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### ORIGINAL ARTICLE



# Granulocyte colony-stimulating factor attenuates liver damage by M2 macrophage polarization and hepatocyte proliferation in alcoholic hepatitis in mice

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### Abstract

Massive inflammation and liver failure are main contributors to the high mortality in alcohol-associated hepatitis (AH). In recent clinical trials, granulocyte colony-stimulating factor (G-CSF) therapy improved liver function and survival in patients with AH. However, the mechanisms of G-CSF-mediated beneficial effects in AH remain elusive. In this study, we evaluated effects of in vivo G-CSF administration, using a mouse model of AH. G-CSF treatment significantly reduced liver damage in alcohol-fed mice even though it increased the numbers of liver-infiltrating immune cells, including neutrophils and inflammatory monocytes. Moreover, G-CSF promoted macrophage polarization toward an M2-like phenotype and increased hepatocyte proliferation, which was indicated by an increased Ki67-positive signal colocalized with hepatocyte nuclear factor 4 alpha (HNF-4 $\alpha$ ) and cyclin D1 expression in hepatocytes. We found that G-CSF increased G-CSF receptor expression and resulted in reduced levels of phosphorylated β-catenin in hepatocytes. In the presence of an additional pathogen-associated molecule, lipopolysaccharide (LPS), which is significantly increased in the circulation and liver of patients with AH, the G-CSF-induced hepatoprotective effects were abolished in alcohol-fed mice. We still observed increased Ki67-positive signals in alcohol-fed mice following G-CSF treatment; however, Ki67 and HNF-4a did not colocalize in LPS-challenged mice. Conclusion: G-CSF treatment increases immune cell populations, particularly neutrophil counts, and promotes M2-like macrophage differentiation in the liver. More importantly, G-CSF treatment reduces alcohol-induced liver injury and promotes hepatocyte proliferation in alcohol-fed mice. These data provide new insights into the understanding of mechanisms mediated by G-CSF and its therapeutic effects in AH.

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## INTRODUCTION

Excessive alcohol consumption causes hepatocyte damage and compromises liver functions. In the spectrum of alcohol-associated liver diseases, alcoholassociated hepatitis (AH) is characterized by jaundice, massive liver inflammation, and high risk of infection that often leads to liver failure and increased mortality.<sup>[1]</sup> Currently, steroid treatment is the first line of standard medical therapy for patients with AH. While steroid treatment improves 28-day mortality and can immediately suppress immune activation and thus dampen alcohol-induced inflammation, it also increases susceptibility to infection, which is a major risk factor for high mortality in patients with AH.<sup>[2]</sup> In addition, based on recent analysis, only approximately 50% of patients with AH respond to corticosteroids.<sup>[3,4]</sup> Therefore, there is an urgent need for more reliable and safer therapeutic approaches to reduce inflammatory responses and restore liver functions. Recently, granulocyte colonystimulating factor (G-CSF) has been evaluated in small clinical trials with patients with severe AH and has shown promising outcomes on liver function recovery and survival.<sup>[5,6]</sup>

G-CSF is a hormone-like cytokine that promotes production and differentiation of granulocytes and their precursors in the bone marrow.<sup>[7]</sup> Under homeostasis, the serum level of G-CSF is undetectably low, but its production is significantly up-regulated following infection or inflammation.<sup>[8]</sup> Increased expression of G-CSF enhances neutrophil counts/functions and facilitates mobilization of stem cells and progenitor cells as a trans effect resulting from a significant increase of neutrophils.<sup>[9,10]</sup> Hence, exogenous G-CSF administration has been considered a therapeutic approach in various disease conditions and is routinely used in chemotherapyinduced neutropenia.<sup>[7]</sup> Clinical trials have shown that 5 days of G-CSF treatment improved liver functions and histologic features as well as increased survival in patients with AH.<sup>[5,11,12]</sup> Because CD34<sup>+</sup> hematopoietic stem cells increase in the circulation following G-CSF administration, it has been postulated that these hematopoietic stem cells contribute to liver regeneration in AH. However, it was also reported that most of these G-CSF-induced hematopoietic stem cells and progenitors found in the circulation are in G0 or G1 phase of the cell cycle and are not actively proliferating cells.<sup>[13]</sup>

The liver is a unique organ that has tremendous regeneration capacity. It has long been investigated to identify cellular sources of liver repopulation. Recent studies using lineage-tracing techniques reported that mature adult hepatocytes have proliferating capacity and repopulate the liver under homeostasis and injury/disease conditions.<sup>[14,15]</sup> In a steady state, hepatocyte turnover rate is as slow as 12-15 months. Following liver injury, hepatocytes can accelerate proliferation to replace injured hepatocytes and lead to liver repopulation.<sup>[14,15]</sup> In addition to hepatocytes, liver progenitor cells (LPCs; or oval cells) also contribute to damage-induced liver regeneration, particularly when the magnitude of damage exceeds hepatocyte self-repair capacity or under conditions when hepatocyte proliferation is inhibited.<sup>[16,17]</sup> These studies indicate that the predominant proliferating cell type in liver regeneration is largely determined by the injury type and extent of liver damage. Therefore, alcohol-induced liver regeneration needs to be understood in a disease-specific context to develop proper therapeutic approaches.

In this study, we examined the effects of G-CSF administration on regulating inflammation and liver repair capacity using a murine model of AH. We found that G-CSF treatment increased neutrophils and lymphocyte antigen 6 complex. locus C1 (Lv6C)<sup>high</sup> inflammatory monocytes in bone marrow and the liver. Furthermore, G-CSF administration significantly diminished alcoholinduced liver injury and inflammatory cytokine/chemokine production in alcohol-fed mice. In addition, we observed a significant increase in M2 macrophage marker expression, suggesting that G-CSF treatment promotes macrophage differentiation to M2 macrophages. Finally, we report that G-CSF promotes hepatocyte proliferation that may ameliorate impaired liver regeneration capacity in AH. Together, our data provide molecular insights into the mechanisms of G-CSF-mediated inflammation regulation and liver repair.

### MATERIALS AND METHODS

### Mice

All mice used in animal experiments were 8-12-week-old female C57BL/6 mice and purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen-free mouse facility at the University of Massachusetts Medical School (UMMS), and all animal handling was performed in compliance with institutional guidelines. Additional procedures were approved by the UMMS Institutional Animal Care and Use Committee.

# Mouse model of AH with chronic alcohol or chronic alcohol with lipopolysaccharide

For chronic alcohol diet feeding, mice received either the Lieber DeCarli liquid diet (BioServ) containing 5% ethanol (EtOH) (volume [vol]/vol) or calorie-matched liquid diet (pair fed [PF]) *ad libitum* for 4 weeks after 1 week of an acclamation period with the Lieber DeCarli with 1%-5% EtOH (vol/vol). Some mice in both PF and alcohol diet-fed groups received 200 µL of lipopolysaccharide

(LPS; 0.5 mg/kg body weight) or saline through intraperitoneal injection 15 hours before being sacrificed.

## **G-CSF** administration

Some mice in both PF and alcohol diet-fed mice groups received either 200  $\mu$ L of saline or G-CSF (Neupogen; 200  $\mu$ g/kg body weight) through intraperitoneal injection. Daily G-CSF injection was performed for 7 consecutive days in the last week of the 4-week chronic-alcohol-diet feeding.

# *In vitro* G-CSF treatment in RAW 264.7 cells and bone marrow-derived macrophages

RAW 264.7 cells were treated first with 50 mM of in vitro EtOH. After 48 hours of alcohol treatment, the culture media was changed and G-CSF (100 ng/mL) was added with 50 mM of EtOH into the culture media. Cells were harvested after 48 hours of treatment and stained with antibodies targeting CD163, CD206, CD86, and CD80 for flow cytometry analysis. Bone marrow cells were harvested from 10-week-old female C57BL/6 mice (n = 3) and differentiated with macrophage colony-stimulating factor (M-CSF; 20 µM) for 6 days. After 6 days of M-CSF treatment, macrophage differentiation was confirmed through a morphology change under microscopy. These bone marrow-derived macrophages (BMDMs) were treated with in vitro EtOH (50 mM) and G-CSF (100 ng/mL) for 24 hours and harvested for flow cytometry analysis of surface markers, including CD206.

# *In vitro* G-CSF treatment in primary mouse hepatocytes

Mouse hepatocytes were harvested as described<sup>[18]</sup> and seeded in collagen-coated, six-well plates. After 4 hours of resting, G-CSF (100 ng/mL) was added into the culture media. The hepatocytes were harvested after 12 hours of treatment for western blotting.

# Statistical analysis

For animal experiments, the minimum sample size of each group was determined by power analysis with the z test, which was based on our preliminary data using a measurement of liver injury following alcohol feeding. All presented data were analyzed using GraphPad 8.0 (Prism) and shown as mean  $\pm$  SEM. Statistical significance was determined using either the Student *t* test, one-way analysis of variance (ANOVA), or Brown-Forsythe and Welch ANOVA test. p < 0.05 was considered to be statistically significant. Additional information can be found in the section of Supporting Materials and Methods.

# RESULTS

First, we evaluated whether G-CSF administration could ameliorate alcohol-induced liver damage using a mouse model of AH. Mice received liquid diet containing 5% (vol/vol) alcohol (Lieber DeCarli, EtOH) or calorie-matched liquid diet (PF) for 4 weeks after 1 week of acclimation. Each group of mice received either G-CSF (200 µg/kg of body weight) or saline intraperitoneal injections for 7 consecutive days in the last week of the 4-week alcohol diet. Alcohol consumption induced liver damage, which was indicated by elevated serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (Figure 1A,B). We found that G-CSF treatment significantly reduced alcoholinduced liver damage in alcohol-fed mice, whereas ALT and AST levels remained unchanged following G-CSF administration in PF control mice (Figure 1A,B). Moreover, the levels of inflammatory cytokines and chemokines, such as interleukin-6 (IL-6) and monocyte chemoattractant protein 1 (MCP-1), were increased in the circulation of alcohol-fed mice and were reduced following G-CSF treatment (Figure 1C,D). In the livers of alcohol-fed mice, we also observed that inflammatory cytokine IL-6 as well as chemokines, such as MCP-1 and C-X-C-motif ligand 1, were increased following alcohol consumption and decreased after G-CSF treatment (Figure 1E-G). The expression of these inflammatory molecules remained unchanged in PF mice (Figure 1E–G). In addition, lipocalin 2 (LCN2), indicating neutrophil infiltration, was significantly increased following G-CSF treatment in the livers of both PF and alcohol-fed mice (Figure 1H). In line with elevated LCN2 levels, we found increased immune cell populations through hematoxylin and eosin staining in the livers of both PF and alcohol-fed mice following G-CSF administration (Figure 1I, black arrow), indicating increased immune cell infiltration. These data indicate that G-CSF reduces alcohol-induced inflammation and liver damage, despite increased infiltration of immune cells into the liver.

As expected, G-CSF administration significantly increased Ly6G<sup>high</sup>CD11b<sup>high</sup> mature neutrophil counts in the bone marrow of both PF and alcohol-fed mice compared to the mice receiving saline (Figure S2A,B). Next, we quantified liver-infiltrating neutrophils (F4/80<sup>-</sup>Ly6G<sup>high</sup>CD11b<sup>high</sup>), using flow cytometry and Ly6G immunofluorescent staining. Neutrophil infiltration to the liver, which is one of the characteristic histologic findings in AH, was significantly increased after 4 weeks of chronic alcohol consumption compared





**FIGURE 1** *In vivo* G-CSF administration ameliorates liver injury and reduces production of inflammatory mediators in alcohol-fed mice. Female mice (8-10 weeks old) were fed with calorie-matched liquid diet (PF) or Lieber DeCarli diet (EtOH) for 4 weeks. Each group of mice received either 200  $\mu$ L saline or recombinant G-CSF (Neupogen; 200  $\mu$ g/kg body weight) for 7 consecutive days in the last week of 4-week alcohol feeding through intraperitoneal injection. (A,B) ALT and AST activities in mouse serum. Graph shows combined data from two independent experiments. (C-H) Evaluation of inflammatory mediators in mouse serum and liver. Combined data from two independent experiments. Serum levels of (C) MCP-1, (D) IL-6. Protein expressions of (E) IL-6, (F) MCP-1, (G) CXCL1, and (H) lipocalin 2 in the livers of each group of mice. (I) Representative images of H&E staining with liver tissue from PF and EtOH-fed mice. Infiltrating immune cells are marked with a black arrow; magnification ×200. Scale bars, 50  $\mu$ m. Data from PF groups (n = 6-8/condition) and EtOH groups (n = 10-12/condition) of mice. Data represent mean with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001. CXCL1, C-X-C-motif ligand 1; H&E, hematoxylin and eosin

to PF control mice (Figure 2A). G-CSF treatment significantly increased neutrophil infiltration in the livers of alcohol-fed mice as determined by flow cytometry analysis (Figure 2A) and immunofluorescence staining (Figure S2C,D). In addition to the neutrophils, the number of liver-infiltrating monocytes/macrophages, gated as F4/80<sup>low</sup>CD11b<sup>high</sup> (black circle), were markedly increased after alcohol consumption. G-CSF administration further elevated the numbers of liverinfiltrating monocytes/macrophages in both PF and alcohol-fed mice (Figure 2B,C). Next, we further separated F4/80<sup>low</sup>CD11b<sup>high</sup>-infiltrating monocytes to Ly6C<sup>high</sup> and Ly6C<sup>low</sup> monocytes using flow cytometry (Figure 2D). Ly6C<sup>high</sup> and Ly6C<sup>low</sup> murine monocytes correspond to CD14<sup>high</sup> CD16<sup>-</sup> classical monocytes and CD14<sup>low</sup> CD16<sup>+</sup> nonclassical monocytes, respectively, in humans. The majority of circulating monocytes are Ly6C<sup>high</sup> inflammatory monocytes that differentiate into macrophages when they migrate into tissues. A small portion of Ly6C<sup>high</sup> monocytes differentiate into Ly6C<sup>low</sup> monocytes that actively participate in patrolling.<sup>[19]</sup> Within the liver-infiltrating monocytes, we found that G-CSF treatment significantly increased Ly6C<sup>high</sup> inflammatory/classical monocytes (Figure 2E) but significantly reduced Ly6C<sup>low</sup> nonclassical monocytes in both PF and alcohol-fed mice (Figure 2F). We also observed significant elevation of Ly6Chigh monocytes, gated as CD11b<sup>high</sup> F4/80<sup>low</sup>Ly6C<sup>high</sup>, and reduced Ly6C<sup>low</sup> monocytes in bone marrow of both PF and alcohol-fed mice following G-CSF treatment (Figure 2G-I). These observations raised the question whether G-CSF treatment inhibits monocyte differentiation from Ly6C<sup>high</sup> to Ly6C<sup>low</sup> cells. Several factors, including M-CSF receptor (M-CSFR), regulate monocyte differentiation.<sup>[20,21]</sup> Interestingly, we found that M-CSFR expression was markedly reduced in Ly6C<sup>high</sup> monocytes found in bone marrow of mice treated with G-CSF (Figure 2J,K). Thus, the significant decrease of Ly6C<sup>low</sup> nonclassical monocytes may result from diminished monocyte differentiation from Ly6C<sup>high</sup> monocytes due to G-CSF-induced M-CSFR reduction.

To investigate whether G-CSF treatment affected macrophage differentiation, we explored macrophage phenotypic changes in mouse liver. First, we observed that RNA and protein expressions of CD68, a marker of macrophage activation, were significantly increased following G-CSF treatment in the livers of both PF and alcohol diet-fed mice (Figure 3A,B). Similar to monocytes, macrophages are a heterogeneous cell population with remarkable phenotypic plasticity. Activated macrophages obtain specific phenotypes in the tissue microenvironment where they are located and exposed to stimuli.<sup>[22]</sup> M1 or classically activated macrophages show increased expression of inflammatory mediators, including inflammatory cytokines,<sup>[23]</sup> while M2 macrophages have anti-inflammatory and repair

characteristics.<sup>[22,23]</sup> Along with enhanced CD68 expression, we found that G-CSF treatment significantly increased RNA levels of macrophage mannose receptor (CD206) in the livers of both PF and alcohol-fed mice (Figure 3C). Expression of the high-affinity scavenger receptor (CD163) was significantly increased by alcohol and further up-regulated by G-CSF treatment in alcohol-fed livers (Figure 3D).

Next, we examined if G-CSF treatment promotes macrophage differentiation toward M2 macrophages in vitro. We treated RAW 264.7 cells with in vitro EtOH (50 mM) for 4 days and added G-CSF (100 ng/mL) at day 2 of alcohol treatment to mimic the in vivo experimental setting. Both CD206 and CD163 expressions were increased after alcohol exposure, and G-CSF treatment further elevated their expression in these alcohol-treated cells (Figure 3E-H). Consequently, macrophages that were treated with both in vitro alcohol and G-CSF significantly up-regulated M2 macrophage marker expression on their surface compared to nontreated control macrophages (Figure 3E-H). We also assessed expression of CD86 and CD80 in these RAW cells, which are used to characterize M1 macrophages. We found that 4 days of in vitro alcohol treatment significantly down-regulated CD86 expression (Figure 3I; Figure S3A) and significantly up-regulated CD80 (Figure 3J; Figure S3B) in the macrophages. G-CSF treatment over 48 hours did not induce additional changes in these M1 macrophage markers (Figure 3I,J; Figure S3A,B) in the alcoholtreated macrophages.

We also examined BMDMs treated with G-CSF (100 ng/mL) for 24 hours with or without in vitro alcohol (50 mM, 24 hours). Similar to the data shown in the RAW cells, in vitro alcohol exposure significantly increased CD206 expression in BMDMs, and G-CSF induced a further significant increase in CD206 expression in alcohol-treated BMDMs (Figure S3C,D). In the presence of alcohol, the BMDMs that were treated with G-CSF also significantly up-regulated CD86 expression on their surface (Figure S3E,F) compared to nontreated control BMDMs, while expression of another M1 macrophage marker, CD80, remained unchanged (Figure S3G). These data together suggest that alcohol induces macrophage activation with an M1/M2 mixed phenotype and G-CSF promotes further macrophage activation/differentiation to an M2-like phenotype in the presence of alcohol.

Next, we sought to investigate the G-CSFmediated mechanisms that may prevent or ameliorate alcohol-induced liver damage. Several mechanisms of G-CSF-mediated hepatoprotective effects have been reported. First, G-CSF treatment prevents liver damage by an increase of anti-apoptotic molecules or a decrease of pro-apoptotic molecules.<sup>[24]</sup> In our experimental setting, although there are differences between RNA and protein levels, G-CSF treatment



failed to increase expressions of B-cell lymphoma extra large (Bcl-xL), an anti-apoptotic molecule, at both transcription and protein levels in hepatocytes (Figure 4A–C).

G-CSF treatment could promote liver regeneration by increasing hematopoietic stem cell (HPSC) mobilization or oval cell proliferation.<sup>[5,17,25]</sup> We found that proliferating cells, indicated by a Ki67-positive signal, **FIGURE 2** G-CSF treatment increases the numbers of neutrophils and monocytes in bone marrow and liver. (A) Liver-infiltrating neutrophils within total live nonparenchymal cells in liver after hepatocyte separation (gated as F4/80<sup>-</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>) were quantified through flow cytometry analysis. (B,C) Quantification of liver-infiltrating monocytes following G-CSF injection. Representative image of (B) monocyte gating (F4/80<sup>low</sup>CD11b<sup>high</sup>) and (C) quantification. (D-F) Quantification of Ly6C<sup>high</sup> monocyte (CD11b<sup>+</sup>Ly6C<sup>high</sup>) counts in the livers of PF and EtOH-fed mice. (D) Gating strategy of inflammatory or anti-inflammatory monocytes within the total infiltrating monocytes. (E) Quantification of inflammatory monocytes (F4/80<sup>low</sup>CD11b<sup>high</sup>Ly6C<sup>high</sup>) and (F) patrolling or noninflammatory monocytes (F4/80<sup>low</sup>CD11b<sup>high</sup>Ly6C<sup>low</sup>) in the liver of PF and EtOH-fed mice following G-CSF treatment. (G-I) Quantification of Ly6C<sup>high</sup> monocyte counts in bone marrow. (G) Representative image of monocyte gating and quantification of (H) Ly6C<sup>high</sup> and (I) Ly6C<sup>low</sup> monocytes. (J,K) Representative image and quantification of mean fluorescent intensity of M-CSFR expression in Ly6C<sup>high</sup> monocytes isolated from bone marrow after G-CSF treatment. Data from PF groups (n = 7-10/condition) and EtOH-fed groups (n = 10-12/condition) of mice. Data are mean with SEM. Statistical significance was determined using Brown-Forsythe and Welch ANOVA tests followed by Dunnett's T3 multiple comparison test; \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001. APC, allophycocyanin; BM, bone marrow; comp, compensation; FITC, fluorescein isothiocyanate; MFI, mean fluorescent intensity; PerCP, peridinin chlorophyll protein

were substantially reduced in the livers of alcoholfed mice compared to PF control mice (Figure 4D,F). However, we observed that Ki67<sup>+</sup> cells were significantly increased in the liver of alcohol-fed mice following G-CSF treatment (Figure 4D,F). The Ki67<sup>+</sup> cells were distributed throughout the entire liver section not just located in a particular area, such as the ductular region (Figure 4E). Interestingly, positive signals were mostly observed in hepatocytes, which are differentiated by big and multiploidy nuclei not in the cells with oval shapes (oval cells, LPCs) (Figure 4D, red box). Therefore, we hypothesized that Ki67-positive proliferating cells are hepatocytes and G-CSF promotes hepatocyte proliferation. It has been reported that alcohol induces cellcycle arrest in hepatocytes and thus halts hepatocyte proliferation and interrupts self-repair systems in the liver.<sup>[26,27]</sup> We found that protein levels of cyclin D1 in hepatocytes were significantly reduced following an alcohol diet, and this reduction was restored following G-CSF treatment in alcohol-fed mice (Figure 4G,H). Interestingly, cyclin-dependent kinase inhibitor (p21) expression was significantly up-regulated in PF mice following G-CSF administration. Given that p21 overexpression inhibits hepatocyte proliferation, [28,29] our data suggest that p21 could be a negative regulator of hepatocyte proliferation in PF mice (Figure 4I).

In clinical trials, a significant increase of CD34<sup>+</sup> cells was observed in the circulation of patients with AH who received G-CSF treatment for 5 days; this was speculated as a source of liver regeneration. Intriguingly, we found a substantial increase in CD34<sup>+</sup> cells in livers of alcohol-fed compared to control diet-fed mice (Figure 4J,K; Figure S4A). Quantitative polymerase chain reaction analysis revealed an additional increase of CD34 expression following G-CSF treatment in alcohol-fed mice; however, this was not statistically significant (Figure S4A). Flow cytometry analysis did not show an additional increase in CD34<sup>+</sup> cells after G-CSF administration in the livers of alcohol-fed mice (Figure 4J,K).

LPCs express LPC-specific progenitor cell markers, including alpha-fetoprotein (AFP) and SRY-box transcription factor 9 (SOX9),<sup>[30,31]</sup> which were increased

following G-CSF in PF control mice (Figure S4B,C). However, these markers were substantially downregulated and remained unchanged even after G-CSF treatment in alcohol-fed mice (Figure S4B,C). Taken together, our data suggest that G-CSF administration facilitates hepatocyte-driven liver repair in alcohol-fed mice and that HPSCs or LPCs are less likely involved in G-CSF-induced liver repair.

We then sought to confirm that hepatocytes are indeed a source of proliferating cells following G-CSF/G-CSF receptor (G-CSFR) engagement. We performed co-immunofluorescent staining with Ki67 and markers of hepatocytes (hepatocyte nuclear factor 4 alpha [HNF-4 $\alpha$ ]), LPC (SOX9), and HPSC (CD45) in mouse liver. Consistent with data from cyclin D1 expressions in hepatocytes, we observed Ki67-positive signals in HNF-4 $\alpha$ -positive cells in the liver of mice receiving G-CSF (Figure 5A). However, we did not observe co-localization of Ki67 and either SOX9- or CD45-positive cells (Figure 5B,C). These data together suggest that G-CSF administration promotes hepatocyte proliferation rather than HPSCs or LPCs in liver.

G-CSF transduces signals following binding to its receptor G-CSFR. G-CSFR expression is observed mainly in granulocytes and their precursor cells. In addition to neutrophils, other cell types, such as endothelial cells, also express functional G-CSFR and respond to G-CSF.<sup>[32]</sup> Hepatocytes express various growth factor receptors, including epidermal growth factor receptor (EGFR), and these receptors have been studied in the context of liver regeneration. However, G-CSFR expression in hepatocytes has not been widely studied. Here, we found that G-CSF significantly increased G-CSFR expression in hepatocytes at the messenger RNA level (Figure 5D). We also observed that protein expression of G-CSFR was significantly increased in hepatocytes following G-CSF administration in both PF and alcohol-fed mice (Figure 5E-G).

Under G-CSFR engagement, activation of the following three major downstream pathways have been described: extracellular signal-regulated kinase (ERK), signal transducer and activator of transcription 3 (STAT3), and phosphatidylinositol 3 kinase (PI3K)/



protein kinase B (Akt) pathways.<sup>[7]</sup> We observed that ERK1/2 activation, indicated by phosphorylation of ERK1/2, is significantly diminished (p42) following alcohol consumption and was markedly restored following

G-CSF treatment in hepatocytes isolated from alcoholfed mice (Figure S5A–C). In contrast to ERK, chronic alcohol already enhanced STAT3 activation, which was shown by increased phosphorylated STAT3 **FIGURE 3** In vivo G-CSF treatment promotes macrophage activation with an M2-like phenotype in liver of alcohol-fed mice. (A, B) RNA and protein expression levels of macrophage activation marker CD68 in liver following G-CSF treatment. (C,D) RNA expression levels of M2 noninflammatory macrophage markers. (C) Mannose receptor or CD206, (D) high-affinity scavenger receptor of human hemoglobin or CD163 expression in mouse liver. Data from PF groups (n = 5-8/condition) and EtOH-fed groups (n = 6-9/condition) of mice. (E-J) Protein expressions of M1/M2 macrophage markers on the surface of RAW 264.7 cells (n = 6) in the presence of *in vitro* alcohol (4 days, 50 mM) and G-CSF (100 ng/mL, 2 days). Representative images and quantification of MFI changes in (E,G) CD206 and (F,H) CD163. MFI changes of M1 macrophage markers (I) CD86 and (J) CD80 in the RAW cells were also quantified through flow cytometry. Data are mean with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.001. comp, compensation; MFI, mean fluorescent intensity; NT, not treated; PE, phycoerythrin

(Figure S5D,E). We did not observe additional STAT3 activation following G-CSF treatment in hepatocytes isolated from alcohol-fed mice (Figure S5D,E). Moreover, we found that chronic alcohol slightly reduced glycogen synthase kinase 3 beta (GSK3 $\beta$ ) phosphorylation at serine 9 (Ser9), which is accompanied by increased phosphorylated  $\beta$ -catenin in hepatocytes (Figure S5F–H). In a steady state, GSK3<sup>β</sup> phosphorylates  $\beta$ -catenin, which induces the  $\beta$ -catenin destruction complex and  $\beta$ -catenin degradation.<sup>[33]</sup> In the PI3K/Akt pathway, activated Akt phosphorylates and inactivates GSK3<sup>β</sup> (phosphorylation at Ser9), preventing  $\beta$ -catenin phosphorylation and allowing  $\beta$ -catenin nuclear translocation.<sup>[33]</sup> Therefore, phosphorylation of GSK3<sup>β</sup> is associated with reduced phosphorylated  $\beta$ -catenin, which results in increased  $\beta$ -catenin nuclear translocation.<sup>[33]</sup> In our experimental setting, we found that G-CSF treatment increased phosphorylation of GSK3<sup>β</sup> (Ser9) followed by reduction in phosphorylated β-catenin in alcohol-fed mice (Figure S5F–H).

To further examine if G-CSF can activate ERK1/2 and  $\beta$ -catenin in hepatocytes, we treated mouse primary hepatocytes and Hepa1-6 cells with G-CSF (100 ng/mL) for 12 hours in vitro. Consistent with our in vivo findings, we found significantly reduced phosphorylated  $\beta$ -catenin expression in both mouse primary hepatocytes and Hepa1-6 cells following in vitro G-CSF treatment (Figure 5H-K). However, there was no significant change in ERK1/2 phosphorylation in hepatocytes (data not shown). This could result from differences between in vivo and in vitro experimental settings or treatment condition, such as long-term and short-term treatment. Altogether, these data suggest that G-CSF increases G-CSFR expression in hepatocytes and promotes hepatocyte proliferation. Our data also show that β-catenin is activated in hepatocytes following G-CSF administration. Further studies may identify downstream pathways regulating hepatocyte proliferation following G-CSF/G-CSFR ligation.

Next, we examined if G-CSF treatment is beneficial to regulate inflammation in the presence of a challenge with the toll-like receptor 4 ligand, LPS, in AH. In the circulation and liver of patients with AH, gut-derived pathogen-associated molecules (PAMPs), including LPS, are significantly elevated due to an increased leaky gut. To mimic this PAMP-enriched environment, mice

received LPS (0.5 mg/kg body weight) (Figure 6A). As expected, G-CSF administration in the LPS-challenged mice resulted in a significant increase of neutrophils and monocyte infiltrations to the liver in both PF and alcohol-fed mice (Figure S6A,B). We found that ALT and AST significantly increased following G-CSF treatment in alcohol-fed mice, indicating that G-CSF administration increases liver damage in alcohol-fed mice following LPS challenge (Figure 6B,C). Similar to the ALT and AST increases, G-CSF administration significantly increased IL-6 production in alcohol-fed mice (Figure 6D). We also observed that MCP-1 production was substantially increased in alcohol-fed mice following G-CSF treatment (Figure 6E). Moreover, G-CSF administration increased the number of proliferating cells in the liver of alcohol-fed mice, indicated by an increased Ki67-positive signal in cells (Figure 6F,G). Interestingly, Ki67-positive signals were not observed in HNF-4 $\alpha^+$  cells, indicating that the proliferating cells are not hepatocytes after an LPS challenge (Figure 6H).

Overall, in the presence of a PAMP, such as LPS, exogenous G-CSF increases inflammation and exacerbates liver damage in alcohol-fed mice. However, G-CSF treatment increases the number of proliferating cells in the liver, although cellular types of these proliferating cells may be shifted.

### DISCUSSION

G-CSF has been used in several clinical settings. Its recent use in patients with severe AH showed improvement on liver functions and survival. However, mechanisms by which G-CSF exerts the beneficial effect have not been completely established. Here, we report that G-CSF attenuates liver damage and inflammation and promotes macrophage polarization toward M2like macrophages (Figure 7A). We found that G-CSF treatment up-regulates its receptor G-CSFR expression in hepatocytes and increases the number of proliferating hepatocytes in alcohol-fed mice. However, following LPS stimulation, the G-CSF-induced protective effect on liver damage was no longer observed in alcohol-fed mice. We still observed increased Ki67<sup>+</sup> cells in alcohol-fed mice following G-CSF administration (Figure 7B); however, Ki67-positive signals were not colocalized with HNF-4 $\alpha$  in the presence of an LPS



challenge. Our data suggest that dominant proliferating cell type (hepatocytes) following G-CSF treatment may be shifted, with increased liver injury triggered by LPS in alcohol-fed mice.

**CD34** 

Comp-PE-A

Generally expected outcomes of in vivo G-CSF administration are increased production and mobilization of granulocytic cells. We observed that productions of neutrophils and Ly6C<sup>high</sup> monocytes were **FIGURE 4** *In vivo* G-CSF administration increases proliferating hepatocytes in mice receiving an alcohol diet. (A-C) RNA and protein expressions of Bcl-2 family Bcl-xL in hepatocytes from PF and EtOH-fed mice with or without G-CSF treatment. (D-F) Proliferating cells in the liver were evaluated with IHC staining with anti-Ki67 antibody. (D,E) Representative image of Ki67 IHC-stained mouse liver tissue, and (F) quantification of Ki67-positive signals on hepatocytes, which are indicated by large cellular size and multiple lobules. Average counts of five different fields in a slide; scale bar, 50 µm. (G,H) Protein expression levels of cyclin D1 in hepatocytes. (G) Representative image of western blotting and (H) its quantification. (I) RNA expression level of cyclin-dependent kinase inhibitor p21 in the livers of PF and EtOH-fed mice. (J) Representative image and (K) quantification of flow cytometry analysis of CD34<sup>+</sup> cells in livers of each group of mice. Data from PF groups (n = 5-8/condition) and EtOH-fed groups (n = 6-9/condition) of mice. Western blotting experiments with biologically independent samples. Data are mean with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; \**p* < 0.05, \*\**p* < 0.01. comp, compensation; FSC-A, forward scatter area; IHC, immunohistochemistry; PE, phycoerythrin

significantly increased in the bone marrow of both PF and alcohol-fed mice following G-CSF administration. Consistently, we found significantly increased liver-infiltrating neutrophils and monocytes following G-CSF. However, the levels of ALT and AST in serum as well as inflammatory cytokine/chemokine productions significantly diminished following G-CSF administration in alcohol-fed mice. These data suggest that the G-CSF induced increase of immune cells in the liver did not necessarily induce liver injury or inflammation in either PF or alcohol-fed mice. Therefore, we speculated that G-CSF suppresses activation of the infiltrating neutrophils and inflammatory monocytes. In fact, the G-CSF-mediated immunomodulatory effect is controversial because contrasting outcomes have been reported in human and animal models of several diseases. Following bacterial infection or LPS stimulation in healthy subjects, in vivo G-CSF administration ameliorates inflammation responses through suppressing proinflammatory cytokine production in various immune cells. In in vitro experiments with isolated human blood cells, G-CSF treatment also reduces inflammatory cytokine production, including tumor necrosis factor alpha, following LPS stimulation.<sup>[34]</sup> These results suggest that G-CSF plays an anti-inflammatory role in acute infection or inflammation. However, G-CSF treatment exhibits a detrimental effect on certain disease conditions, such as chronic obstructive pulmonary disease<sup>[35]</sup> and rheumatoid arthritis.<sup>[36]</sup> In these disease conditions, immune cell infiltration and chronic inflammation are highlighted, and blockade of G-CSF reduces inflammation and ameliorates disease progression. Considering that G-CSF enhances immune cell production and mobilization, exogenously administered G-CSF may exacerbate chronic inflammatory diseases that are full of damageassociated and/or pathogen-associated molecules. In our experimental setting, elevated liver-infiltrating immune cells after G-CSF treatment could be involved in G-CSF-mediated immunomodulation and thus reduce alcohol-induced inflammation and liver injury in alcohol-fed mice. When these immune cells are exposed to additional stimulation (LPS), the increased immune cells can elicit inflammatory responses and contribute to tissue damage. However, considering

that immune reactions are orchestrated with peak and resolution phases, G-CSF treatment could provide benefit in the late phase of LPS-induced inflammation even though it results in robust inflammatory reactions in the early phase of inflammation. Follow-up studies are required to examine inflammation and liver injury at multiple time points after LPS challenge to evaluate G-CSF effects/outcomes in patients with AH.

The liver has unique regeneration capacity. Adult mature hepatocytes are mostly found quiescent and stay in G0 phase in the cell cycle under homeostasis.<sup>[37]</sup> Following injury, various mitogenic stimuli trigger G0/ G1 transition, and hepatocytes enter the cell cycle and replace damaged hepatocytes.<sup>[15,38]</sup> We observed that proliferating hepatocytes were significantly increased following G-CSF treatment in alcohol-fed mice, indicated by colocalization of Ki67 and HNF-4 $\alpha$  staining. Although hepatocytes are considered a major contributor in liver repopulation, other cells, including LPCs and HPSCs, are also described as a source of proliferating cells and are actively involved in liver repair under certain conditions. LPCs possess the capacity to be differentiated to both hepatocytes and biliary epithelial cells (or cholangiocytes).<sup>[39,40]</sup> However, LPCs are rarely observed in normal adult liver under homeostasis. LPCs are believed to be located within the Canals of Hering, where intracellular bile ductules are observed in periportal areas.<sup>[39]</sup> Following its activation in an injury setting, several LPC-specific markers, such as AFP and SOX9, would be up-regulated in the liver. We did not observe AFP up-regulation in alcohol-fed mice following G-CSF treatment. Consistently, we found the SOX9-positive signals near the bile duct area, but these SOX9-positive cells did not show Ki67-positive signals, suggesting that LPCs or biliary epithelial cells are not the source of proliferating cells following G-CSF administration in alcohol-fed mice.

In small clinical studies with patients with severe AH, G-CSF administration significantly increased bone marrow-derived stem cells (CD34<sup>+</sup> cells) in the circulation, which was speculated as a source of liver repopulation following G-CSF treatment. Interestingly, we observed that CD34<sup>+</sup> cells were increased following 4-week alcohol consumption, and additional increase of CD34<sup>+</sup> cells following G-CSF treatment was



**FIGURE 5** G-CSF treatment increases proliferating hepatocytes and up-regulates G-CSFR in hepatocytes in alcohol diet-fed mice. (A-C) Assessment of proliferating cells in the livers of each group of mice through co-immunofluorescence staining with anti-Ki67 antibody and antibodies targeting HNF-4 $\alpha$  (hepatocytes), SOX9 (LPCs), and CD45 (HPSCs). Representative images of coimmunofluorescence staining with Ki67 and (A) HNF-4 $\alpha$ , (B) SOX9, or (C) CD45. Scale bar, 20 µm. (D-G) Assessment of G-CSFR in hepatocytes. RNA expression levels of G-CSFR in (D) mouse hepatocytes and (E) representative image of protein expression measured by immunofluorescence staining with mouse liver. Representative image of isotype antibody used in the liver of EtOH + G-CSF. Scale bar, 50 µm. (F) Representative image and (G) quantification of western blotting presenting G-CSFR expressions in mouse hepatocytes. Data from PF groups (n = 5-8/condition) and EtOH-fed groups (n = 6-9/condition) of mice. (H-I) Western blotting image and quantifications of phosphorylated  $\beta$ -catenin in mouse primary hepatocytes (n = 3) following *in vitro* G-CSF treatment (100 ng/mL, 12 hours). (J,K) Western blotting image and quantification of phosphorylated  $\beta$ -catenin in Hepa1-6 cells (n = 3) following *in vitro* G-CSF treatment (100 ng/mL, 12 hours). Western blot images shown are representative of two independent western blotting experiments with biologically independent samples. Data are mean with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; \*p < 0.05, \*\*p < 0.01. DAPI, 4',6-diamidino-2-phenylindole; IF, immunofluorescence; NT, not treated



### FIGURE 5 (Continued)

marginal (RNA expression) in the livers of alcohol-fed mice. Moreover, we did not find that Ki67-positive signals overlapped with CD45<sup>+</sup> cells, representing bone marrow-derived cells.

However, in the presence of LPS, Ki67-positive signals were rarely colocalized with HNF-4 $\alpha$  in alcohol-fed mice. Considering that liver damage was exacerbated following G-CSF administration in the combination of



FIGURE 5 (Continued)

chronic alcohol and LPS, it is tempting to speculate that the dominant cellular source of liver repopulation may be switched from hepatocytes to another when the degree of liver injury exceeds hepatocyte self-repair capacity. Additional experiments are required to identify which cell populations are mainly contributing to G-CSF-mediated liver repair in the presence of additional PAMPs, such as LPS.

Numerous studies have tried to identify mechanisms regulating liver regeneration. There are several receptors and mitogens that show a hepatotropic effect, such as EGFR and its ligands.<sup>[37]</sup> However, G-CSF/G-CSFR-mediated hepatocyte proliferation has not been extensively studied. We showed in this study that hepatocytes express G-CSFR and that its transcriptomic and protein expression increase following G-CSF administration. With G-CSF treatment, we investigated activation of G-CSFR downstream signaling pathways. Of these, we observed substantial changes following G-CSF in ERK1/2 and  $\beta$ -catenin in vivo. The ERK pathway is considered one of the major cellular signaling cascades governing cell proliferation and differentiation. ERK activation can directly increase cyclin D1 expression and accelerate the cell cycle.<sup>[38]</sup> In addition to ERK,  $\beta$ -catenin is a component of Wnt/ $\beta$ -catenin signaling, which is a major pathway of liver regeneration. In the steady state, phosphorylated  $\beta$ -catenin is degraded. Following stimulation,  $\beta$ -catenin phosphorylation is inhibited, which allows  $\beta$ -catenin nuclear translocation. We found significant reduction in  $\beta$ -catenin phosphorylation in mouse primary hepatocytes and Hepa1-6 cells following *in vitro* G-CSF treatment. We also observed a trend of reduction in  $\beta$ -catenin phosphorylation in hepatocytes of alcohol-fed mice following G-CSF treatment, although this change was not statistically significant. Follow-up studies need to investigate whether ERK or  $\beta$ -catenin is directly activated and regulates hepatocyte proliferation following G-CSF/G-CSFR ligation.

Taken together, we revealed that G-CSF plays an immune modulatory and hepatotropic role in AH, which provides insights to identify the mechanism of G-CSF-mediated therapeutic effects. Our results also indicate that the effect of G-CSF on the liver might be different in patients with AH in the absence or presence of an additional infectious signal, such as LPS. These results highlight clinically relevant challenges in administration of G-CSF in human patients with AH.



**FIGURE 6** G-CSF administration exacerbates liver damage and production of inflammatory mediators in alcohol-fed mice in the presence of LPS. (A) Schematic explanation for the mouse model of AH with 4-week chronic alcohol plus LPS (15 hours) at the last week of alcohol feeding. (B,C) ALT and AST activities in serum of mice. (D,E) IL-6 and MCP-1 in serum of mice. (F-H) Assessment of proliferating cell in livers of each group of mice. (F) Representative images and (G) quantification of Ki67 staining with mouse liver tissue. Ki67-positive signals are indicated with a black arrow and circle. Scale bar, 50 µm. (H) Representative image of co-immunofluorescent staining with Ki67 and HNF-4 $\alpha$ in mouse liver tissue. Scale bar, 20 µm. Representative scale bar added to Merge of PF + Saline group and carries through panel h. Inset in Merge of Ethanol + GCSF group shows 3X enlarged image. Data from PF groups (n = 5-8/condition) and EtOH-fed groups (n = 6-9/condition) of mice. Data are mean with SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparisons test; \**p* < 0.05, \*\**p* < 0.01, \*\*\*\**p* < 0.0001. DAPI, 4',6-diamidino-2-phenylindole; IF, immunofluorescence; Sac, sacrifice



**FIGURE 7** Summary of G-CSF effects on AH. (A,B) Graphical summary of *in vivo* G-CSF treatment on AH using a mouse model in the (A) absence and (B) presence of LPS. Created in Biorender

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### CONFLICT OF INTEREST

Dr. Szabo is a paid consult for Allergan, Alnylam, Arrow, Durect Corporation, Generon, Glympse Bio, Terra Firma, Quest Diagnostics, Pandion Therapeutics, Surrozen, and Zomagen; she holds intellectual property rights with Up to Date. The other authors have nothing to report.

### AUTHOR CONTRIBUTIONS

Gyongyi Szabo and Yeonhee Cho conceived and designed the study. Yeonhee Cho, Radhika Joshi, Patrick Lowe, Christopher Copeland, and Marcelle Ribeiro performed experiments, and Yeonhee Cho, Radhika Joshi, and Gyongyi Szabo analyzed the data. Yeonhee Cho, Caroline Morel, and Donna Catalano performed mouse housing and feeding. Gyongyi Szabo obtained funding for this project. Yeonhee Cho and Gyongyi Szabo wrote the manuscript, and all authors read the manuscript and approved it before submission.

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### SUPPORTING INFORMATION

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