capacity to injured sites in a mice model of traumatic brain injury, which could improve the therapeutic efficiency of these cells following infusion.[28] Acute alcohol intake was found to result in increased induction of FGF21 in serum in both humans and mice.[49] In a mouse model of chronic alcohol-induced liver injury, FGF21-KO mice had increased mortality and more severe histological damage, whereas exogenous supplementation with rhFGF21 attenuated hepatic steatosis and inflammatory responses in the liver.[50] Therefore, we hypothesized that MSCs overexpressing FGF21 might be a novel cell therapy approach for treating ALD. FGF21-MSCs treatment significantly decreased neutrophil infiltration in the livers of alcohol-fed mice as determined by immunohistochemical staining. Except for neutrophils, the number of liver-infiltrating KC/monocyte-derived macrophages was markedly increased after alcohol consumption. Administration of FGF21-MSCs could reduce the infiltration of macrophages in alcohol-fed mice. Macrophages are crucial to processes of liver injury and exhibit a high degree of plasticity, adjusting their phenotype in response to signals of the hepatic microenvironment. The

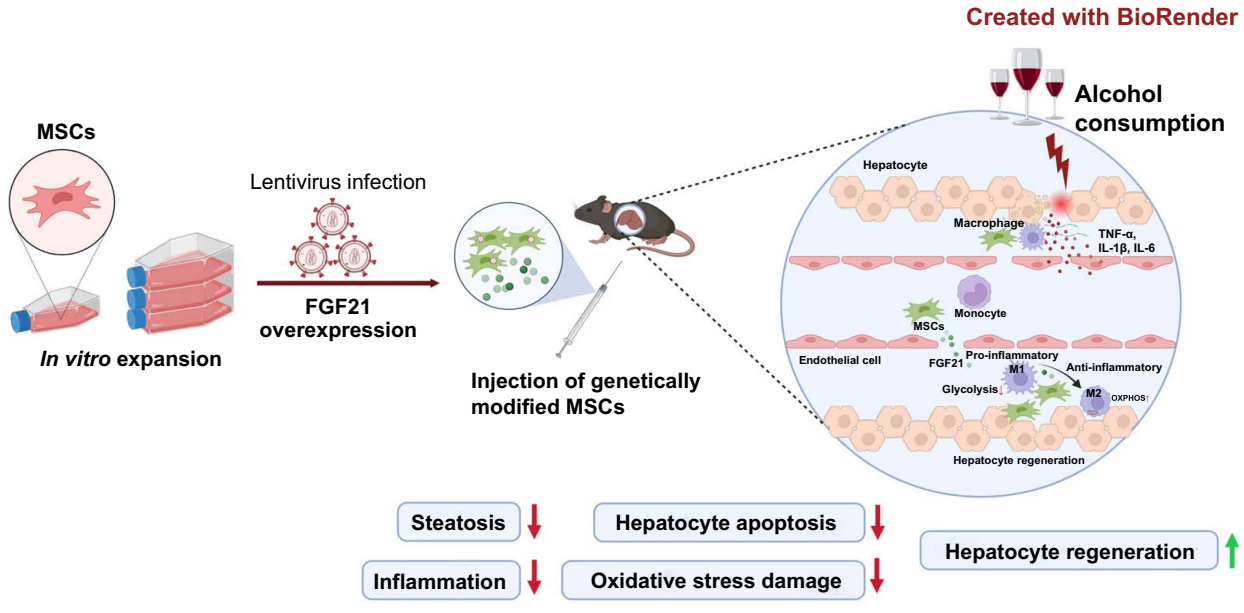

FIGURE 7 The therapeutic potential of MSCs overexpressing FGF21 in ALD models. Briefly, FGF21-MSCs administration greatly ameliorated alcohol-induced liver injury in both Gao-binge and chronic Lieber-DeCarli models of ALD, as demonstrated by reduced steatosis, reduced hepatocyte death, reduced hepatic inflammation and oxidative stress, and increased liver regeneration. Mechanistically, FGF21 could facilitate the immunomodulatory function of MSCs on macrophages by setting metabolic commitment for oxidative phosphorylation, which enables macrophages to exhibit anti-inflammatory inclination. Abbreviations: ALD, Alcohol-associated liver disease; MSCs, mesenchymal stem/stromal cells.

adaptability elucidates their manifold and even contradictory functions during liver injury. Traditionally, macrophage functions have been assigned as inflammatory, labeled as M1, and anti-inflammatory, termed M2. In the injured liver, macrophages frequently express markers of inflammation or resolution simultaneously, showcasing their ability to rapidly change their phenotype depending on the dynamic hepatic microenvironment. In our study, we collected supernatants from MSCs or FGF21-MSCs and co-cultured them with BMDMs, respectively. We found a significant increase in M2 macrophage marker expression, suggesting that FGF21-MSC treatment promotes macrophage differentiation to M2 macrophages. Mechanistically, we found that FGF21 could facilitate the immunomodulatory function of MSCs on macrophages by setting metabolic commitment for oxidative phosphorylation, which enables macrophages to exhibit anti-inflammatory inclination. We speculated that overexpression of FGF21 enhanced the therapeutic effect of MSCs on ALD, possibly through a paracrine mechanism. However, the detailed mechanism of this effect still needs to be further validated by in vivo experiments.

Taken together, our present study has demonstrated that FGF21-MSCs were preferentially able to alleviate liver injury over natural MSCs, presumably due to their ability to more effectively reduce hepatic steatosis, downregulate the expression of inflammatory chemokines, attenuate hepatic inflammatory cells infiltration, suppress the production of pro-inflammatory factors, decrease hepatic apoptosis and oxidative stress levels, and promote liver regeneration. Our findings offer novel insights into the role of FGF21-MSCs in the pathogenesis of ALD and may shed light on the development of MSC-based cell therapies ameliorating ALD.

DATA AVAILABILITY STATEMENT

All data generated and/ or analyzed in this study are included in this published article and its supplemental information files, http://links.lww.com/HC9/A836. The RNA-seq data were deposited and can be found in the NCBI Sequence Read Archive (SRA) repository under accession numbers PRJNA1066704 and PRJNA1037595. Additional experimental details and more data used or analyzed in the current study are available from the corresponding author upon reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All animal experiments were approved by the Animal Ethics Committee of Anhui Medical University. The study adhered to the Declaration of Helsinki and was approved under the project, entitled “Mechanistic investigation of human umbilical cord mesenchymal stem cells ameliorating alcoholic liver injury” (No. LLSC20231222). The approved date was April 10th, 2023.

AUTHOR CONTRIBUTIONS

Qian Huai: Drafted the manuscript. Qian Huai, Cheng Zhu, Xu Zhang, Hanren Dai, and Xiaolei Li: Conducted the experiments and analyzed the data. Qian Huai, Xiaolei Li, and Hua Wang: Participated in the research design. Qian Huai and Xiaolei Li: Revised the manuscript. All authors discussed the results and contributed to the final manuscript.

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CONFLICTS OF INTEREST

The authors have no conflicts to report.

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