# Exercise changes the intrahepatic immune cell profile and inhibits the progression of nonalcoholic steatohepatitis in a mouse model

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# Exercise changes the intrahepatic immune cell profile and inhibits the progression of nonalcoholic steatohepatitis in a mouse model

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## Abstract

**Background:** NASH is an increasingly common cause of chronic liver disease and can progress to cirrhosis and HCC. Although exercise suppresses inflammation during acute hepatitis, its impact on the progression of chronic liver disease remains unclear. Here, we investigated the effects of exercise on disease progression and intrahepatic immune cell composition in a mouse model of NASH.

**Method:** Mice were assigned to 4 groups: 2 control groups (normal diet) and 2 NASH groups (western diet and low-dose carbon tetrachloride injection). One of each group remained sedentary and one was exercised on a treadmill for 12 weeks (60 min/d, 5 times/wk). All mice were then analyzed for liver histomorphology, steatosis, inflammation, and fibrosis; liver, adipose tissue, and skeletal muscle expression of genes related to metabolism and inflammation; and intrahepatic immune cell composition.

**Result:** Compared with the normal diet mice, NASH mice exhibited enhanced liver steatosis, inflammation, and fibrosis; upregulated expression of liver lipogenesis-related and inflammation-related genes; and increased frequencies of intrahepatic F4/80$^{int}$ CD11b$^{hi}$ bone marrow-derived macrophages and

**Abbreviations**: Acaca, acetyl-CoA carboxylase alpha; Acox1, acyl-CoA oxidase; ALT, alanine aminotransferase; α-SMA, alpha-smooth muscle actin; BMDMs, bone marrow-derived macrophages; CCL2, C-C motif ligand 2; $CCl_4$, carbon tetrachloride; Cpt1a, carnitine palmitoyltransferase 1A; CTSB, cathepsin B; CX3CL1, fractalkine; exe, exercise; FABP3, fatty acid-binding protein 3; Fasn, fatty acid synthase; H&E, hematoxylin-eosin; KO, knockout; MCP-1, monocyte chemoattractant protein-1; ND, normal diet; PD-1, programmed death receptor-1; Pparα/γ, peroxisome proliferator-activated receptor alpha/gamma; RT-qPCR, reverse transcription-quantitative PCR; Scd1, stearoyl CoA desaturase 1; sed, sedentary.

Yuriko Tsutsui and Taizo Mori contributed equally.

Additional information can be found online in the Supporting Information section for this article.

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programmed death receptor-1 (PD-1)$^+$ CD8$^+$ T cells. Expression of inflammatory cytokines and the frequencies of bone marrow-derived macrophages and PD-1$^+$ CD8$^+$ T cells correlated positively with liver steatosis, inflammation, and fibrosis. Exercise was shown to reduce NASH-induced hepatic steatosis, liver inflammation, and fibrosis; induce alterations in metabolism-related genes and inflammatory cytokines in the liver; and suppress accumulation of liver bone marrow-derived macrophages and PD-1$^+$ CD8$^+$ T cells. In addition, we showed that exercise induced increased expression of IL-15 in muscle and its deficiency exacerbated the pathology of NASH.

**Conclusions:** Exercise alters the intrahepatic immune cell profile and protects against disease progression in a mouse model of NASH.



## INTRODUCTION

NAFLD is an increasingly common cause of liver disease worldwide[1] and represents a major global public health burden,[2] a prevalence of NAFLD is about 30% in Asia.[3] NAFLD includes a spectrum of conditions ranging from simple hepatic steatosis to NASH. NASH is characterized by persistent liver inflammation, injury, and fibrosis and can progress to liver cirrhosis and HCC,[4] both of which can be lethal.[5] Therefore, there is an urgent need to identify novel methods to inhibit or prevent the progression of simple hepatic steatosis to NASH and thus improve patient prognosis.

Pharmacological therapies for lifestyle-related disorders such as hyperglycemia, hyperlipidemia, and hypertension, along with antioxidants such as vitamin E, are currently the only treatments for NASH recommended by the Japanese Society of Hepatology.[6] Lifestyle improvements (dietary interventions and exercise) are generally regarded as the only treatment for suppressing the progression from simple hepatic steatosis to NASH. Aerobic and resistance exercises reduce hepatic steatosis in NAFLD patients,[7,8] and exercise can prevent sarcopenia, which is an independent risk factor for NASH and significant fibrosis.[9]

In a mouse model of acute hepatic failure induced by N-galactosamine and lipopolysaccharide, voluntary distance running prevented upregulation of inflammatory pathways in the liver.[10] In a mouse model of liver ischemia/reperfusion injury, preoperative treadmill exercise decreased hepatic tissue injury and liver metastases of murine colorectal adenocarcinoma by inhibiting neutrophil recruitment and extracellular trap formation in the liver.[11] In addition, irisin as a cytokine produced in muscle by exercise improved energy metabolism and prevented fibrosis in a model of chronic kidney injury.[12] However, the impact of exercise on the immunological status of the liver and disease progression in NAFLD is unclear.

Hepatic macrophages have been suggested to play an important role in the pathogenesis of NAFLD. KCs are the main macrophage population in the healthy liver, but in NAFLD, their numbers are reduced and they are replaced by bone marrow-derived macrophages (BMDMs),[13] which express higher levels of inflammatory pathway-related genes than KCs.[14] Activated KCs and BMDMs produce a range of inflammatory cytokines and chemokines, including TNF$\alpha$, IL-1$\beta$, and monocyte chemoattractant protein-1 (MCP-1, also known as C-C motif ligand 2 [CCL2]), which contribute to hepatocyte injury and inflammatory necrosis and promote fibrosis by activation of HSCs.[15] In addition, recent studies have shown that CD8$^+$ T cells play a critical role in obesity-induced chronic inflammation and metabolic disorders.[16] In particular, CD8$^+$ T cell populations that express the immune checkpoint protein programmed death receptor-1 (PD-1) are known to be increased and to directly correlate with body mass index and liver damage in patients with NASH.[17,18]

In the present study, we investigated the effects of treadmill exercise on changes in liver immunology, lipogenesis, inflammation, fibrosis, and skeletal muscle in a mouse model of NASH. We found that exercise suppressed intrahepatic accumulation of BMDMs and PD-1$^+$ CD8$^+$ T cells and prevented disease progression.

## METHODS

### Animals

Animals were treated in accordance with the Guidelines for Animal Experiments of the Research Institute, National Center for Global Health and Medicine, and the animal experimental protocol was approved (Approval No: A041).

Male C57BL/6J and *IL-15* knockout (KO) mice were purchased from Japan SLC (Shizuoka, Japan) and the Jackson Laboratory (Bar Harbor, ME). As for *IL-15* KO mice, after backcrossing with *IL-15* KO mice for 3 generations, KO of *IL-15* was confirmed. Studies were initiated when mice were 6–8 weeks of age.

## Exercise protocol

After an acclimatization period, mice were tested on a treadmill and those animals that refused to run were excluded from the experiments. The remaining mice were assigned to exercise (exe) or sedentary (sed) groups. Mice in the exe group were trained on a motorized treadmill (Muromachi, Tokyo, Japan) during the light phase at 15 m/min, 60 min/day, 5 times per week, for 12 weeks. Mice attempting to rest were encouraged to move by gently tapping them on the tail and back. Sed mice remained in their cages. Exe and sed mice were provided food and water *ad libitum* and were sacrificed 3–6 days after the final exercise training session under free feeding condition.

The final 4 experimental groups were ND + oil + sedentary, ND + oil + treadmill exercise, western diet + $CCl_4$ + sedentary, and western diet + $CCl_4$ + treadmill exercise. In addition, in the study of *IL-15* KO mice, the final 4 experimental groups were wild type (WT) + ND, WT + NASH, *IL-15* KO + ND, and *IL-15* + NASH.

## Induction of NASH

We used a previously reported protocol to induce NASH.[19] Mice were fed a western diet containing 21.1% fat, 34% sucrose, and 0.21% cholesterol (Research Diets, New Brunswick, NJ, D12079B); were provided with a high-sugar drinking solution [23.1 g/L D-fructose (G8270, Sigma-Aldrich, St. Louis, MO) and 18.9 g/L D-glucose (G0127 Sigma-Aldrich)]; and were administered an i.p. injection of 0.2 μL (0.32 μg)/g body weight of carbon tetrachloride ($CCl_4$; Wako, Osaka, Japan, diluted with corn oil to total 100 μL per animal) once per week for 12 weeks. The normal diet (ND) control mice were fed standard chow (CLEA Japan, Tokyo, Japan), provided with tap water, and received i.p. injections of 100 μL per animal of corn oil (C8267, Sigma-Aldrich) on the same schedule. The ND and NASH mice were then assigned to 2 groups and subjected to the treadmill exercise protocol or remained sedentary for 12 weeks, as described above. The NASH group began exercise with the start of the NASH induction procedure. Various experimental methods are explained in supporting information (Supplemental method, http://links.lww.com/HC9/A454). And lists of primers for reverse transcription-quantitative PCR (RT-qPCR) were described in Supplemental Table 1 and 2, http://links.lww.com/HC9/A454.

## Statistical analysis

Prism version 7 software (GraphPad, San Diego, CA) was used for data analysis. Data are presented as the mean ± SD of the indicated number of replicates. Reverse transcription-quantitative PCR (RT-qPCR) analysis was performed in duplicate. Data were compared using Student *t* test and 1-way ANOVA. Correlations were assessed using Pearson rank correlation coefficient. A $p$-value $<0.05$ was considered significant.

# RESULTS

## Exercise suppresses hepatic steatosis, inflammation, and fibrosis in the NASH model

The effects of a treadmill exercise were examined in a mouse model that recapitulates the stages of NASH in humans from simple steatosis to inflammation and fibrosis. Treadmill exercise suppressed body weight gain over the 12-week exercise period (Figure 1A) and showed trends toward decreasing the ratio of liver weight to body weight (Figure 1B) and of epididymal adipose weight to body weight (Figure 1C) after 12 weeks. Liver steatosis was assessed by hematoxylin-eosin and Oil Red O staining of tissue sections (Figure 1D) and showed that, although steatosis developed in the livers of NASH mice compared with the control mice, it was significantly suppressed by exercise (Figure 1D–F). Similarly, serum alanine aminotransferase (ALT) levels (Figure 2A) and expression of inflammation-related genes [*Il-1b*, *Tnf*, *Mcp-1,* and fractalkine (*Cx3cl1*)] in the liver (Figure 2B) were increased in NASH/sed mice compared with ND/sed mice, but these increases were also suppressed by exercise (Figure 2A, B). These results indicate that exercise had an inhibitory effect on hepatic injury and inflammation in the NASH mouse model. We next determined the effects of exercise on liver fibrosis, the terminal pathology of NASH, by staining of liver sections with Sirius Red, which detects collagen deposition, and with an anti-alpha-smooth muscle actin (α-SMA) antibody. Imaging of sections (Figure 2C) and quantification of positively stained areas (Figure 2D, E) showed that tissues from NASH/sed mice had significantly greater areas of Sirius Red and α-SMA staining than did tissues from ND/sed mice, but the extent of staining was significantly reduced in the livers of NASH/exe mice. To confirm these findings, we performed RT-qPCR and showed that exercise effectively suppressed expression of *Collagen1a1,* a fibrosis marker, which was increased in NASH/sed compared with ND/sed mice (Figure 2F). Collectively, these data demonstrate that exercise suppresses hepatic steatosis, inflammation, and fibrosis in the NASH mouse model.

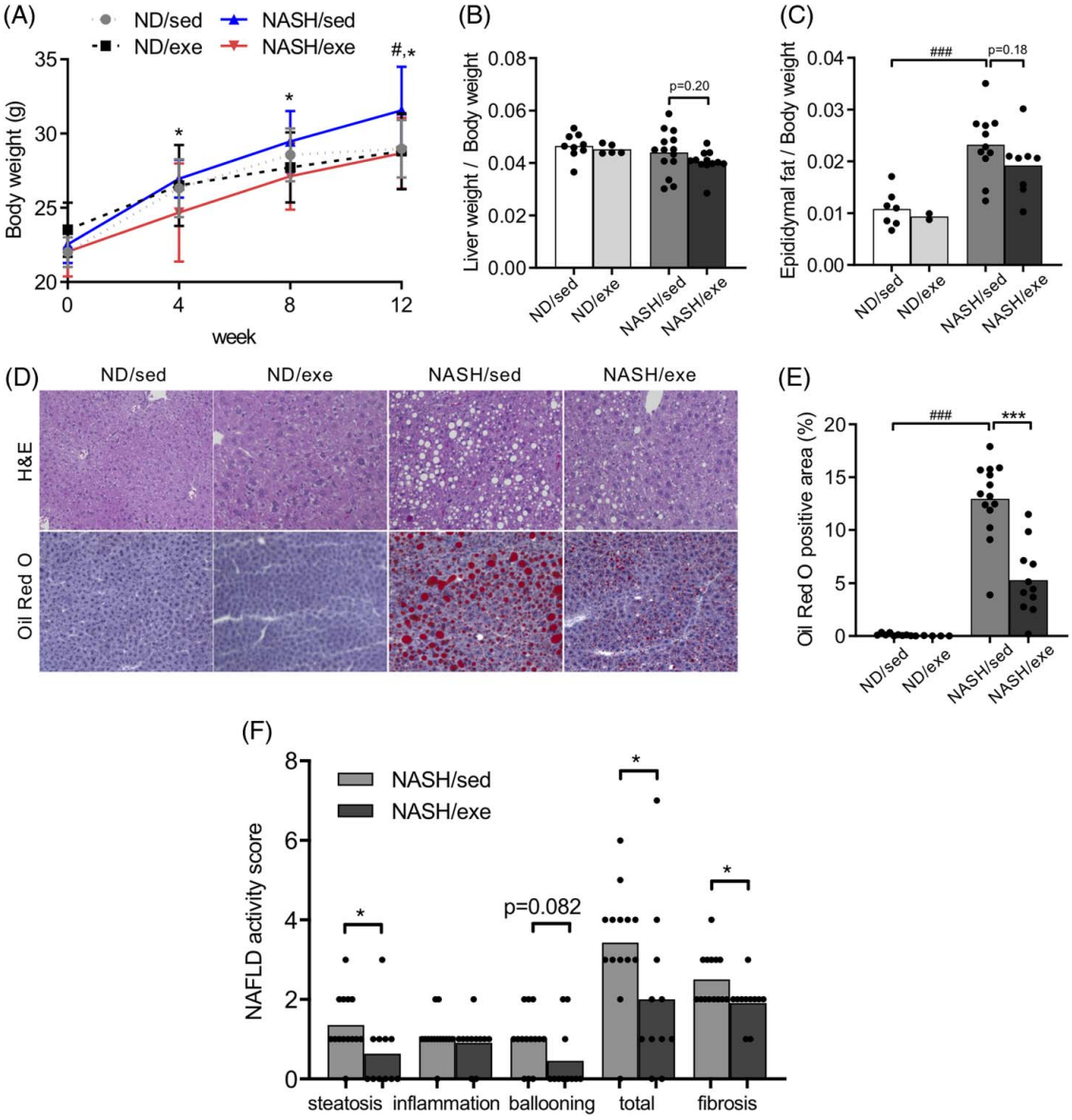


**FIGURE 1** Exercise suppresses obesity and hepatic steatosis in the NASH mouse model. Groups of control mice (normal diet + oil [ND]) or NASH mice (western diet + $CCl_4$) remained sedentary (ND/sed n = 9, NASH/sed n = 14) or exercised on a treadmill (ND/exe n = 5, NASH/exe n = 11) for 12 weeks (except as indicated in A) and analyzed. (A) Body weight. (B) Liver weight-to-body weight ratio. (C) Epididymal fat weight-to-body weight ratio. The number of individuals for this figure only differs from those listed above (ND/sed n = 7, ND/exe n = 2, NASH/sed n = 11, and NASH/exe n = 9). (D) Representative images of liver sections stained with H&E and Oil Red O (original magnification ×40). (E) Quantification of the Oil Red O-positive areas. (F) NAFLD activity score and fibrosis stage at 12 weeks. Results are expressed as the mean ± SD. $^{*}p < 0.05$, $^{\#\#\#,***}p < 0.001$ by Student *t* test for comparison between ND/sed and NASH/sed ($^{\#}$) and between NASH/sed and NASH/exe (*). Abbreviations: $CCl_4$, carbon tetrachloride; exe, exercise; H&E, hematoxylin-eosin; sed, sedentary.

## Exercise alters lipid metabolism in liver, adipose, and muscle tissues

The first step in NASH pathogenesis is conversion of excess energy intake into fat stores, most notably triglycerides. Consistent with this, we observed that hepatic triglyceride levels were increased in NASH/sed compared with ND/sed mice, but this increase was suppressed by exercise (Figure 3A). To determine the mechanism by which hepatic steatosis was reduced by exercise, we performed RT-qPCR analysis of selected genes involved in *de novo* lipogenesis and triglyceride production (acetyl-CoA carboxylase alpha (*Acaca),* stearoyl CoA desaturase *(Scd-1)*, fatty acid synthase *(Fasn),* and peroxisome proliferator-activated receptor gamma (*Pparg*)][20] and of genes involved in lipolysis by means of β-oxidation [peroxisome proliferator-activated receptor alpha (*Ppara*), acyl-CoA oxidase 1 (*Acox1*), and carnitine palmitoyltransferase 1A (*Cpt1a*)][20] in liver, adipose, and muscle tissue samples. Notably,

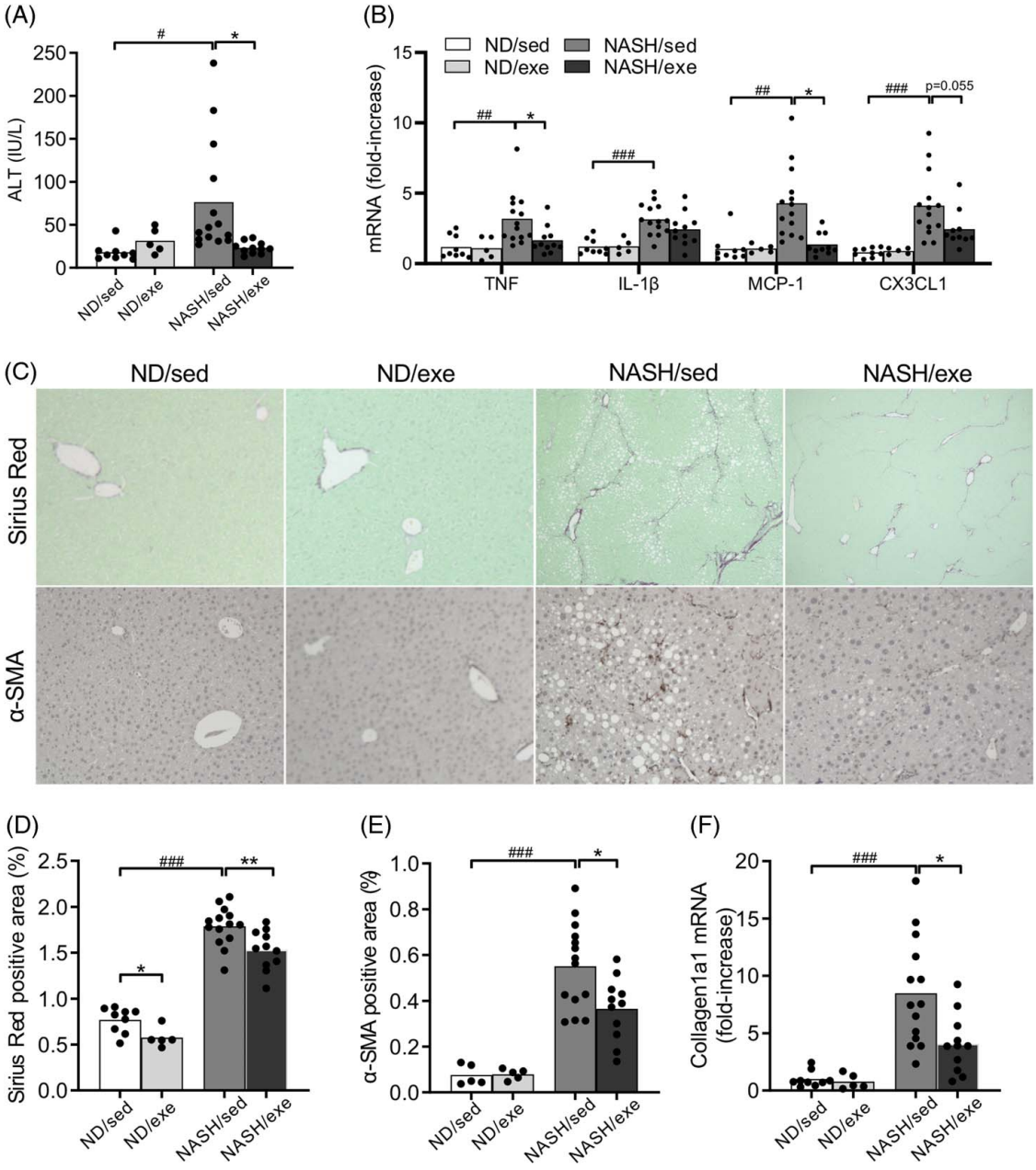


**FIGURE 2** Exercise suppresses hepatic inflammation and fibrosis in the NASH mouse model. Mice were treated as described in Figure 1. (A) Serum ALT levels. (B) RT-qPCR analysis of the indicated mRNA levels in the liver. Gene expressions are calculated as fold increase of average of ND/sed as 1 and shown. (C) Representative images of liver sections stained with Sirius Red (original magnification ×40) or immunostained for α-SMA (original magnification ×100). (D, E) Quantification of the Sirius Red-positive (D) and α-SMA-positive (E) areas. (F) RT-qPCR analysis of *Collagen1a1* expression in the liver. Results are expressed as the mean ± SD. $^{\#,*}p < 0.05$, $^{\#\#,**}p < 0.01$, $^{\#\#\#}p < 0.001$ by Student *t* test for comparison between ND/sed and NASH/sed ($^{\#}$) or between NASH/sed and NASH/exe ($^{*}$). Abbreviations: α-SMA, alpha-smooth muscle actin; ALT, alanine aminotransferase; CX3CL1, fractalkine; exe, exercise; MCP-1, monocyte chemoattractant protein-1; ND, normal diet.

in the liver, expression of the lipogenic genes *Acaca, Scd-1*, and *Fasn* and the lipolytic genes *Ppara* and *Cpt1a* were upregulated in NASH/sed mice compared with ND/sed mice (Figure 3B), and upregulation of *Scd-1* and *Pparg* was reduced by exercise (Figure 3B). In adipose tissue of NASH mice, expression of the lipogenic gene *Fasn* was significantly downregulated by exercise, whereas the lipolytic genes *Ppara* , *Acox1*, and *Cpt1a* were upregulated by exercise, albeit not significantly (Figure 3C). In the muscle of NASH mice, expression of the lipogenic gene *Acaca* was decreased by exercise (Figure 3D). We also assessed the expression of two additional genes, fatty acid-binding protein 3 (*Fabp3)* and cathepsin B (*Ctsb),* in muscle tissue. FABP3 participates in the uptake, intracellular metabolism, and transport of long-chain fatty acids[21]

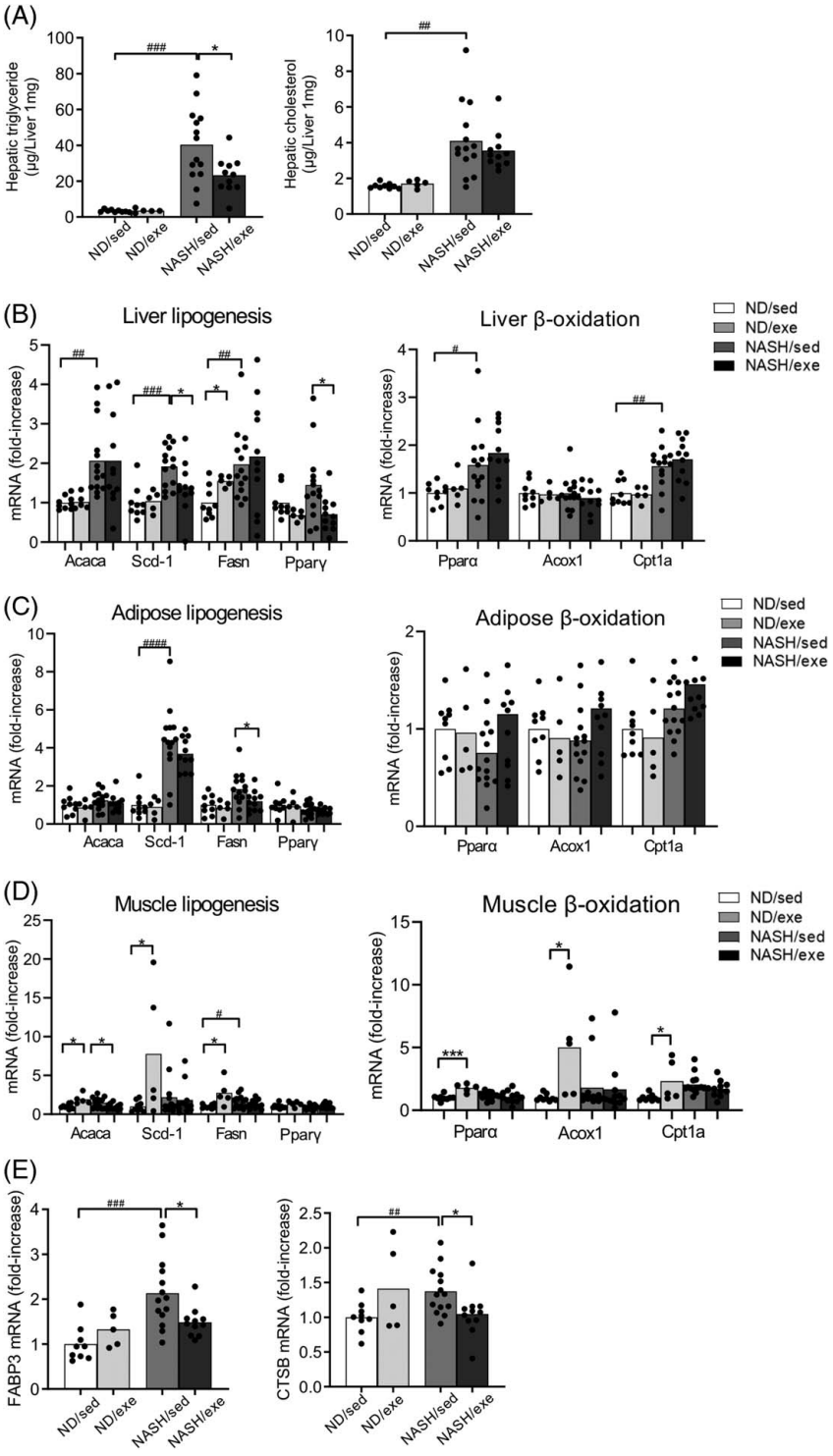


**FIGURE 3** Exercise alters lipid metabolism in the liver, adipose tissue, and muscle in the NASH mouse model. Mice were treated as described in Figure 1. (A) Hepatic triglyceride and cholesterol levels. (B–D) RT-qPCR analysis of genes related to lipogenesis and β-oxidation in the liver (B), adipose tissue (C), and muscle (D). Gene expressions are calculated as fold increase of average of ND/sed as 1 and shown. (E) RT-qPCR analysis of *Fabp3* and *Ctsb* expression in muscle tissue. Results are expressed as the mean ± SD. $^{*,\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ by Student *t* test for comparison between ND/sed and NASH/sed ($^{\#}$) and between ND/sed and ND/exe or NASH/sed and NASH/exe ($^{*}$). Abbreviations: Acaca; acetyl-CoA carboxylase alpha; Acox1, acyl-CoA oxidase; Cpt1a, carnitine palmitoyltransferase 1A; CTSB, cathepsin B; exe, exercise; FABP3, fatty acid-binding protein 3; Fasn, fatty acid synthase; ND, normal diet; Pparγ/α, peroxisome proliferator-activated receptor gamma/alpha; Scd1, stearoyl CoA desaturase 1; sed, sedentary.

and CTSB is a lysosomal protease and that supports lipid deposition in the liver.[22] Of note, the expression of both *Fabp3* and *Ctsb* mRNA in the muscle was induced by NASH, and the increase in both genes was suppressed by exercise (Figure 3E). Taken together, these data suggest that exercise inhibits lipogenesis in the liver and tends to promote lipolysis in the liver and adipose tissues in this NASH mouse model.

## Exercise inhibits intrahepatic accumulation of BMDMs and PD-1$^+$ CD8$^+$ T cells

Next, we investigated the impact of exercise on the dynamics of intrahepatic immune cells in the NASH model. To this end, we performed flow cytometry of total intrahepatic CD45$^+$ leukocytes as well as of subsets of B cells (CD19$^+$), neutrophils (Gr1$^+$), KCs (F4/80$^{hi}$ CD11b$^{int}$), BMDMs (F4/80$^{int}$ CD11b$^{hi}$), NK cells (NK1.1$^+$ TCR-β$^-$), NKT cells (NK1.1$^+$ TCR-β$^+$ ), CD4$^+$ T cells (CD4$^+$ TCR-β$^+$ ), and CD8$^+$ T cells (CD8$^+$ TCR-β$^+$ ) in ND/sed, ND/exe, NASH/sed, and NASH/exe mice after 12 weeks. Representative plot was shown in Figure 4A. The overall frequency of CD45$^+$ immune cells was significantly higher in the NASH/sed mice compared with the ND/sed mice, and although exercise suppressed the increase in NASH mice, the change did not reach the level of statistical significance (Figure 4A, B). Analysis of the average percentage of each cell subset (Figure 4C) showed that NASH was associated with a decreased frequency of KCs and a corresponding increase in BMDMs compared with the livers of ND control mice (Figure 4C–E). In contrast, NASH mice had significantly higher frequencies of intrahepatic NK cells, CD8$^+$ T cells, and PD-1$^+$ CD8$^+$ T cells compared with the control mice (Figure 4F–H). Induction of NASH had no effect on the frequencies of intrahepatic B cells, neutrophils, NKT cells, or CD4$^+$ T cells (Figure 4C). Notably, the NASH-induced changes in the frequencies of intrahepatic BMDMs and PD-1$^+$ CD8$^+$ T cells were significantly reduced by exercise (Figure 4E, H), whereas the NASH-induced increases in NK cells and CD8$^+$ T cells were unaffected by exercise (Figure 4F, G).

## Intrahepatic accumulation of BMDMs and PD-1$^+$ CD8$^+$ T cells is associated with liver inflammation and fibrosis

To determine whether NASH-induced changes in intrahepatic immune cell composition might be related to NASH pathology, we performed correlation analyses of key disease metrics and the frequencies of liver BMDMs and PD-1$^+$ CD8$^+$ T cells. Indeed, the percentage of BMDMs in the liver of NASH mice correlated significantly with body weight, epididymal adipose weight-to-body weight ratio, hepatic steatosis, ALT levels, inflammatory cytokine and chemokine expression (*Tnf*, *Il-1b*, and *Mcp-1*), and liver fibrosis (Figure 5A–H). Moreover, RT-qPCR analysis of *Tnf*, *Il-1b*, and *Mcp-1* mRNA levels in sorted intrahepatic BMDMs and KCs from the NASH mice showed that exercise reduced the expression of these genes in both cell types, but only the reduction of *Il-1b* in BMDMs reached the level of statistical significance (Figure 5I). A similar analysis of correlations between inflammation and disease progression and NASH-induced changes in the frequency of liver PD-1$^+$ CD8$^+$ T cells revealed significant positive correlations between cell frequency and hepatic steatosis, *Tnf* and *Il-1b* mRNA levels, and liver fibrosis (Figure 6A–H). Taken together, these results suggest that exercise reduces the accumulation of body fat, including the liver, resulting in a decrease in infiltration of BMDMs and PD-1$^+$ CD8$^+$ T cells into the liver, thereby attenuating liver inflammation and suppressing the progression of liver fibrosis. Next, to assess the relationship between CD8$^+$ T cells and macrophages in the progression of NASH, we examined the effects of CD8 depletion for 4 weeks during NASH formation. Administration of CD8 antibody to NASH mice markedly reduced the frequency of CD8$^+$ T cells (Supplemental Figure S2A, http://links.lww.com/HC9/A454) and increased the frequency of KCs, but did not alter the frequency of BMDMs in the liver compared to IgG-treated NASH mice (Supplemental Figure S2B, C, http://links.lww.com/HC9/A454). Furthermore, in NASH mice, 4 weeks of CD8 depletion did not affect liver steatosis and fibrosis (Supplemental Figure S2D, E, http://links.lww.com/HC9/A454).

## IL-15 deficiency accelerated liver steatosis and BMDM infiltration

We examined effect of *IL-15*, one of the myokines upregulated by exercise (Supplemental Figure S1, http://links.lww.com/HC9/A454). Under ND, WT and *IL-15* KO mice showed no phenotypic differences (Figure 7A–I). In the NASH condition, *IL-15* KO mice showed no difference in body weight compared to WT mice, but the ratio of liver weight/body weight (Figure 7B) and epididymal fat weight/body weight (Figure 7C) were increased in *IL-15* KO mice. *IL-15* KO mice also had higher serum ALT level than WT mice (Figure 7D) and increased hepatic triglyceride level and hepatic *Cd36* expression (Figure 7E, F) and worse fatty liver (Figure 7G, H). On the other hand, there was no significant difference in the degree of fibrosis (Figure 7G, I). Hepatic immune cell analysis revealed an increased frequency of KCs and BMDMs in *IL-15* KO mice compared to WT mice with NASH (Figure 7J, K). Since IL-15 is known to play an important role in homeostasis of NK cell and memory cytotoxic CD8$^+$ T cells,[23] the percentage of NK cells and

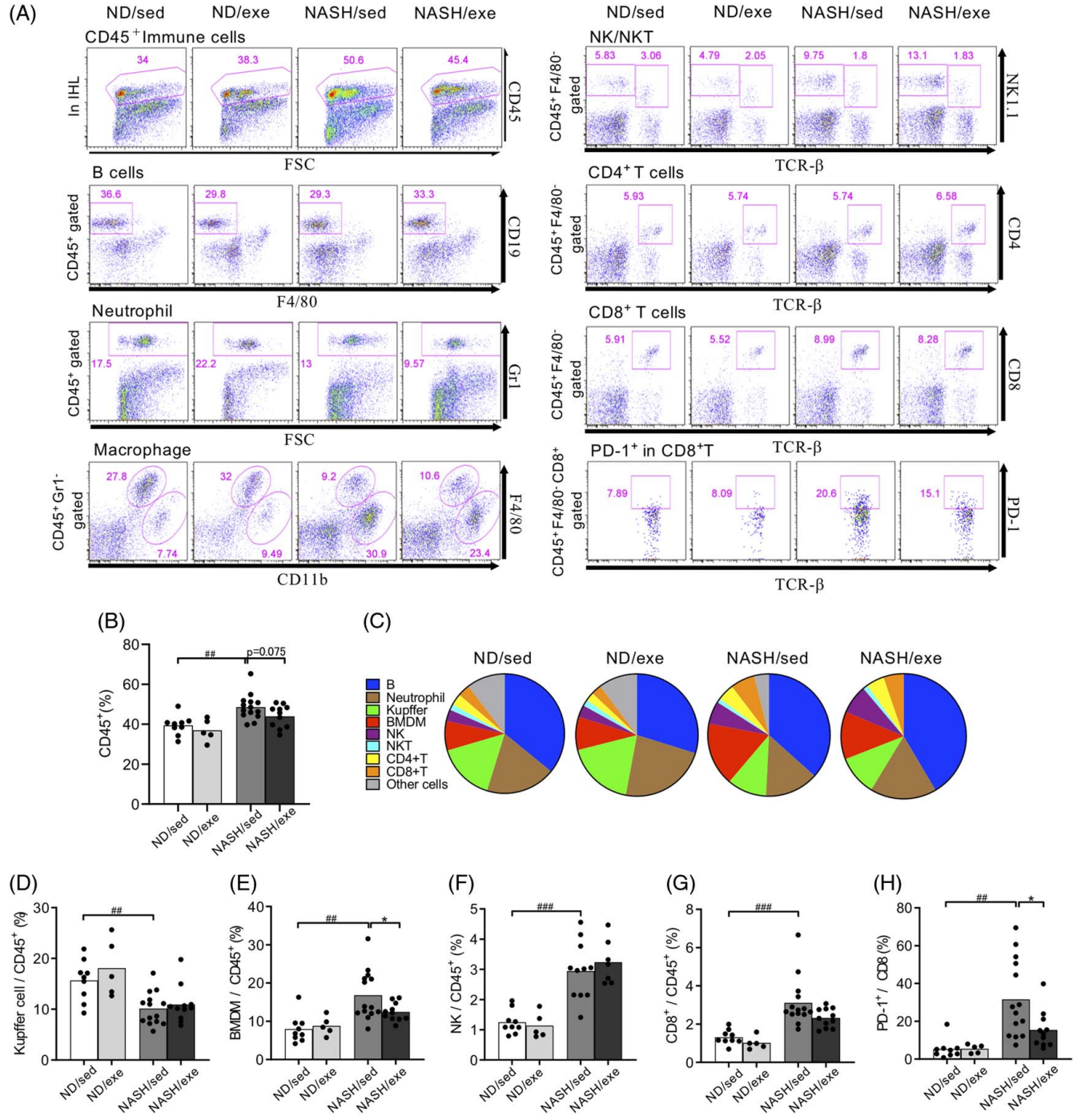


**FIGURE 4** Exercise inhibits NASH-induced intrahepatic accumulation of BMDMs and PD-1 + CD8+ T cells. Mice were treated as described in Figure 1 for 12 weeks and analyzed. (A) Representative flow cytometry dot plots of total CD45+ intrahepatic immune cells and intrahepatic subpopulations of B cells, neutrophils, macrophages, NK/NKT cells, CD4+ T cells, CD8+ T cells, and PD-1 + CD8+ T cells. (B) Percentage of total CD45+ intrahepatic immune cells. (C) Pie charts showing the average proportion of immune cell subpopulations in the livers of each mouse group. (D–G) Percentage of KCs (D), BMDMs (E), NK cells (F), and CD8+ T cells (G) among the CD45+ intrahepatic immune cells. Percentage of PD-1 + among the CD8+ T cells (H). Results are expressed as the mean ± SD. $^{*}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ by Student *t* test for comparison between ND/sed and NASH/sed ($^{\#}$) and between NASH/sed and NASH/exe ($^{*}$). Abbreviations: BMDMs, bone marrow-derived macrophages; exe, exercise; ND, normal diet; PD-1, programmed death receptor-1; sed, sedentary.

CD8+ T cells in the liver was severely reduced in *IL-15* KO mice, with no difference between mice with and without NASH (Figure 7L, M). These results indicated that deficiency of *IL-15* increases the uptake of fatty acids into the liver and exacerbates fatty liver and liver injury caused by NASH.

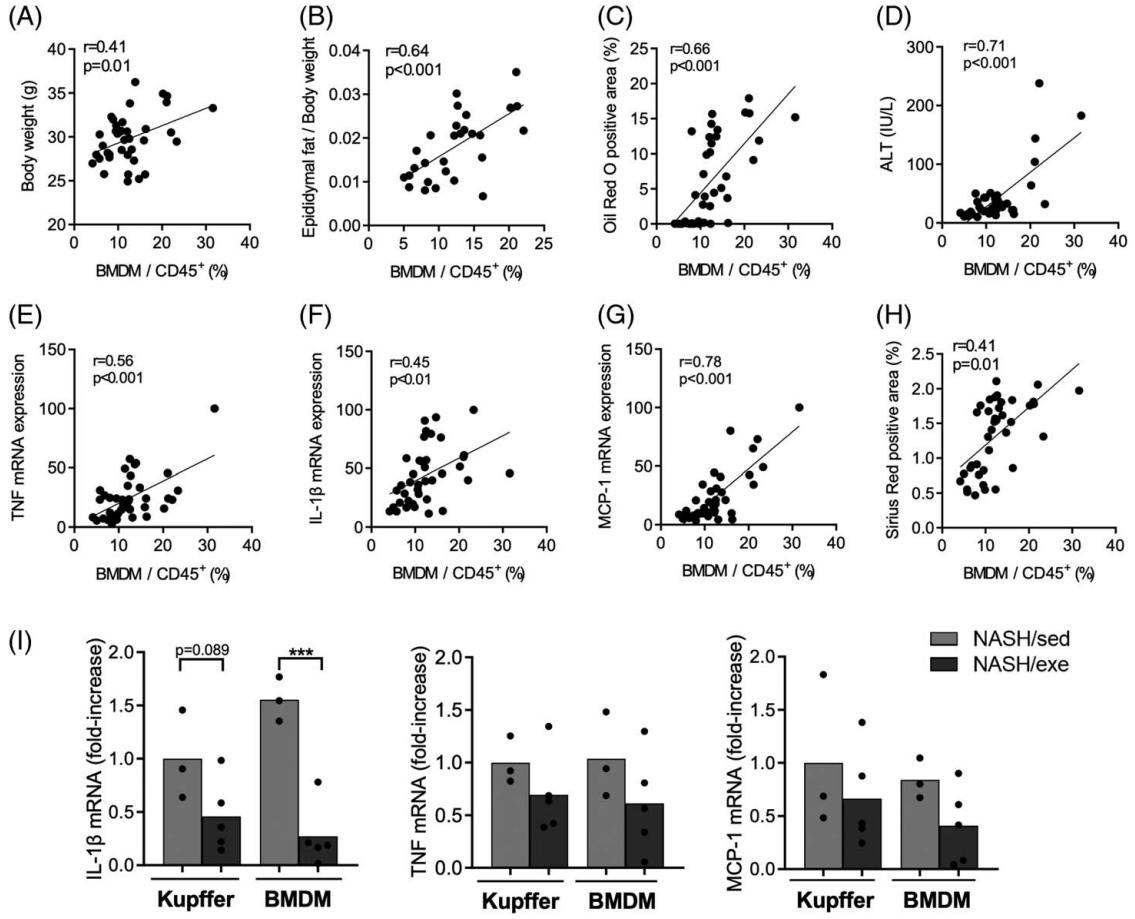


**FIGURE 5** Frequency of intrahepatic BMDM induced by NASH correlates with disease progression. Mice were treated as described in Figure 1 for 12 weeks and analyzed. (A–H) Correlations between the percentage of BMDMs in CD45+ intrahepatic immune cells and body weight (A), epididymal fat weight-to-body weight ratio (B), Oil Red O-positive area in the liver (C), serum ALT levels (D), liver *Tnf* mRNA expression value (E), liver *Il-1b* mRNA expression value (F), liver *Mcp-1* mRNA expression value (G), and Sirius Red-positive area in the liver (H). (I) RT-qPCR analysis of *Il-1b*, *Tnf*, and *Mcp-1* mRNA in sorted liver KCs or BMDMs (NASH/sed n = 3, NASH/exe n = 5 mice). Gene expressions are calculated as fold increase of average of KCs in NASH/sed as 1 and shown. Results are expressed as the mean ± SD. ***$p < 0.001$ by Student *t* test for comparison between NASH/sed and NASH/exe. *r* values represent Pearson rank correlation coefficients. Abbreviations: ALT, alanine aminotransferase; BMDMs, bone marrow-derived macrophages; exe, exercise; MCP-1, monocyte chemoattractant protein-1; ND, normal diet; sed, sedentary.

## DISCUSSION

The importance of exercise for patients with NASH has been advocated in clinical practice, but little evidence is available to support its effectiveness. In the present study, we showed that exercise suppressed NASH progression, including liver steatosis, inflammation, injury, and fibrosis, in a mouse model. We also demonstrated that exercise suppressed the body weight gain and increased fat content of adipose tissue and liver induced by NASH and that exercise altered lipid metabolism and inhibited the accumulation of BMDMs and PD-1$^+$ CD8$^+$ T cells in the liver of NASH mice.

Our study showed that mice with NASH had increased frequencies of intrahepatic BMDMs, CD8+ T cells and NK cells compared with control mice maintained on a normal diet. However, we have found that antibody-mediated depletion of CD8+ T cells had no effect on the frequency of intrahepatic BMDMs in our model, suggesting that BMDMs accumulation in the NASH liver occurs independently of CD8+ T cells.

During the pathogenesis of NASH, hepatic injury due to lipotoxicity causes the release of hepatocyte-derived factors such as damage-associated molecular patterns, extracellular vesicles (exosomes), and inflammatory cytokines, all of which can activate KCs.[24] In turn, activated KCs increase the secretion of inflammatory cytokines and chemokines, such as CCL2, TNF, and IL-1β, leading to recruitment and activation of BMDMs.[25,26] CCL2 is produced not only by macrophages but also by hepatocytes on stimulation by excessive lipid levels and bacterial lipopolysaccharide[27] and induces recruitment of CCR2-expressing BMDMs into the liver. Thus, exercise-induced attenuation of lipid signaling to hepatocytes reduces BMDMs accumulation by suppressing CCL2

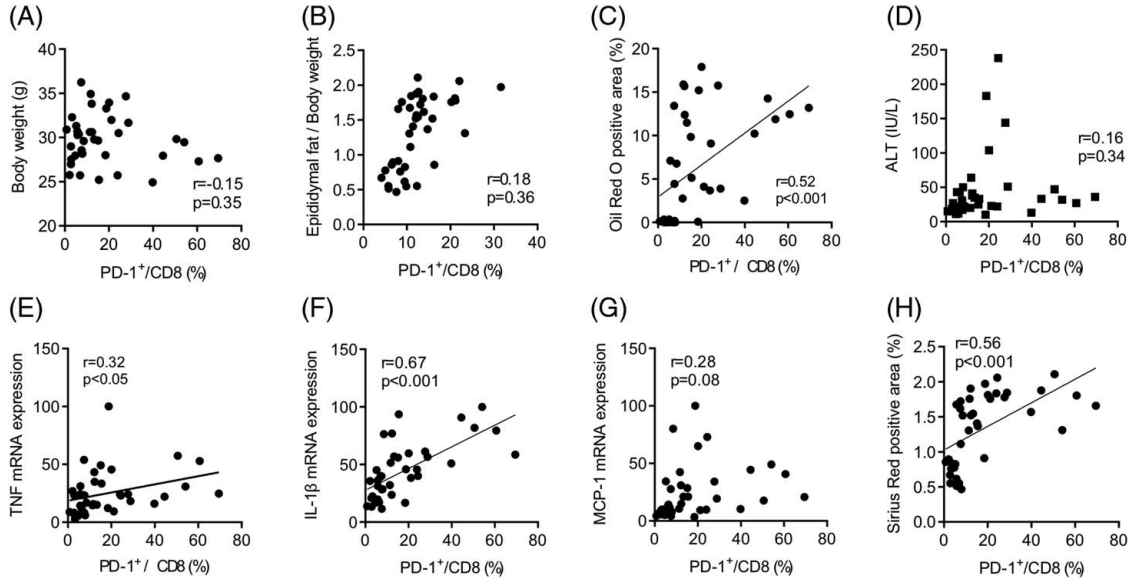


**FIGURE 6** NASH-induced PD-1 positivity in intrahepatic CD8+ T cell correlates with liver inflammation and fibrosis. Mice were treated as described in Figure 1. (A–H) Correlations between the percentage of PD-1 positivity in intrahepatic CD8+ T cells and body weight (A), epididymal fat weight-to-body weight ratio (B), Oil Red O-positive area in the liver (C), serum ALT levels (D), liver *Tnf* mRNA expression value (E), liver *Il-1b* mRNA expression value (F), liver *Mcp-1* mRNA expression value (G), and Sirius Red-positive area in the liver (H). *p*-values were determined by Student *t* test. *r* values represent Pearson rank correlation coefficients. Abbreviations: ALT, alanine aminotransferase; exe, exercise; PD-1, programmed death receptor-1; MCP-1, monocyte chemoattractant protein-1; ND, normal diet; sed, sedentary.

production in the liver. BMDMs also produce TNF and IL-1β and promote liver inflammation,[25] and we found that exercise reduced the accumulation of BMDMs and suppressed hepatic inflammation, which also reduced macrophage activation and IL-1β expression.

Gene set enrichment analysis has shown that mice with NASH induced by choline deficiency and a high-fat diet exhibit increased numbers of PD-1$^+$ CD8$^+$ T cells that involved in ongoing T cell activation and differentiation, TNF signaling, and NK cell–like cytotoxic properties.[18] A progressive accumulation of PD-1$^+$ CD8$^+$ T cells is also observed in the liver of NASH patients,[17,18] and interestingly, patients with NASH-driven HCC who received anti-PD-1 or anti-PD-L1 treatment showed reduced overall survival compared with patients with non-NASH HCC.[18] Our NASH mouse model induced by a high-fat diet and $CCl_4$ also resulted in an increased frequency of PD-1$^+$ CD8$^+$ T cells in the liver that correlated with TNF and IL-1β expression and liver fibrosis, and importantly, this increase may have been reduced by the suppression of hepatic inflammation by exercise.

Exercise is thought to be useful both as a treatment for NASH,[7,8] and for prevention of sarcopenia,[28] although its regulatory mechanism is under investigation. In a mouse model of high-fat diet and high-fructose water, treadmill exercise attenuated hepatic inflammation, injury, and fibrosis by suppressing macrophage infiltration.[29] Furthermore, treadmill exercise reduces the accumulation of inflammatory BMDMs in the NASH liver, and high-intensity interval training improved NASH progression more than moderate-intensity continuous training.[30] However, there is no established mechanism for the myokines produced by muscle during exercise and the inhibitory effect of exercise on the progression of NASH.

Myokines are cytokines or peptides secreted by skeletal muscle cells,[31] and exercise-induced changes in myokine production and muscle quality may affect immunity as well as metabolism. In the present study, we found that exercise enhanced the expression of various myokines, including *PGC1α, IL-15* and *BDNF* (Supplemental Figure S1, http://links.lww.com/HC9/A454). IL-15 was upregulated by treadmill in the skeletal muscle of acute exercise in other animal models.[32] IL-15–mediated signaling plays a protective role in the progression of liver fibrosis in a model of chronic liver injury.[23] *IL-15*–deficient mice in our NASH model exhibited exacerbated liver steatosis and injury compared to the normal NASH model, suggesting that increased hepatic CD36 expression by *IL-15* deficiency may have exacerbated fatty liver disease by enhancing fatty acid uptake. In addition, *IL-15* KO mice showed an increased frequency of BMDMs specific to the NASH model, but did not show increased fibrosis compared to control mice. Although these results were surprising, it is possible that the loss of IL-15 signaling promoted liver adipogenesis and enhanced BMDM recruitment, while suppressing BMDM and KC activation.[33,34] These results suggest that IL-15 produced by exercise may be partially responsible for the inhibition of NASH progression by exercise. However, we did not use

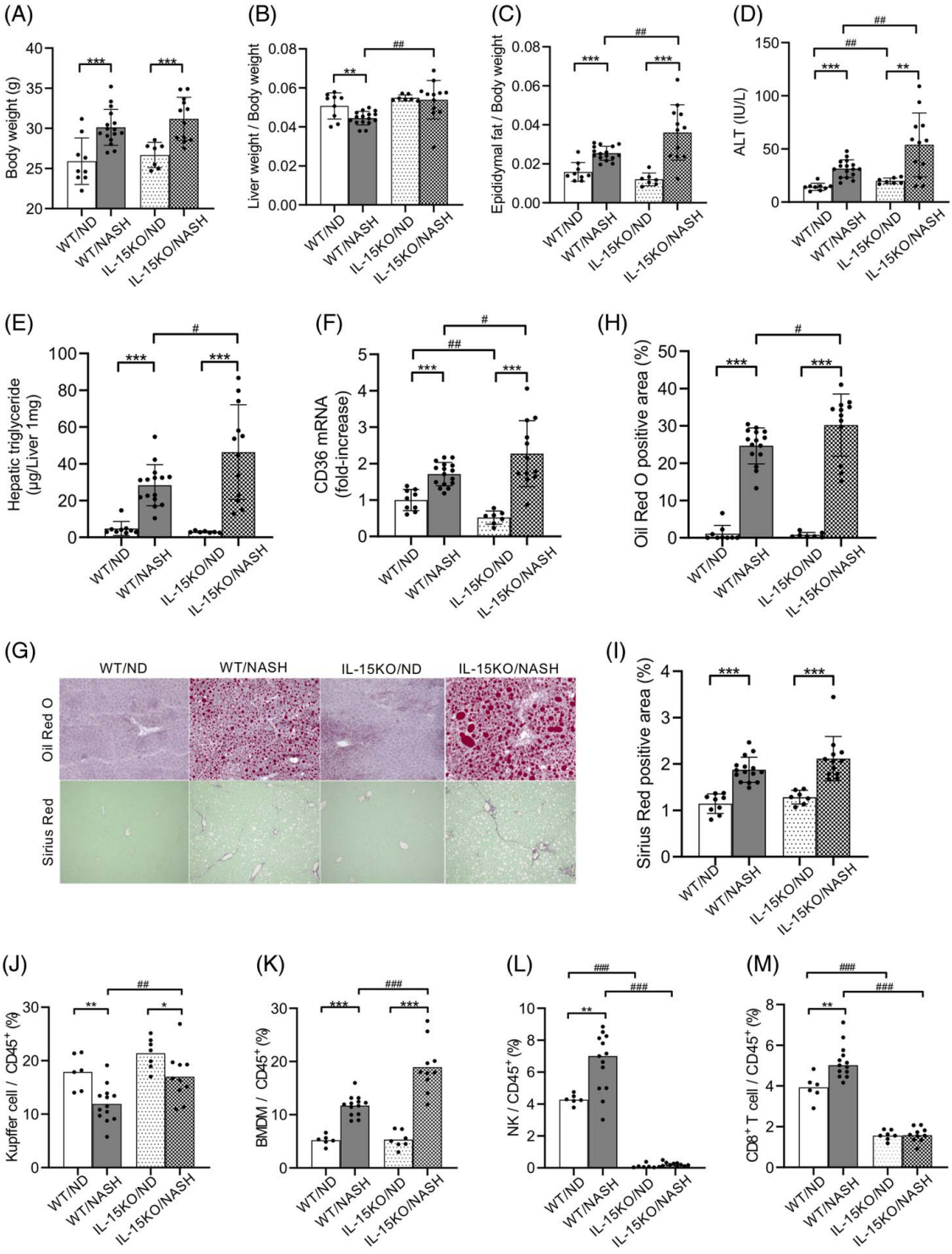


**FIGURE 7** Knockout of *IL-15*–accelerated NASH progression. Groups of WT mice or *IL-15* KO mice in control model (ND + oil; WT/ND n = 9, *IL-15* KO/ND n = 7) or NASH model (western diet + $CCl_4$; WT/NASH n = 16, *IL-15* KO/NASH n = 12) for 12 weeks and analyzed. We assessed the following parameters; (A) body weight, (B) liver weight-to-body weight ratio, (C) epididymal fat weight-to-body weight ratio, (D) serum ALT levels, (E) hepatic triglyceride levels, (F) liver *CD36* mRNA expression value, and (G) representative images of liver sections stained with Oil Red O and Sirius Red (original magnification ×40). Quantification of the Oil Red O-positive (H) and Sirius Red-positive (I) areas. (J–M) percentage of KCs (J), BMDMs (K), NK cells (L), and CD8+ T cells (M) among the CD45+ intrahepatic immune cells. The number of individuals for these figures only differs from those listed above (WT/ND n = 6, WT/NASH n = 13, *IL-15* KO/ND n = 7, and *IL-15* KO/NASH n = 10). Results are expressed as the mean ± SD. $^{\#,*}p < 0.05$, $^{\#\#,**}p < 0.01$ $^{\#\#\#,***}p < 0.001$ by Student *t* test for comparison between WT/ND and *IL-15*KO/ND or WT/NASH and *IL-15* KO/NASH ($^{\#}$), and between WT/ND and WT/NASH or *IL-15* KO/ND and *IL-15* KO/NASH ($^{*}$). Abbreviations: BMDMs, bone marrow-derived macrophages; $CCl_4$, carbon tetrachloride; KO, knockout; ND, normal diet; WT, wild type.

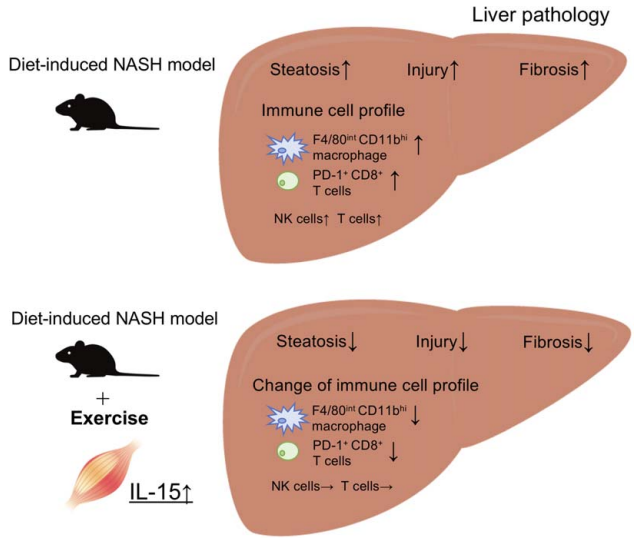


**FIGURE 8** Working model of exercise effects in NASH model. In this NASH mouse model, liver steatosis, liver injury, and fibrosis were progressed. Exercise changed intrahepatic immune cell profile, and suppressed liver steatosis, liver injury, and fibrosis, and that the beneficial effects correlated with reductions in intrahepatic BMDMs ($F4/80^{int}CD11b^{hi}$ macrophage) and PD-$1^+$ $CD8^+$ T cells. Exercise mice had increased *IL-15* expression in muscle and fatty liver and liver injury were exacerbated in *IL-15* KO mice with NASH, suggesting that IL-15 may be involved in the inhibitory effect of exercise on NASH progression. Abbreviations: BMDMs, bone marrow-derived macrophage; KO, knockout; PD-1, programmed death receptor-1.

muscle-specific but systemic *IL-15* KO mice, so further investigation is needed to determine the extent to which muscle-derived IL-15 contributes to the pathogenesis of NASH.

In summary, the results of our study show that exercise ameliorated hepatic steatosis, inflammation, and fibrosis in a mouse model of NASH, and that the beneficial effects correlated with reductions in intrahepatic BMDMs and PD-$1^+$ $CD8^+$ T cells. Therefore, one of the many beneficial effects of exercise on NASH pathogenesis may be an impact on the intrahepatic immune cell profile. In addition, it is possible that IL-15 produced by exercise is involved in the inhibition of NASH progression by exercise (Figure 8).

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## CONFLICTS OF INTEREST

Tatsuya Kanto is on the speakers' bureau for AbbVie and Gilead. Takumi Kawaguchi is on the speakers' bureau for EA Pharma, Janssen Pharmaceutical K.K, Otsuka Pharmaceutical, and Taisho Pharmaceutical. The remaining authors have no conflicts to report.

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