Original Article

MOLECULAR METABOLISM

Exercise of high intensity ameliorates hepatic inflammation and the progression of NASH

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ABSTRACT

Objective: Non-alcoholic fatty liver disease (NAFLD) covers a wide spectrum of liver pathology ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH). Notably, immune cell-driven inflammation is a key mechanism in the transition from fatty liver to the more serious NASH. Although exercise training is effective in ameliorating obesity-related diseases, the underlying mechanisms of the beneficial effects of exercise remain unclear. It is unknown whether there is an optimal modality and intensity of exercise to treat NAFLD. The objective of this study was to determine whether high-intensity interval training (HIIT) or moderate-intensity continuous training (MIT) is more effective at ameliorating the progression of NASH.

Methods: Wild-type mice were fed a high-fat, high-carbohydrate (HFHC) diet for 6 weeks and left sedentary (SED) or assigned to either an MIT or HIIT regimen using treadmill running for an additional 16 weeks. MIT and HIIT groups were pair-fed to ensure that energy intake was similar between the exercise cohorts. To determine changes in whole-body metabolism, we performed insulin and glucose tolerance tests, indirect calorimetry, and magnetic resonance imaging. NASH progression was determined by triglyceride accumulation, expression of inflammatory genes, and histological assessment of fibrosis. Immune cell populations in the liver were characterized by cytometry by time-of-flight mass spectrometry, and progenitor populations within the bone marrow were assessed by flow cytometry. Finally, we analyzed the transcriptional profile of the liver by bulk RNA sequencing.

Results: Compared with SED mice, both HIIT and MIT suppressed weight gain, improved whole-body metabolic parameters, and ameliorated the progression of NASH by reducing hepatic triglyceride levels, inflammation, and fibrosis. However, HIIT was superior to MIT at reducing adiposity, improving whole-body glucose tolerance, and ameliorating liver steatosis, inflammation, and fibrosis, without any changes in body weight. Improved NASH progression in HIIT mice was accompanied by a substantial decrease in the frequency of pro-inflammatory infiltrating, monocyte-derived macrophages in the liver and reduced myeloid progenitor populations in the bone marrow. Notably, an acute bout of MIT or HIIT exercise had no effect on the intrahepatic and splenic immune cell populations. In addition, bulk mRNA sequencing of the entire liver tissue showed a pattern of gene expression confirming that HIIT was more effective than MIT in improving liver inflammation and lipid biosynthesis.

Conclusions: Our data suggest that exercise lessens hepatic inflammation during NASH by reducing the accumulation of hepatic monocyte-derived inflammatory macrophages and bone marrow precursor cells. Our findings also indicate that HIIT is superior to MIT in ameliorating the disease in a dietary mouse model of NASH.

Keywords NASH; NAFLD; Exercise; HIIT; Inflammation

1. INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is one of the leading causes of abnormal liver function and is estimated to affect approximately 25% of the population [1]. Obesity is a major risk factor for the development of NAFLD that, along with associated genetic polymorphisms, dictates the severity of the disease [2]. NAFLD is a spectrum of liver disease that can progress from simple fatty liver to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma [3–5]. Immune cell-driven inflammation is a key trigger of the transition from fatty liver to the more serious NASH in a process involving the innate and adaptive immune systems [3]. Our understanding of the pathogenesis of NASH has grown in recent years. However, there are no current FDA-approved therapeutics for its treatment [6]. Only lifestyle interventions, such as physical exercise, have been shown to be effective at ameliorating the progression of NASH in humans [7,8]. The protective effects of physical activity against NAFLD and NASH can be ascribed to different mechanisms, including decreased hepatic fat content, fibrosis, inflammation, apoptosis, and oxidative stress [9,10]. Exercise has been shown to dampen the immune cell-driven inflammation typical of NASH through

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Abbreviations

NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
HIIT	High-intensity interval training
MIT	Moderate-intensity continuous training
BM	Bone marrow
HFHC	High-fat high-carbohydrate
SED	Sedentary
MoMF	Monocyte-derived macrophage
ITT	Insulin tolerance test
GTT	Glucose tolerance test
ALT	Alanine aminotransferase
AST	Aspartate transaminase
CyTOF	Time of flight mass spectrometry
LPS	Lipopolysaccharide
αSMA	α-smooth muscle actin
BW	Bodyweight
EE	Energy expenditure
RER	Respiratory exchange ratio
KCs	Kupffer cells
LSK	$Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
TLR	Toll-like receptor

the downregulation of pro-inflammatory cytokines and a reduction of infiltrating macrophages [10–13]. Recent findings suggest that habitual physical exercise alters the hematopoietic bone marrow (BM) niche by reducing the output of inflammatory leukocytes, which lead to reduced cardiovascular inflammation [14]. However, the mechanisms by which exercise reduces hepatic inflammation in NASH remain unclear.

Current guidelines for managing NAFLD consist of lifestyle modifications such as a hypocaloric diet and regular physical activity or exercise to achieve weight loss [15], although there is no consensus on the most effective modality or intensity [16]. Several human studies have shown that high-intensity interval training (HIIT) is superior at improving cardiorespiratory fitness, glucose control, and insulin sensitivity, compared with a moderate-intensity continuous training (MIT) modality [17,18]. Indeed, a meta-analysis of 17 studies with 953 participants showed that, compared to MIT, HIIT was more effective at improving cardiorespiratory fitness in patients with coronary artery disease [19]. To our knowledge, there is only one randomized controlled study that has compared the effects of energy-matched MIT and HIIT exercise on NAFLD patients, which failed to detect differences in the intrahepatic lipid content following 4 weeks of exercise intervention [20]. Whether the intensity and modality of exercise influence the efficacy of exercise in mitigating the progression from fatty liver to NASH and the potential mechanisms underlying this beneficial effect remain unclear. Thus, the objective of this study was to compare the efficacy of HIIT and MIT in ameliorating the progression of NASH in a dietary mouse model of disease. We hypothesized that high-intensity exercise is superior in dampening the hepatic inflammatory process of NASH compared to an iso-caloric, moderate-intensity exercise modality.

Our findings show that, compared to sedentary (SED) mice, both HIIT and MIT improved whole-body metabolic parameters and lessened the progression of NASH by decreasing hepatic triglycerides, inflammation, and fibrosis. However, HIIT was superior to MIT at improving glucose and insulin tolerance, reducing hepatic steatosis and fibrosis, and decreasing the frequency of infiltrating monocyte-derived macrophages (MoMF). In addition, bulk mRNA sequencing of liver tissue showed a pattern of gene expression consistent with HIIT being more effective than MIT in improving hepatic metabolic parameters. Notably, an acute bout of MIT or HIIT exercise had no effect on the immune cell-driven progression of NASH. Collectively, our data suggest that exercise improves NASH and reduces hepatic inflammation, particularly by decreasing the accumulation of pro-inflammatory macrophages. These findings also indicate that HIIT is superior to MIT in ameliorating NASH in a pre-clinical mouse model of disease.

2. MATERIALS AND METHODS

2.1. Animals

Five-week-old C57BL/6J mice were purchased from The Jackson Laboratory and maintained in a pathogen-free, temperature-controlled environment. At six weeks of age, mice were fed a high-fat, high-carbohydrate diet (HFHC; 40% kcal palm oil, 20% kcal fructose, and 2% cholesterol supplemented with 23.1 g/L D-fructose and 18.9 g/L D-glucose in the drinking water) ad-libitum for 6 weeks. Subsequently, they remained SED or were exercised either on an MIT regimen or a HIIT regimen for an additional 14 weeks while on the HFHC diet. The SED and MIT groups were fed ad-libitum. To achieve iso-caloric intake between HIIT and MIT groups, mice were group-pair fed during the exercise period of the study. The amount of HFHC fed to the HIIT group was based on the mean consumption of diet by the MIT group from the preceding day. The HIIT regimen consisted of running on a 6-lane mouse treadmill (Columbus Instruments) three days a week with one or two days of rest between each exercise session and 2 min of running, followed by 2 min of rest for a total of 60 min. The MIT regimen consisted of continuous treadmill running to match the distance ran by the HIIT group three days a week. The speed of running was increased weekly or bi-weekly (Table S1). To avoid circadian rhythm influences all groups were exercised at the same time in the evening (4 pm CDT). The treadmill was not equipped with a shock grid, and the mice were encouraged to run using gentle tapping with a tongue depressor, as needed. We performed a maximal running capacity test on all mice before and during the final week of the exercise regimen. The test consisted of determining the distance run by the mice on the 6-lane treadmill, beginning at a speed of 5 m/s, which was increased by 2 m/s every 2 min until the mice would refuse to run after two consecutive gentle taps with a tongue depressor. For the acute exercise intervention, the mice were fed the HFHC diet for 6 weeks and assigned to a single bout of HIIT or MIT followed by euthanasia 3 h after the exercise intervention. All animal experiments were approved by the University of Minnesota Institutional Animal Care and Use Committee.

2.2. Metabolic assessments

Insulin tolerance tests (ITT) and glucose tolerance tests (GTT) were performed as previously described [21]. To determine energy metabolism, mice were placed in automated metabolic cages for 48 h. An assessment of energy expenditure (EE) was obtained via indirect calorimetry in free-moving animals housed in individual cages consisting of an indirect open circuit calorimeter that provides measures of O_2 consumption and CO_2 production (Oxymax, Columbus Instruments,

Ohio). The ambulatory activity was assessed by the breaking of infrared laser beams in the x-y plane. The cages were provided with ad libitum access to food and water throughout the procedure. These procedures were performed by the IBP Phenotyping Core at the University of Minnesota. Calorimetry data were analyzed using Calr [22]. All metabolic assessments were completed at least 48 h post-exercise.

2.3. Body and tissue composition

Body composition (fat mass, fat-free mass, total water, and free water) was assessed in conscious mice using an Echo-MRI 3-in-1 (Echo Medical Systems LLC, Houston, TX). Mice were restrained in a custom restraint tube and placed in the MRI for a maximum of 60–80 s. Liver triglyceride content was measured using a colorimetric assay (Cayman Chemical). Serum concentrations of alanine aminotransferase (ALT) and aspartate transaminase (AST) were determined enzymatically by IDEXX BioAnalytics using the AU680 Chemistry System (Beckerman Coulter).

2.4. Immune cell characterization

In the chronic exercise experiments, mice were euthanized 48 h after their last training session to avoid any acute effects of exercise. Intrahepatic immune cells were isolated from perfused livers using enzymatic digestion and density gradient centrifugation, as previously described [23]. Splenic, BM, and blood immune cells were isolated, as previously described [21]. For cytometry by time-of-flight mass spectrometry (CyTOF), cells were stained with 0.5 μg of metal-conjugated primary antibodies (Fluidigm) for 30 min at 4 °C [24]. Data were collected on a CyTOF 2 instrument (DVS Sciences) and analyzed using Cytobank. For flow cytometry, cells were incubated with fluorophore-conjugated primary antibodies for 30 min at 4 °C. Flow cytometry data were acquired on a Fortessa flow cytometer (BD Biosciences) and analyzed using Flowjo (TreeStar) software. Antibodies are listed in Table S2.

2.5. LPS stimulation

For the *ex vivo* characterization of cytokine-producing leukocytes, 8×10^5 intrahepatic immune cells were cultured in 10% FBS RPMI and stimulated with 5 μg/mL of lipopolysaccharide (LPS, eBioscience) and 2 μL/mL of protein transport inhibitor (eBioscience) for 8 h in a humidified incubator set at 37C, 5% CO_2. After incubation, the LPS and protein transport inhibitor were washed off with PBS, and the cells were stained with 1:200 zombie aqua (Biolegend) to discriminate between viable and dead cells. Staining for cell surface markers was performed with fluorophore-conjugated primary antibodies for 30 min at 4C. For intracellular staining, a BD Cytofix/Cytoperm kit (BD Bioscience) was used to fix and permeabilize cells prior to staining with intracellular fluorophore-conjugated antibodies. Flow cytometry data were acquired on Fortessa X-30H0081 (BD Biosciences) cytometer and analyzed using Flowjo software (TreeStar). Fluorophore-conjugated antibodies are listed in Table S2.

2.6. Real Time-PCR

Total RNA was extracted from livers using an RNeasy Plus Mini kit (Qiagen). cDNA was prepared using the iScript cDNA Synthesis kit (Bio-Rad). Gene expression was calculated using the 2 (-ΔΔCT) method and normalized to GAPDH. Primers are listed in Table S3.

2.7. Bulk RNA sequencing

Total RNA was extracted from livers using an RNeasy Plus Mini kit (Qiagen). Samples were sequenced on a Novaseq 6000 using a 150 PE flow cell at the University of Minnesota Genomics Center. The SMARTer Stranded RNA Pico Mammalian V2 kit (Takara Bio) was used to create Illumina sequencing libraries. Differential gene expression analysis was performed using edgeR (Bioconductor). A gene ontology and pathway analysis were completed using DAVID Bioinformatics Resources 6.8 (NIAID/NIH). The upstream regulator analysis was performed using Ingenuity Pathway Analysis (Qiagen).

2.8. Histology

Liver sections fixed in 10% formalin were histologically assessed for steatosis by hematoxylin and eosin staining, fibrosis by Masson's trichrome, and α-smooth muscle actin (αSMA) by immunohistochemistry (Biorepository & Laboratory Services at the UMN Clinical and Translational Science Institute). The collagen and αSMA areas were quantified in stained images at 10X resolution using the Weka Trainable Segmentation plugin in FIJI software (ImageJ, version 1.52p). Approximately ten different sections per histological slice were analyzed. The staining of the collagen deposition and αSMA expression was segmented from the other colors using the plugin classifier. Images were then converted to RGB color, and the segmented color of collagen was isolated using color thresholding. Using binary processing, the percent area positive for collagen was calculated for each section. Interstitial fibrosis scoring (0 = no fibrosis; 1 = mild, focal, and delicate; 2 = moderate, focal and coarse; and 3 = severe and diffusely coarse) was performed by a blinded liver pathologist.

2.9. Statistical analysis

Statistical significance was determined using a one-way ANOVA with a Tukey's multiple comparison test using GraphPad Prism 8.3. All data analyzed by ANOVA followed a Gaussian distribution (Shapiro–Wilk test) and had equal variances (Brown-Forsythe). For datasets that failed the normality test, such as scoring data, statistical significance was determined using multiple nonparametric Mann–Whitney tests. Indirect calorimetry data were analyzed by analysis of covariance (ANCOVA) to adjust for the influence of body composition [22]. Mouse studies were repeated in two independent experiments. Data are presented as means ± SEM with statistical significance denoted by $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$.

3. RESULTS

3.1. HIIT is superior to MIT in decreasing adiposity and glucose tolerance in NASH mice

To investigate whether HIIT is more effective at ameliorating the progression of NASH, we fed C57BL/6J mice an HFHC diet that causes human-relevant features of obesity-induced NASH [25]. After 6 weeks of HFHC feeding, mice were acclimated to treadmill running, and their maximal running capacity was assessed. Mice were then assigned to SED, MIT, or HIIT exercise regimens for 14 weeks. All three groups remained on the HFHC diet for the duration of the exercise treatments (Figure 1A). Mice in the MIT and HIIT groups ran the same treadmill distance (Table S1). As HIIT was expected to result in higher free-food intake than MIT (Figure S1A), we pair-fed HIIT and MIT animals to ensure iso-caloric conditions. Throughout the duration of exercise regimens, SED, MIT, and pair-fed HIIT mice showed a similar food intake (Figure S1B). After 14 weeks of exercise, HIIT mice showed the longest distance run during a maximal running capacity test (Figure 1B), suggesting that HIIT is more effective at improving fitness than MIT in our mouse model of treadmill running. Compared to SED mice, which continued to gain weight, both MIT and HIIT exercise regimens mitigated the weight gain of HFHC-fed mice, although no differences were detected in BW between pair-fed MIT and HIIT mice (Figure 1C). Despite

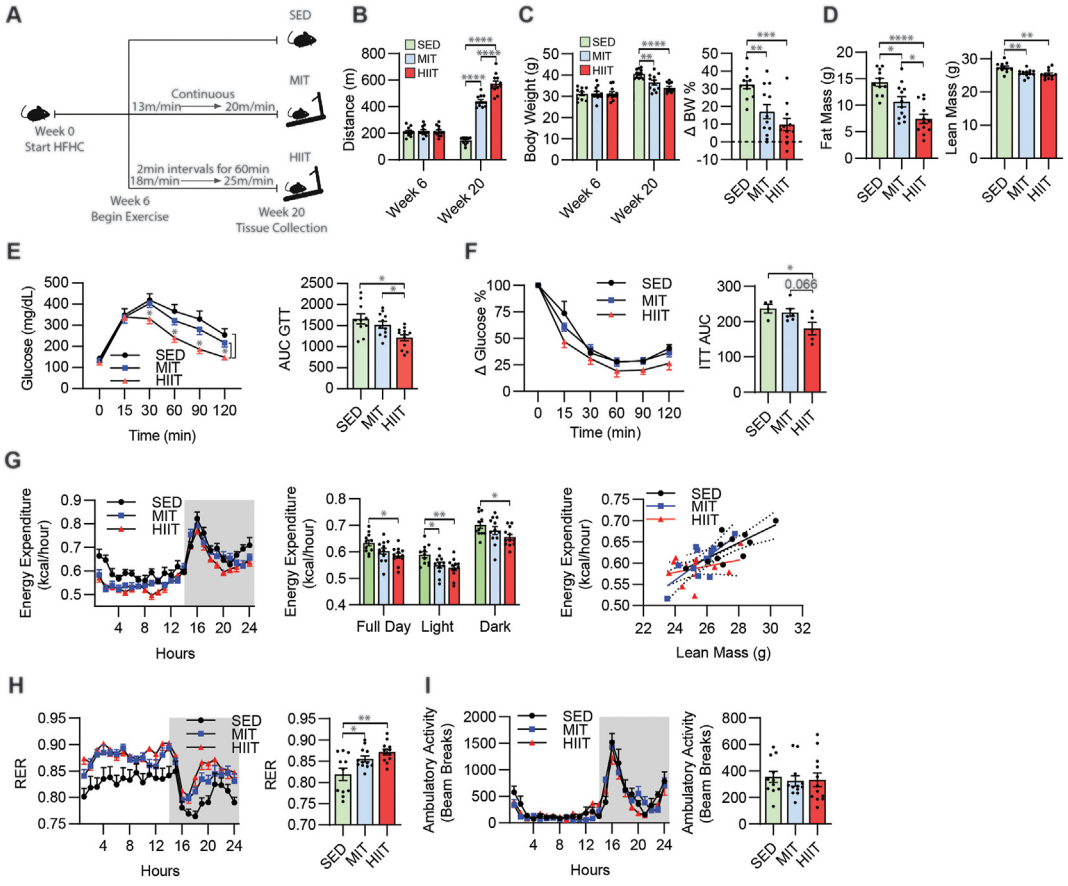

Figure 1: HIIT is superior to MIT in decreasing adiposity and glucose tolerance in NASH mice. (**A**) Experimental design. (**B**) Distance covered in a maximal running capacity test at week 6 (pre-exercise intervention) and week 20 (post-exercise intervention; n = 12 mice per group). (**C**) Bodyweight at weeks 6 and 20 (left) and percent change in body weight (right; n = 12 mice per group). (**D**) Fat mass (left) and lean mass (right) determined by MRI (n = 12 mice per group). (**E**) Glucose tolerance test (GTT, left) with corresponding areas under the curves (AUC, right; n = 11–12 mice per group). (**F**) Insulin tolerance test (ITT, left) with corresponding areas under the curves (AUC, right; n = 5–6 mice per group). (**G**) Energy expenditure (left) during the full day, light phase, and dark phase (middle) and linear regression by lean mass (right, n = 12 mice per group). (**H**) Respiratory exchange ratio (RER, left) with corresponding daily RER (right; n = 12 mice per group). (**I**) Ambulatory activity (left) with corresponding hourly ambulatory activity per day (right; n = 12 mice per group). Data are presented as mean ± SEM. Statistical significance is denoted by (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, and ****p ≤ 0.0001).

similar BW, HIIT mice showed a larger reduction in total fat mass than the MIT group (Figure 1D, left). No differences in lean mass between MIT and HIIT mice were detected, though both exercise regimens showed a slight, but statistically significant, lower lean mass than SED controls (Figure 1D, right). Represented as a proportion of BW, however, HIIT mice had the highest lean mass and lowest body fat percentage, compared to both MIT and SED (Figure S1C). Next, to determine the effects of exercise intensity on systemic glucose metabolism, we performed a GTT and ITT in SED, MIT, and HIIT mice. Compared with the SED and MIT mice, the HIIT group showed an overall improved GTT (Figure 1E) and ITT (Figure 1F), while no differences were detected between the SED and MIT groups. Together, these findings suggest that HIIT was superior to MIT in reducing adiposity and improving glucose and insulin tolerance under iso-caloric conditions.

To better understand the potential mechanisms by which HIIT reduced adiposity in HFHC-fed mice, we assessed their basal EE, substrate utilization, and ambulatory activity 48 h after their last bout of exercise using indirect calorimetry. Compared to SED, both MIT and HIIT decreased EE, with HIIT resulting in the greatest effect (Figure 1G, left). This decrease in raw EE seen in HIIT mice occurred during both the light and dark phases, whereas MIT only showed reductions in EE primarily during the light phase (Figure 1G, middle). To normalize EE for differences in BW and body composition, we analyzed the data by ANCOVA using BW, lean mass, and fat mass as covariates [22]. Analysis of EE data by ANCOVA (Table S4) revealed that the lower EE in exercise groups was driven by the changes in lean mass (Figure 1G, right), total mass (Figure S1D), and fat mass (Figure S1E). Similarly, basal O_2 consumption was the lowest in HIIT mice, compared with MIT and SED groups (Figure S1F). For basal O_2 consumption, ANCOVA determined a slight group effect for HIIT ($P = 0.04$) (Table S4) after correcting for the effect of lean mass (Figure S1G). This result suggests that HIIT may alter basal O_2 consumption, independent of differences in lean mass. There were no differences in basal CO_2 production between groups (Figure S1H). We then determined the respiratory exchange ratio (RER) to estimate substrate utilization. While MIT induced a moderate increase in RER, HIIT resulted in the highest RER of all three groups, suggesting that the exercise of mice fed an HFHC diet leads to increased use of glucose as a source of energy (Figure 1H). No differences in ambulatory activity were detected between any groups of mice (Figure 1I). Together, these data show that HIIT was the most effective exercise regimen at decreasing adiposity and improving glucose tolerance in mice with continuous access to an HFHC diet. Notably, these improvements were independent of BW and cannot be explained by changes in EE after correcting for body composition.

3.2. HIIT is more effective in ameliorating NASH progression

Given that HIIT was superior to MIT in reducing adiposity and improving the glucose tolerance of HFHC-fed mice, we sought to determine the effects of exercise intensity on the progression of NASH in the liver. Compared to SED mice, HIIT resulted in decreased liver weight (Figure 2A) and triglyceride content (Figure 2B), while the effect of MIT was milder and not statistically significant. Consistently, hematoxylin and the eosin staining of liver sections showed a substantial decrease in macrovesicular steatosis in MIT and HIIT livers (Figure 2C). To determine whether exercise can dampen the hepatic inflammatory process during the progression of NASH, we determined the expression of pro-inflammatory genes in the liver of SED, MIT, and HIIT mice using RT-PCR. Both exercise groups reduced the expression of the pro-inflammatory genes monocyte-chemoattractant protein 1 (*Mcp1*), e-selectin (*Sele*), intercellular adhesion molecule 1 (*Icam1*), interleukin 1 beta (*Il1b*), and tumor necrosis factor-alpha (*Tnfa*) in comparison to SED, although there were no differences between MIT and HIIT mice (Figure 2D). In addition to improvements in lipid accumulation and inflammatory gene expression, both MIT and HIIT markedly reduced fibrosis, as determined by trichrome staining (Figure 2E). Quantification of collagen deposition revealed that HIIT was more effective at reducing fibrosis than MIT (Figure 2F, S2A). We assessed the areas with fibrosis in trichrome-stained slides and, while perivascular fibrosis was not detected, interstitial fibrosis was the primary form of collagen deposition. Blinded scoring indicated that HIIT was more effective than MIT at reducing interstitial fibrosis (Figure 2G). Accordingly, the expression of the fibrogenesis genes collagen type 1 alpha 1 (C*ol1a1*), matrix metallopeptidase 2 (*Mmp2)*, TIMP metallopeptidase inhibitor 1 (*Timp1*), and transforming growth factor-beta 1 (*Tgfb1*) was decreased in MIT and HIIT mice, compared with SED controls (Figure 2H). As liver fibrosis is associated with the activation of αSMA^{+} hepatic stellate cells during NASH, we performed αSMA immunohistochemistry staining. In agreement with reduced collagen deposition, both MIT and HIIT livers showed reduced αSMA compared to SED (Figure 2I). Furthermore, HIIT was more effective than MIT in reducing the expression of αSMA, suggesting decreased activation of hepatic stellate cells (Figure 2I). To determine whether these improvements in NASH progression influenced liver function, we measured AST and ALT in the serum. HIIT mice showed decreased serum levels of ALT and AST, while MIT only showed a numerical decrease in ALT (Figure 2J). Overall, these data indicate that both exercise regimens ameliorate the progression of NASH but that HIIT is more effective than MIT at reducing steatosis, fibrosis, and liver injury under isocaloric conditions. Our finding that HIIT reduced fat mass more than MIT, despite similar weight loss, suggests that the improved progression of NASH in HIIT mice can be partially ascribed to decreased adiposity.

3.3. Chronic HIIT reduces the accumulation of inflammatory MoMFs in the NASH liver

To better understand how exercise modulates the inflammatory process in the NASH liver, we isolated intrahepatic immune cells from SED, MIT, and HIIT mice and characterized their phenotype by CyTOF. We performed unsupervised viSNE clustering by high-dimensional single-cell analysis [26], which allowed us to simultaneously identify the major intrahepatic immune cell populations based on the expression of 20 cell surface markers (Figure 3A, S3A). Quantification of gated immune cell populations revealed that HIIT resulted in a larger reduction in the frequency of intrahepatic myeloid cells (e.g., monocytes, MoMFs, conventional dendritic cells) than MIT. However, neither regimen altered neutrophils, plasmacytoid DCs, or Kupffer cells (KCs; Figure 3B). In contrast, HIIT increased the frequency of natural killer T cells, CD4 T cells, and CD8 T cells compared to SED and MIT (Figure 3C). B cells, NK cells, and double-negative T cells were not affected by exercise, regardless of intensity (Figure 3C). MoMFs can be classified into pro-inflammatory or anti-inflammatory subsets according to the expression of CCR2 and Ly6C [27]. As HIIT resulted in a substantial decrease in MoMFs, we determined the frequency of MoMFs subsets. While MIT did not alter MoMFs, HIIT mice showed decreased intrahepatic pro-inflammatory CCR2+ $Ly6C^{hi}$ MoMFs and a higher proportion of anti-inflammatory CCR2- $Ly6C^{lo}$ MoMFs, compared to SED and MIT (Figure 3D). To determine whether exercise intensity alters the cytokine production of MoMFs, we stimulated intrahepatic immune cells *ex vivo* with LPS for 8 h. Then, we measured the expression of intracellular INFγ, IL-6, TNFα, and IL-10 within MoMFs by flow cytometry. Compared with MIT, MoMFs from HIIT mice showed a decreased expression of INFγ, TNFα, and IL-6 in response to the LPS stimulation (Figure 3E). Overall, these data indicate that decreased hepatic inflammation in HIIT mice is associated with a reduction in the frequency of inflammatory MoMFs and a decrease in their expression of pro-inflammatory cytokines.

To examine whether a single bout of exercise would acutely alter the intrahepatic immune cells populations in NASH mice, we fed mice the HFHC diet for 6 weeks and subjected them to a single bout of MIT or HIIT. Three hours after the exercise intervention, the mice were euthanized, and their intrahepatic immune cells were assessed by CyTOF (Figure S4A). There were no differences in BW between the three groups (Figure S4B), and all immune cells in the liver were largely unaffected (Figure S4C), suggesting that long-term exercise is required to blunt the inflammatory process of NASH.

3.4. HIIT reduces hematopoiesis and the systemic supply of leukocytes

Regular exercise has been shown to alter the hematopoietic niche by reducing the hematopoietic activity and the production of inflammatory leukocytes [14]. To determine whether the reduced accumulation of immune cell populations in the liver was associated with altered hematopoiesis and subsequent supply of cells, we quantified the leukocyte progenitor populations in the BM, as well as the mature immune cell populations in the spleen and blood of SED, MIT, and HIIT mice. Compared to the SED condition, HIIT resulted in a severe decrease in BM leukocytes (Figure 4A) and a decrease in the hematopoietic stem $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ cells (LSKs), from which all leukocyte progenitor populations are derived (Figure 4B). Compared to SED, HIIT decreased the number of common myeloid progenitors, megakaryocyte-erythrocyte progenitors (Figure 4C), granulocyte-monocyte progenitors, and monocyte-dendritic cell progenitors (Figure 4D). In contrast, the effects of MIT on progenitor cells were milder than HIIT and did not reach statistical significance compared to SED mice (Figure 4A–D). Notably, chronic MIT and HIIT exercise did not alter the circulating leukocyte populations in the blood (Figure 4E) or a secondary lymphoid organ, such as the spleen (Figure 4F). Furthermore, a single bout of HIIT or MIT had no effects on the frequency of major progenitor populations within the BM (Figure S4D). Together, these data suggest that long-term exercise, especially HIIT, reduces hematopoiesis at the stage of leukocyte progenitors of myeloid lineage, which may partially explain the changes in intrahepatic immune cell accumulation.

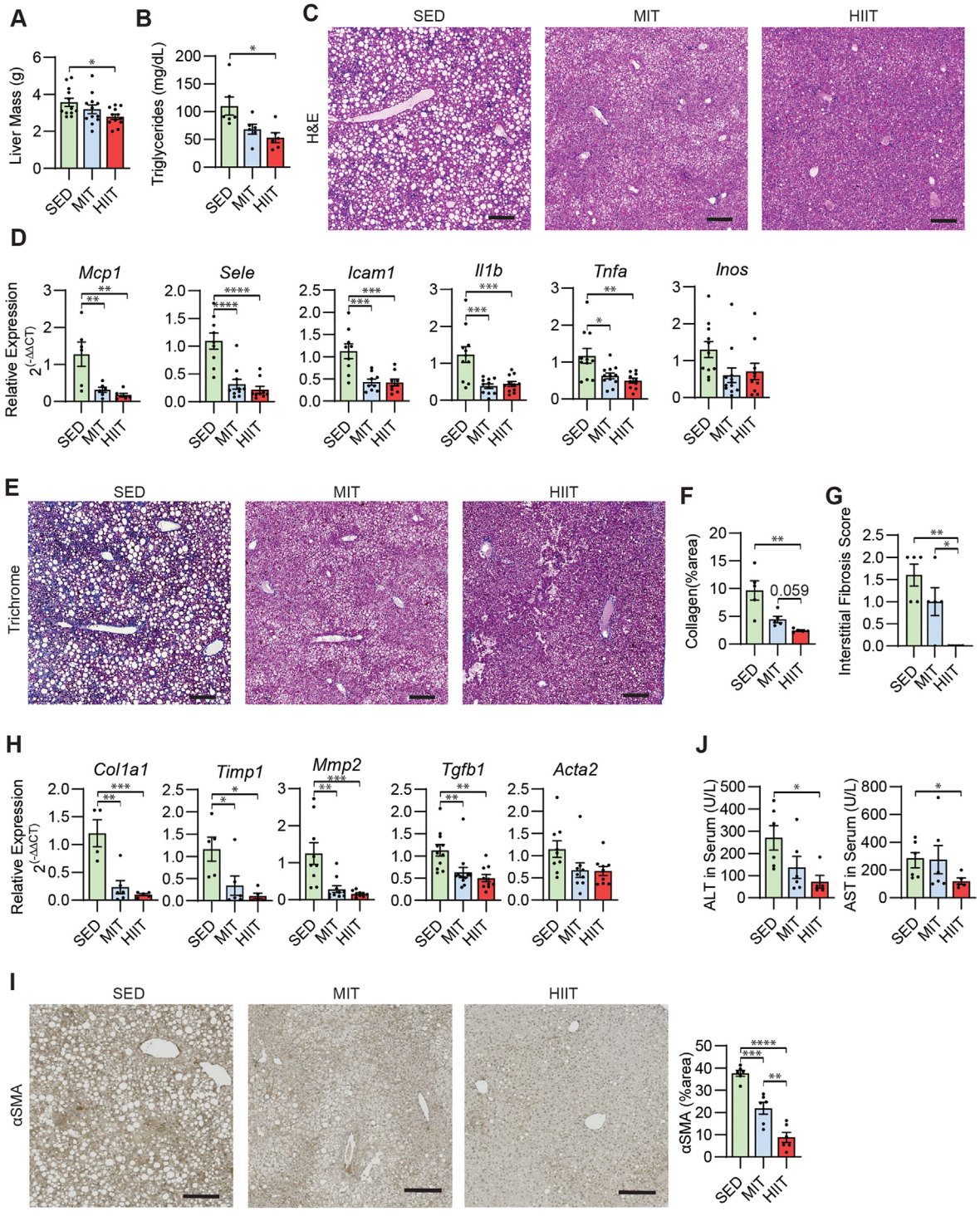

Figure 2: HIIT is more effective in ameliorating NASH progression. (**A**) Liver mass (n = 12 mice per group). (**B**) Liver triglyceride content (n = 6 mice per group). (**C**) Representative hematoxylin and eosin (H&E) liver stain (scale bar, 200 μm). (**D**) RT-PCR gene expression analysis of liver inflammatory genes (*Mcp1, Sele, Icam1, IL1B, TNFa, and Inos*; n = 6–12 mice per group). (**E**) Representative Mason's trichrome liver stain (scale bar, 200 μm). (**F**) Quantification of the area with collagen deposition (n = 3 mice per group). (**G**) Interstitial fibrosis scoring (n = 3 mice per group). (**H**) RT-PCR expression analysis of liver pro-fibrogenesis genes (*Col1a1*, *Timp1, Mmp2, Tgfb1*, and *Acta2*; n = 5–12 mice per group). (**I**) Representative immunohistochemistry staining (left) and quantification (right) of αSMA expression (n = 6 mice per group). (**J**) Serum ALT and AST levels (n = 5–6 mice per group). Data are presented as mean ± SEM. Statistical significance is denoted by (*p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001).

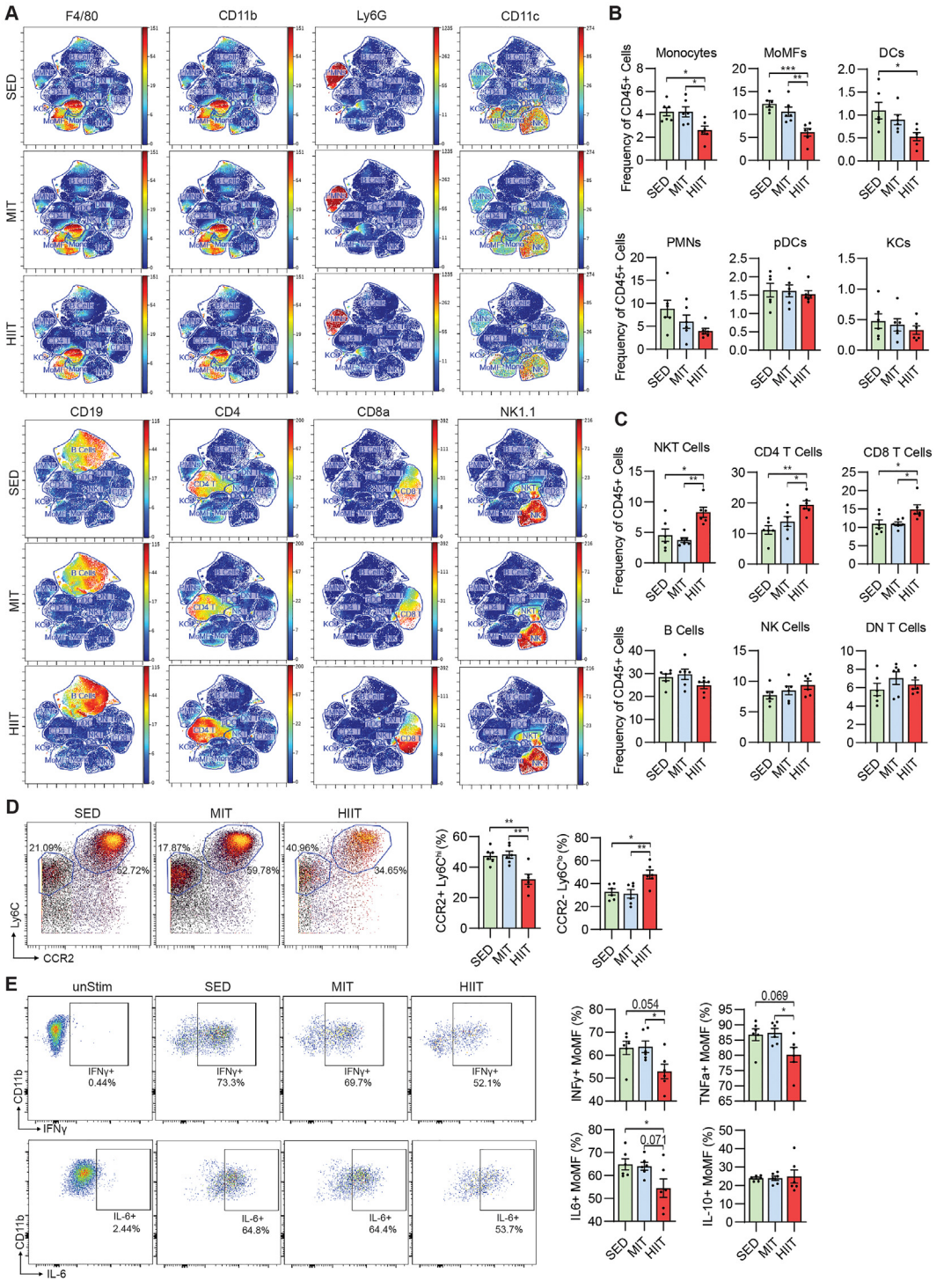

Figure 3: Chronic HIIT reduces the accumulation of inflammatory MoMFs in the NASH liver. (**A**) Representative viSNE plots from liver CyTOF data showing unsupervised clustering and expression of markers used in gating. Gates were drawn based on the expression of commonly used markers to distinguish B cells, monocytes (Mono), Kupffer Cells (KC), monocyte-derived macrophages (MoMF), dendritic cells (DC), neutrophils (PMN), natural killer (NK) cells, natural killer T Cells (NKT), double-negative T Cells (DN T), and CD4 and CD8 T cells. (**B**) Quantification of hepatic non-lymphocyte immune cell subsets from CyTOF data in A, shown as a frequency of hepatic CD45+ cells (n = 6 mice per group). (**C**) Quantification of hepatic lymphocyte immune cell subsets from CyTOF data in A, shown as a frequency of hepatic CD45+ cells (n = 6 mice per group). (**D**) Representative gates for hepatic MoMF subsets: Pro-inflammatory (CCR2+ Ly6C^{hi}) and anti-inflammatory (CCR2- Ly6C^{lo}). Gated on MoMF population from viSNE plot A (left). Quantification of hepatic MoMF subsets, shown as a frequency of hepatic MoMFs (right, n = 6 mice per group). (**E**) Representative gates for LPS stimulation Flow data showing IFNγ+ and IL-6+ MoMF populations. Gated on MoMF population (left). Quantification of IFNγ+, IL-6+, TNFα+, and IL-10+ MoMFs as a frequency of total MoMFs from LPS stimulation Flow data (right, n = 6 mice per group). Data are presented as mean ± SEM. Statistical significance is denoted by (*p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001).

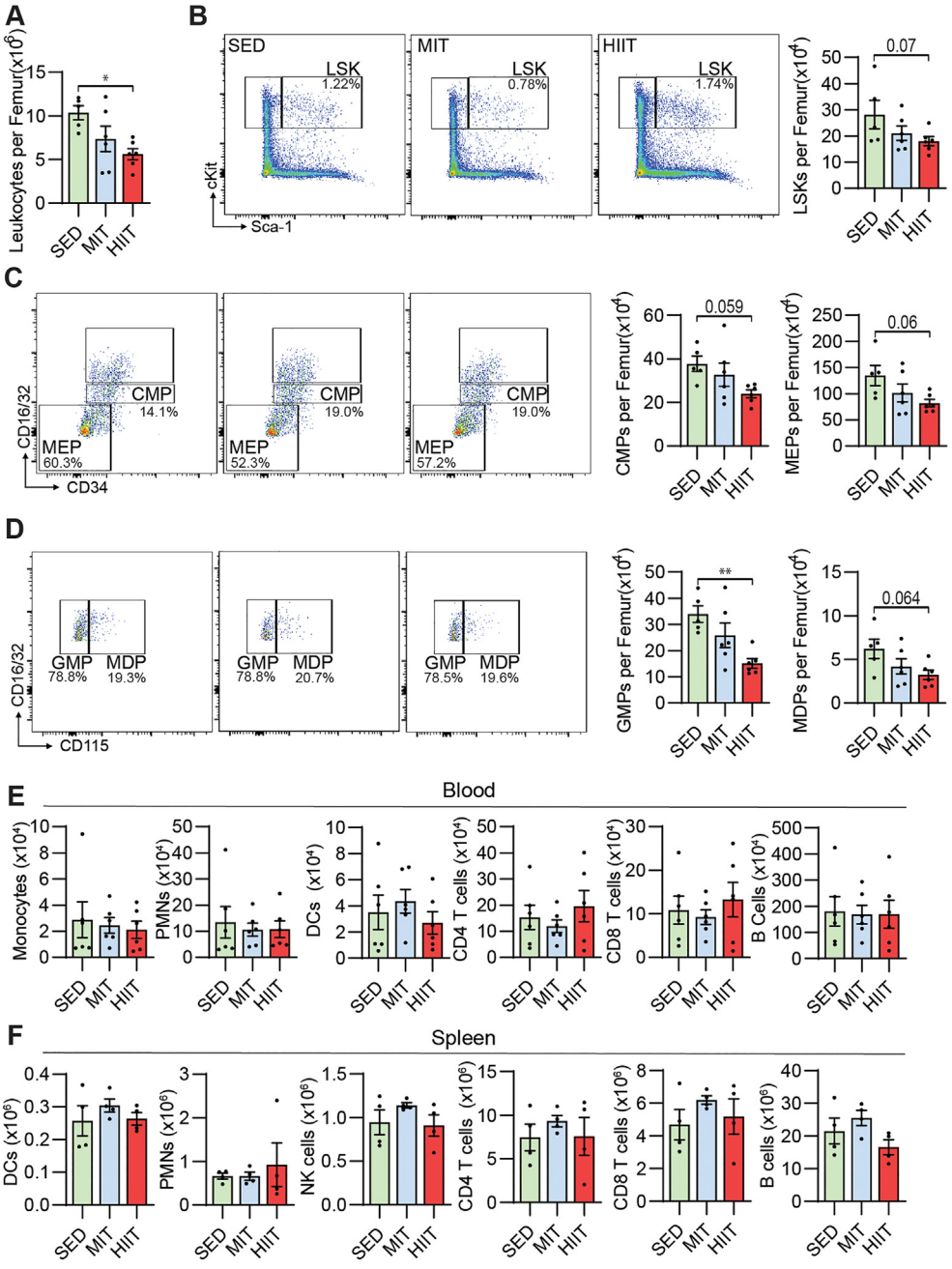

Figure 4: HIIT reduces hematopoiesis and the systemic supply of leukocytes. (**A**) Quantification of total bone marrow leukocytes (n = 6 mice per group). (**B**) Representative gating strategy for lin$^-$ Sca-1$^+$ cKit$^+$ cells (LSKs, left). Quantification of total bone marrow LSKs (right, n = 6 mice per group). (**C**) Representative gating strategy for megakaryocyte-erythrocyte progenitors (MEPs) and common myeloid progenitors (CMPs) gated from the cKit$^+$ and Sca-1$^-$ population in panel B (left). Quantification of total bone marrow MEPs and CMPs (right, n = 6 mice per group). (**D**) Representative gating strategy for granulocyte-monocyte progenitors (GMPs) and monocyte-dendritic progenitors (MDPs) gated from the CD16/32^{hi} and CD34$^+$ population in panel C (left). Quantification of total bone marrow GMPs and MDPs (right, n = 6 mice per group). (**E**) Quantification of monocytes, neutrophils (PMNs), dendritic cells (DCs), CD4 T cells, CD8 T cells, and B cells in blood shown as the total cell number per 200 μL (n = 6 mice per group). (**F**) Quantification of dendritic cells (DCs), neutrophils (PMNs), natural killer cells (NK), CD4 T cells, CD8 T cells, and B cells in the spleen shown as the total number of cells (n = 6 mice per group). Data are presented as mean ± SEM. Statistical significance is denoted by (*p ≤ 0.05 and **p ≤ 0.01).

3.5. Exercise of different intensities alters the hepatic transcriptional landscape

Given the profound effects of exercise, particularly HIIT, on the immune cell composition of the liver during NASH, we next explored changes in whole-tissue hepatic gene expression patterns in response to different exercise modalities. We performed mRNA sequencing of liver tissue isolated from SED, MIT, and HIIT mice. Compared with SED controls, differential gene expression analysis (>1.5-fold change, FDR-corrected P < 0.05) revealed 237 and 26 differentially expressed genes (DEGs) in HIIT and MIT livers, respectively (Figure 5A). We detected 10 overlapping DEGs in the MIT and HIIT exercise groups that included 3 genes encoding major urinary proteins (Figure S5A).

Compared with SED mice, HIIT increased the expression of genes involved in the unfolded protein response, such as homocysteine inducible ER protein with ubiquitin-like domain 1 (*herpud1*), synoviolin 1 (*Syvn1*), and cysteine rich EGF-like domains 2 (*Creld2*), while reducing the expression of mitogen-activated protein kinase 13 (*Mapk13*), P21 activated kinase 6 (*Pak6*), and b cell linker (*Blnk*), which regulate mitogen-activated protein kinase signaling (Figure 5B, S4B). In contrast, MIT increased the expression of genes involved in retinol metabolism, such as cytochrome P450 4A12B (*Cyp4a12b*) and UDP-glucuronosyltransferase 2B1 (*Ugt2b1*), while downregulating the expression of immunoglobulin kappa constant (*Igkc*) and immunoglobulin heavy chain mu (*Ighm*), which regulate B cell-mediated antigen recognition (Figure 5C, S5C). Only 9 DEGs were detected between the HIIT and MIT groups (Figure S5D).

To interpret the biological meaning of these data, we performed a GO enrichment and pathway analysis on the DEGs lists. The IPA regulator

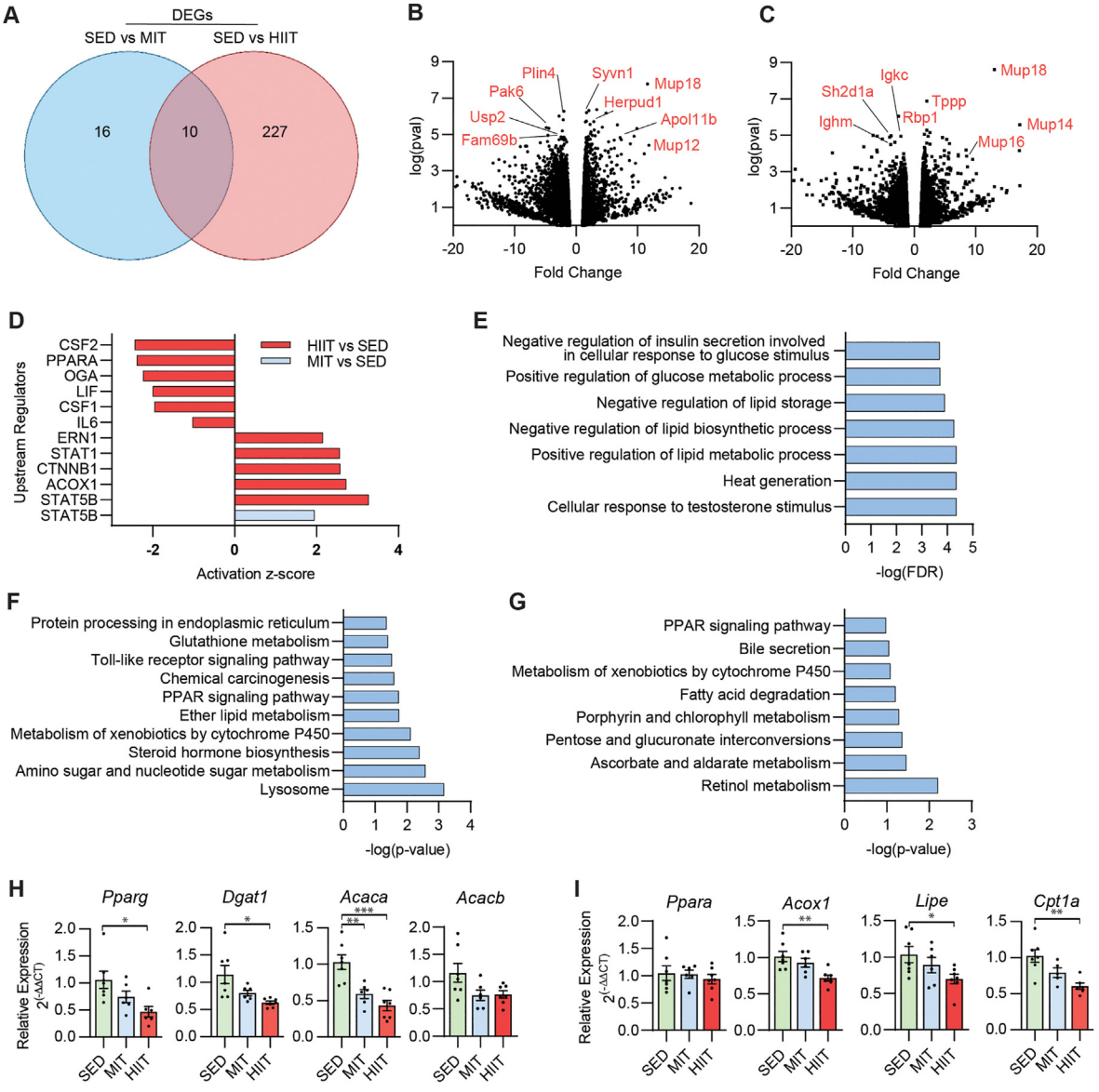

Figure 5: Exercise of different intensities alters the hepatic transcriptional landscape. (**A**) Venn diagram representing the total number of differentially expressed genes (DEGs) for the comparisons of HIIT to SED and MIT to SED from a bulk RNA sequencing of liver tissue (n = 5–6 mice per group). (**B**) Volcano plot of all genes from the comparison of HIIT to SED, shown as the average fold change by the associated log(pval). A positive fold change indicates that HIIT had a higher expression of that gene than SED (n = 5–6 mice per group). (**C**) Volcano plot of all genes from the comparison of MIT to SED, shown as the average fold change by the associated log(pval). A positive fold change indicates that MIT had a higher expression of that gene than SED (n = 5–6 mice per group). (**D**) Top upstream regulators from an upstream regulator analysis of all DEGs. Red indicates the top upstream regulators from the analysis of the DEGs from the comparison of HIIT to SED. Blue indicates the top upstream regulator from the analysis of the DEGs from the comparison of MIT to SED. (**E**) Top gene ontology (GO) terms from a GO enrichment analysis of the DEGs from the comparison of HIIT to SED. (**F**) Top pathways from pathway analysis of the DEGs from the comparison of HIIT to SED. (**G**) Top pathways from pathway analysis of the DEGs from the comparison of MIT to SED. (**H**) RT-PCR analysis of liver lipogenesis genes (*Pparg, Dgat1, Acaca, and Acacb*; n = 6 mice per group). (**I**) RT-PCR analysis of liver lipid catabolism genes (*Ppara, Acox1, Lipe, and Cpt1a*; n = 6 mice per group). Data are presented as mean ± SEM. Statistical significance is denoted by (*p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001).

effects tool predicted that, compared with SED, HIIT activates upstream regulators, such as transcription factors STAT5 and STAT1, and inhibits upstream regulators, such as the inflammatory cytokines IL-6, LIF interleukin 6 family cytokine (*LIF*), and the colony-stimulating factor 1 and 2 (*CSF1, CSF2*; Figure 5D). In contrast to HIIT, MIT was predicted to activate only STAT5B, compared with SED (Figure 5D). The GO terms with the highest enrichment in the HIIT vs. SED comparison included "negative regulation of insulin secretion" and "negative regulation of lipid storage and biosynthetic processes" (Figure 5E). Pathway analysis for the HIIT vs. SED DEGs detected altered metabolic pathways such as "PPAR signaling" as well as toll-like receptor signaling, in agreement with the reduced immune-mediated inflammation (Figure 5F). In the comparison between MIT and SED, IPA revealed altered metabolic pathways, including "retinol metabolism" and "PPAR signaling" (Figure 5G). To better understand how exercise intensity modulates hepatic lipid metabolism, we used RT-PCR to assess the expression of genes involved in lipid synthesis and catabolism in the liver. While both exercise regimens resulted in a decreased expression of the lipid storage and biosynthesis genes, the HIIT group showed the largest effect, concurring with our GO enrichment analysis (Figure 5H). Interestingly, the expression of *Ppara* was unaffected by either exercise regimen, and only HIIT decreased the expression of additional lipid catabolism genes (Figure 5I). Overall, these data suggest that HIIT had a larger impact than MIT in altering the hepatic transcriptional environment toward reduced lipid biosynthesis and hepatic inflammation in agreement with our liver functional assessments.

4. DISCUSSION

Clinical recommendations for patients with NAFLD include reduced caloric consumption and regular physical activity consisting of 150–300 min of moderate-intensity or 75–150 min of vigorous-intensity aerobic exercise per week [15]. However, there is no consensus on an optimal modality or intensity [16]. In the present study, we compared the efficacy of MIT and HIIT under pair-fed conditions in ameliorating the progression of NASH in a mouse model of obesity-associated disease. We found that both exercise intensities successfully ameliorated NASH, though HIIT was more effective than MIT in reducing adiposity, improving glucose homeostasis, and diminishing immune cell-driven inflammation under iso-caloric conditions despite similar BW. A previous study showed that, compared with MIT, obese mice exercised under a HIIT regimen had a substantial reduction in BW, adiposity, hepatic lipid accumulation, and adipose tissue inflammation [28]. Our data suggest that the HIIT-induced improvements in metabolism and NASH progression are independent of caloric intake or BW loss. The finding that HIIT surpasses MIT in improving NASH outcomes without altering BW is consistent with the notion that exercise is more advantageous than simple caloric restriction at preventing NAFLD [29]. In humans, short-term and high- and moderate-intensity can alter the expression of different chemokines on circulatory leukocytes without weight loss or decreased adiposity [30]. While weight loss is associated with a substantial decrease in the risk of obesity-related diseases such as NAFLD, exercise seems to provide a wide range of health effects beyond weight loss [31]. Although it is generally accepted that a single bout of prolonged exercise can depress immunity, regular exercise can be beneficial for the host partly due to ameliorated inflammation [32].

Our data show that MIT and HIIT regimens decreased fat mass but also led to a slight decrease in total lean mass. Previous studies have shown that voluntary wheel running in high fat diet-fed mice reduces adiposity but has no effect on lean mass [33,34]. One interpretation for the slight decrease in lean mass in the MIT and HIIT mice, relative to SED controls, is that obese mice need a larger amount of lean mass to cope with the increased BW and excess adiposity. We also found that the decreased adiposity in MIT and HIIT HFHC-fed mice was not accompanied by a higher resting EE, suggesting that the exercise EE was the largest contributor in reducing BW and adiposity. In contrast with our findings in mice, a study in humans showed that HIIT reduced basal RER, suggesting increased total fatty acid oxidation for substrate utilization [35]. In our study, the decreased expression of genes involved in hepatic lipogenesis, but not lipid catabolism, suggests that reduced fatty acid synthesis in the liver is the main mechanism by which HIIT is superior to MIT at ameliorating steatosis during NASH. A previous study in hyperphagic rats with steatosis showed that voluntary wheel running, equivalent to MIT, suppresses both lipid biosynthesis and enhances fatty acid oxidation [36,37]. Research from the same group compared moderate and vigorous exercise training in steatotic rats and reported a similar mitochondrial adaptation and reduction in steatosis between exercise intensities, though the vigorous exercise group had increased hepatic PPARα [38]. Notably, studies in endurance-trained subjects show that HIIT results in lower total lipid oxidation than MIT, presumably due to a decline in circulating free fatty acids [39]. As changes in resting EE and substrate use can be driven by compensatory adaptations to an exercise-specific EE [40], future studies are needed to determine the total energy metabolism of obese mice considering the metabolic cost of exercise and the contribution of muscle, adipose, and liver.

Hepatic inflammation is a critical component in the initiation and progression of NAFLD and NASH [41]. Particularly, the recruitment of pro-inflammatory MoMFs to the liver via the CCR2/CCL2 axis is key in the progression of NASH [42,43]. Liver macrophages are a heterogeneous population composed of monocyte-derived infiltrating and resident KCs that partially lose their ability to self-renew and are replaced by monocyte-derived KCs during NASH [44]. Notably, a non-KC-like population resembles lipid-associated macrophages that are critical determinants of adipose tissue homeostasis [45]. A previous study showed that the voluntary wheel [46] and continuous treadmill running [11] of NAFLD mice results in a decrease in the frequency of total macrophages in the liver. Here, we demonstrate that HIIT specifically decreases the accumulation of the pro-inflammatory monocyte-derived macrophages in the NASH liver. As activated macrophages secrete harmful cytokines, such as TNFα and IL-1β [47–49], our study suggests that exercise protects the liver against NASH by decreasing the accumulation and the activation of monocyte-derived macrophages. Similarly, HIIT in rats has been shown to decrease the production of TNFα, IL-1β, IL-12, and MCP-1 by peritoneal macrophages [50]. Together, these data suggest that the increased efficacy of HIIT in ameliorating NASH can be partially ascribed to reductions in the frequency of pro-inflammatory MoMFs and the shifting of macrophages toward a less inflammatory phenotype [51].

Obesity-associated chronic inflammation is recognized as a major cause of altered metabolic homeostasis [52]. One mechanism by which obesity contributes to metabolic disease is through increased activity of BM myeloid progenitors, common myeloid, and granulocyte-monocyte progenitors, which leads to monocytosis and ultimately macrophage expansion in metabolic tissues [53]. As NASH is associated with an increased accumulation of monocytes and macrophages within the liver, we reasoned that this process was mediated by an expansion of myeloid progenitors [44]. Our data suggest that the exercise-induced decrease in the accumulation of intra-hepatic

macrophages is associated with a lower production of myeloid progenitors, which diminishes the pool of monocytes available to traffic to the NASH liver. Indeed, a recent work has shown that chronic exercise reduces the production of inflammatory immune cells through the action of leptin [14]. As leptin is positively correlated with BW and adiposity [14], it is possible that some of the beneficial effects of HIIT are induced through leptin-mediated reductions in hematopoiesis. In addition to leptin, a wide array of myokines have been identified as potential mediators of the beneficial effects of exercise [54]. For example, IL-6 is produced in response to muscle contractions [55], and its release is positively correlated with exercise intensity [56]. Exercise-induced IL-6 promotes metabolic adaptations, such as insulin-mediated glucose uptake and increased fatty acid oxidation, but also mediates some of the anti-inflammatory effects of exercise by inhibiting TNFα production [57]. Future studies must determine whether the exercise-driven decrease in macrophage accumulation and activation in the liver is mediated by IL-6.

5. CONCLUSIONS

In conclusion, we show here that high-intensity exercise is more effective than moderate-intensity exercise in improving the progression of NASH disease, including a substantial decrease in hepatic inflammation characterized by a reduced accumulation of pro-inflammatory MoMFs in the liver. These beneficial effects of HIIT may be partially ascribed to the reduction in hematopoiesis, leading to a decreased output of inflammatory cells. Our findings indicate that high-intensity exercise may provide more favorable outcomes to NASH and highlight that intensity needs to be considered when physical exercise is prescribed to manage disturbances associated with obesity, including NAFLD.

AUTHOR CONTRIBUTIONS

X.S.R. and G.F. conceived the study and designed experiments. G.F., F.B., K.D., P.P., S.K., S.R., M.D., H.R., H.W., and X.S.R. performed experiments. O.A. performed histology assessments and fibrosis scoring. G.F. and X.S.R. interpreted results, generated figures and tables, and wrote the manuscript. X.S.R. obtained funding for, supervised, and led the overall execution of the study.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molmet.2021.101270.

CONFLICT OF INTEREST

None declared.

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