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Exercise training attenuates hepatic inflammation, fibrosis and macrophage infiltration during diet induced-obesity in mice

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ABSTRACT

Nonalcoholic steatohepatitis, which is considered the hepatic event in metabolic syndrome, was recently associated with the innate immune system. Although regular exercise reduces hepatic injury markers like serum alanine aminotransferase (ALT) levels, the mechanisms regulating the effects of exercise on steatohepatitis are unclear. This study aimed to clarify whether exercise training suppresses hepatic injury, inflammation, and fibrosis by suppressing macrophage infiltration. Male C57BL/6J (4-week old) mice were randomly divided into four groups: normal diet (ND) control ($n = 7$), ND exercise ($n = 5$), high-fat diet and high-fructose water (HFF) control ($n = 11$), and HFF exercise ($n = 11$) groups. Mice were fed the ND or HFF from 4 to 20 weeks of age. The exercise groups were trained on a motorized treadmill for 60 min/day, five times/week. The nonalcoholic fatty liver disease (NAFLD) activity score and plasma ALT activity, indicators of liver injury, were increased in HFF control mice but were attenuated in HFF exercise mice. Hepatic inflammation, indicated by hepatic tumor necrosis factor (TNF)-α levels and hepatic resident macrophage infiltration, was significantly lower in HFF exercise mice than in HFF control mice. Hepatic fibrosis markers (histological hepatic fibrosis detected by Sirius red and α-smooth muscle actin staining and tissue inhibitor of matrix metalloproteinase-1 mRNA) were attenuated in HFF exercise mice compared with HFF control mice. These results suggest that exercise training reduces hepatic inflammation, injury, and fibrosis by suppressing macrophage infiltration.

1. Introduction

Nonalcoholic steatohepatitis (NASH) is considered the hepatic event in metabolic syndrome (Browning and Horton, 2004). Although the pathogenesis of NASH remains unclear, several factors such as inflammation and fibrosis have been proposed (Argo et al., 2009). Inflammatory cytokines such as tumor necrosis factor (TNF)-α and reactive oxygen species (ROS) exert a fibrotic effect (Tomita et al., 2006; Minicis et al., 2010). Indeed, TNF-α directly up-regulates transforming growth factor (TGF)-β and α-smooth muscle actin (SMA) in hepatocytes (Tomita et al., 2006), and TGF-β subsequently induces tissue inhibitor of metalloproteinase (TIMP) 1, which enhances hepatic fibrosis development via induction of collagen 1α expression (Wang et al., 2009).

The innate immune system is associated with NASH development via activation of hepatic resident macrophages, Kupffer cells (Zhan and An, 2010). Macrophage infiltration was induced in the livers of mice by combined administration of high-fat diet and high-fructose water, and Kupffer cells subsequently induced various pro-inflammatory cytokines, which enhance hepatic inflammation and fibrosis (Kohli et al., 2010). Kupffer cells express toll-like receptors (TLRs) and recognize several molecular patterns that induce inflammatory cytokine production (Szabo et al., 2006). Seki et al. (2007) recently reported that hepatic inflammation and fibrosis are attenuated in TLR4-knockout mice. Therefore, the TLRs in Kupffer cells may play an important role in NASH. Furthermore, it was suggested that T lymphocytes play an essential role in the development of adipose tissue inflammation (Winer et al., 2009; Nishimura et al., 2009). Although it is proposed that cytotoxic T cells are associated with NASH development (Popov and Schuppan, 2010), this has not been conclusively proven.

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Regular exercise may prevent or attenuate NASH development. In addition, higher levels of physical activity are negatively associated with the state of fibrosis in patients with NASH (Kistler et al., 2010), and regular exercise reduces the serum alanine aminotransferase (ALT) activity, which is a marker of hepatic injury (Promrat et al., 2010; Sreenivasa et al., 2006). Furthermore, exercise training reduces serum ALT activity and hepatic steatosis in obese mice (Marques et al., 2010; Schultz et al., 2010). However, it is not known whether exercise training alleviates hepatic injury and fibrosis by suppressing inflammation in obese mice.

It was recently shown that regular exercise can protect against the development of several types of chronic inflammation (Gleeson et al., 2011; Pedersen, 2011). In fact, the concentrations of plasma inflammatory cytokines such as TNF-α and IL-6 are lower in physically active subjects than in physically inactive subjects (Pedersen and Saltin, 2006). Moreover, exercise training is now considered a crucial event leading to reduced inflammation in the adipose tissue. We and another research group have shown that exercise training enhances down-regulation of TNF-α gene expression in the adipose tissue of obese mice (Kawanishi et al., 2010; Vieira et al., 2009). Furthermore, we showed that exercise training attenuates inflammatory macrophage infiltration into adipose tissue by suppressing chemokine and adhesion molecules (Kawanishi et al., 2010). Accordingly, it is possible that exercise training attenuates inflammation in liver tissue by suppressing macrophage infiltration in diet-induced NASH model mice.

Therefore, the purpose of the present study was to clarify whether exercise training reduces hepatic injury, inflammation and fibrosis in diet-induced NASH model mice. We hypothesized that exercise training attenuates hepatic injury, inflammation and fibrosis by suppressing the infiltration of macrophages.

2. Methods

2.1. Animals, diets and exercise training protocol

Male C57BL/6 mice (4 weeks old) were purchased from Kiwa Laboratory Animals (Wakayama, Japan). Four animals were housed together in 1 cage (27 × 17 × 13 cm) in a controlled environment under a light-dark cycle (lights on at 0900 and off at 2100). The experimental procedures followed the Guiding Principles for the Care and Use of Animals in the Waseda University Institutional Animal Care and Use Committee (approved number: 2011-A19). The mice were randomly divided into four groups: normal diet (ND) control ($n = 7$), ND with exercise training ($n = 5$), high-fat diet and high-fructose water (HFF) control ($n = 11$), and HFF with exercise training ($n = 11$) groups. Ethically, this experiment was started with the animal number of 8 (Normal diet group) or 12 (High fat diet group) per respective groups, but the number of exercise training mice were reduced to by three due to injury during the treadmill running. The high-fat diet (D12492; Research Diets, New Brunswick, NJ) was composed of 60% fat, 20% protein, and 20% carbohydrate (of total calories), and the high-fructose water contained 21% (wt/V) fructose (Sigma, St. Louis, MO).

A mix of high fat and fructose has been shown effective for inducing fatty acid liver disease. Overintake of fructose is also associated with the development of NASH (Lim et al., 2010). Animals fed a high-fat diet and high-fructose water *ad libitum* developed hepatic inflammation and fibrosis in mice (Kohli et al., 2010; Sohet et al., 2009). In contrast, animals fed only a high-fat-diet (about 60% kcal from fat) *ad libitum* developed steatosis without any hepatic inflammation or fibrosis (Kohli et al., 2010). Therefore, feeding high-fat diet and high-fructose water is a useful model to develop fatty acid liver disease and NASH.

The mice in the present study were fed the HFF from 4 to 20 weeks of age. All ND mice were fed a standard normal diet containing 10% fat, 20% protein, and 70% carbohydrate (D12450B; Research Diets, New Brunswick, NJ) and fructose-free water. All groups had free access to food and water. Body mass was measured weekly. Food and water intakes per four mice were measured twice from week 15 to week 16.

Four-week old mice were trained on a motorized treadmill (Natsume, Kyoto, Japan) for 60 min/day during their light phase at running speeds of 15–20 m/min, five times/week for 16 weeks. Exercise speed was set initially from 15 m/min per session during the first four weeks, and increased to 20 m/min per session on and after 5 weeks. Electric shock was not used during the treadmill run to avoid noxious stress. Control mice were housed in cages. The exercise-trained and untrained mice (20-weeks old) were sacrificed 3 days after the final exercise training session under light anesthesia with the inhalant isoflurane (Abbott, Tokyo, Japan). Abdominal vein blood samples were collected in heparin tubes, and plasma was stored at −80 °C. Liver tissue was quickly frozen in liquid nitrogen and stored at −80 °C.

2.2. Plasma alanine aminotransferase and hepatic triglyceride measurements

ALT activity was measured using the L type Wako GPT Kit (Wako, Osaka, Japan). The minimum detectable limit of ALT activity was 2.2 U/L. To examine triglyceride levels in the livers of mice, liver tissue (100 mg) was homogenized in a 1 ml solution containing 5% Triton-X100 in water, and heated to 85 °C for 5 min. Hepatic triglyceride content was measured using the triglyceride Quantification Kit (BioVision, Milpitas, CA). The minimum detectable concentration of Triglyceride was 2 pmol/L.

2.3. Hepatic TNF-α and monocyte chemoattractant protein (MCP)-1 measurement

To examine TNF-α and MCP-1 protein levels in the livers of mice, liver tissue (100 mg) was homogenized in 500 μl Tissue Protein Extraction Reagent (T-PER) with Protease inhibitor (Thermo, Rockford, IL). The protein concentration was measured using the BCA Protein Assay (Thermo). The TNF-α level was measured using the Mouse TNF-α Quantikine ELISA kit (R&D Systems, Minneapolis, MN). The MCP-1 level was measured using the Mouse MCP-1 Quantikine ELISA kit (R&D Systems). The minimum detectable concentrations of TNF-α and MCP-1 were 5.1 and 15.6 pg/ml, respectively.

2.4. Isolation of hepatic mononuclear cells and flow cytometry analysis

We isolated mononuclear cells from the liver using previously described methods with some modifications (Kinoshita et al., 2010; Kleiner et al., 2005). After removal of blood from liver tissue, the liver was minced with scissors. Hanks' Balanced Salt Solution (HBSS) (10 ml) containing 0.05% collagenase type 4 (Worthington, Lakewood, NJ) was added to the pieces of liver tissue. The mixture was shaken for 20 min at 37 °C, and the digested tissue was centrifuged at 500 × *g* for 10 min. The resultant pellet containing the mononuclear cells was resuspended in RPMI containing 1% FBS, filtered through a 70-μm stainless steel mesh, and the digested tissue was centrifuged at 500 × *g* for 10 min. The resultant pellet containing the mononuclear cells was suspended in 12 ml 33% Percoll (Sigma) solution containing 10 U/ml heparin and a 15 min centrifugation step at 500 × *g* was performed at room temperature. The resultant pellet containing the mononuclear cells was suspended in 5 ml Red Blood Cell Lysing buffer (Sigma) and filtered through a 40-μm nylon mesh. The cells were washed twice with

Table 1
Primer sequences for real-time RT-PCR analysis.

Gene	Forward	Reverse
GAPDH	TGAAGCAGGCATCTGAGGG	CGAAGGTGGAAGAGTGGGAG
TNF-α	CCTCCCTCTCATCAGTTCTA	ACTTGGTGGTTTGCTACGAC
IL-1β	CTGGAGAGTGTGGATCCCAAG	GGAAGACACGGATTCCATGGTG
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-10	TGAATTCCCTGGGTGAGAAG	TCACTCTTCACCTGCTCCACT
Collagen 1α	GAGCGGAGAGTACTGGATCG	GCTTCTTTTCCTTGGGGTTC
α-SMA	AAACAGGAATACGACGAAG	CAGGAATGATTTGGAAAGGA
TIMP1	TGGGAAATGCCGCAGATATC	TGGGACTTGTGGGCATATCC
TGF-β	GACCCTGCCCCTATATTTGGA	GCCCGGGTTGTGTTGGT
CD11c	CTGGATAGCCTTTCTTCTGCTG	GCACACTGTGTCCGAACTC
TLR4	ATGGCATGGCTTACACCACC	GAGGCCAATTTTGTCTCCACA
MCP-1	CTTCTGGGCCTGCTGTTCA	CCAGCCTACTCATTGGGATCA
CXCL14	CCAAGATTCGCTATAGCGAC	CCTGCGCTTCTCGTTCCAGG
RANTES	CAGAGGAAAGAGAGAAAGTCC	CACACGGTGACAGTGCTGG

Stain Buffer (BD Pharmingen, Franklin Lakes, NJ), and the mononuclear cells were resuspended in Stain Buffer. The mononuclear cells (2.5×10^5 cells) were incubated with Fc-blocker (anti-CD16/CD32) for 20 min followed by staining with PE-Cy7-CD3e, PE-CD4, and PE-Cy5-CD8a for 20 min. All of the above mentioned antibodies were purchased from eBioscience (San Diego, CA). Flow cytometry was performed using a Guava® EasyCyte™ 6HT (Millipore, Long Beach, CA) and InCyte software (Millipore). We validated flow cytometric identification of cytotoxic T cells ($CD8a^+CD3e^+$) and helper T cells ($CD4^+CD3e^+$). Hepatic resident mononuclear cells from mice were gated according to side scatter and forward scatter plots. Cytotoxic T cell populations were gated according to CD3e and CD8a on the SSC/FSC plot. Helper T cell populations were gated according to CD3e and CD4 on the SSC/FSC plot.

2.5. Real-time quantitative PCR

A piece of the liver tissue was quickly immersed in RNAlater (Applied Biosystems, Carlsbad, CA) and stored at −80 °C. Total RNA was extracted from the liver tissue homogenate using Trizol reagent (Invitrogen, Carlsbad, CA) and the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The purity of total RNA was assessed using the NanoDrop system (NanoDrop Technologies, Wilmington, DE). Total RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Polymerase chain reactions (PCR) were performed with the Fast 7500 real-time PCR system (Applied Biosystems) using the Fast SYBR® Green PCR Master Mix (Applied Biosystems). The thermal profiles consisted of 10 min at 95 °C for denaturation followed by 40 cycles of 95 °C for 3 s and annealing at 60 °C for 15 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the housekeeping gene, and all data are represented relative to its expression (i.e., using standard curve methods) as fold change based on the values of the ND control group. Specific PCR primer pairs for each studied gene are shown in Table 1.

2.6. Histological analysis

A piece of the liver tissue was transferred to a plastic mold, covered with OCT compound, and snap frozen by immersing the closed in pre-cooled isopentane at −80 °C. Hematoxylin–Eosin (H&E), Oil-Red O, Sirius red, and Masson trichrome staining were performed. The nonalcoholic fatty liver disease (NAFLD) activity score is an accepted paradigm for the staging and grading of NASH development. The NAFLD activity score was determined as previously described (Kleiner et al., 2005). Scores for steatosis (0–3), lobular inflammation (0–3), and hepatocyte ballooning (0–2) were also summed to produce the NAFLD activity score; thus, the total score ranged from 0 to 8. Oil red O- and Sirius red-staining positive areas were analyzed on four random low power (100×) fields/slide using ImageJ software (National Institutes of Health, Bethesda, USA).

Immunohistochemical staining was applied to frozen sections of liver tissue to examine expression of F4/80 and α-SMA. The 5-μm serial sections were incubated in 4% paraformaldehyde for 7 min at 4 °C. Endogenous peroxidase was inactivated with 1% hydrogen peroxide in methanol for 30 min at 4 °C. α-Smooth muscle actin (Dako, Carpinteria, CA) and F4/80 (Serotec, Kidlington, UK) primary antibodies were added in 1% BSA solution and sections were incubated overnight at 4 °C. Secondary anti-rabbit antibody was added in PBS buffer with normal mouse serum for 30 min at room temperature. Proteins were visualized using the Vectastain Elite ABC Kit (anti-rabbit, Vector, Burlingame, CA) for 30 min at room temperature and further incubation was carried out with diaminobenzidine (DAB) chromogen. F4/80-positive cells were counted on 4 random high power (200×) fields/slide using BZ-2 software (KEYNENCE, Osaka, Japan).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed on frozen sections of liver tissue. Sections were digested with 20 μg/mL proteinase K, reacted with terminal deoxynucleotidyl transferase enzyme, and incubated with anti-digoxigenin conjugate followed by incubation with DAB solution. TUNEL-positive cells were counted on four random high power (200×) fields/slide using BZ-2 software.

Calculations of the NAFLD activity score, and of the Oil red O- and Sirius red-positive areas, and counts of the F4/80- and TUNEL-positive cell number were performed by three independent observers, who were blinded to the diagnosis, and the average value for each section was calculated. The coefficients of variation for the NAFLD activity score was 11.6%.

2.7. Statistical analyses

All data are expressed as means ± SEM. Statistical analyses were performed using SPSS V17.0 except for the NAFLD activity score. Body weight changes were analyzed using a general linear model ANOVA with repeated measures at weekly. The statistical significance of differences in body mass, mRNA expression, protein levels and histological analysis parameters, and T cell populations between groups were determined using 2-way analysis of variance (ANOVA) with diet (ND or HFF) and exercise (control or exercise training). If significant interactions were observed in any of these analyses, and comparisons with the Bonferroni correlated post-hoc test was performed. The NAFLD activity score is a non-continuous scoring system using 0–8 as possible scores, and non-parametric tests were employed on NAFLD activity score analysis. The statistical significance of differences in the NAFLD activity score between groups was determined using the Friedman test, and comparisons with the Steel Dwass post-hoc test were performed subsequently using Ekuseru-Toukei 2010 (Social Survey Research Information Co. Ltd., Tokyo, Japan). The alpha level was set at $p < 0.05$.

3. Results

3.1. Effects of HFF diet and exercise training on characteristic physical parameters

Fig. 1 illustrates the change in body weight that occurred during the intervention, using repeated measures ANOVA, and there were

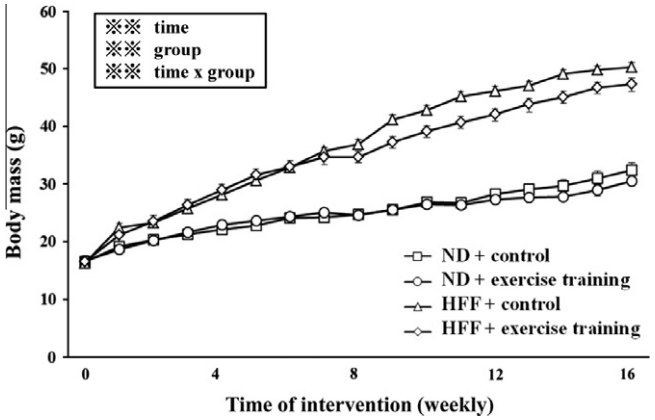

Fig. 1. Body weight change at weekly during intervention. Values represent means ± SEM. Repeated measures analysis of ANOVA revealed significant time, group and time × group effect for body weight gain (boxed text). $^{**}p < 0.01$.

significant time, group and time × group effects on body gain during the intervention. A statistically significant diet × exercise interaction and exercise effect was not found on body mass at 16 weeks using 2-way ANOVA, but a statistically significant effect was found for diet ($F_{1,30} = 208.67$, $p < 0.01$). A significant diet × exercise interaction was observed for liver mass ($F_{1,30} = 6.75$, $p < 0.05$). Post-hoc comparisons revealed that HFF control mice were heavier than ND control and HFF exercise mice ($p < 0.01$, Table 2). Calorie intake, which was measured in terms of diet and water, varied as a main effect of diet ($F_{1,20} = 150.58$, $p < 0.01$), but the ANOVA did not reveal a statistically significant diet × exercise interaction (Table 2).

3.2. Effect of HFF diet and exercise training on hepatic steatosis

To evaluate hepatic steatosis, we performed Oil-red O staining and examined hepatic triglyceride content. The Oil-Red O staining of neutral triglycerides and lipid droplets in the liver (Fig. 2A) was calculated as the percentage of positive-stained areas per square millimeter in each section, and ANOVA revealed a significant diet × exercise interaction ($F_{1,28} = 18.9$, $p < 0.01$). The positive area was significantly greater in HFF control mice than in ND control mice ($p < 0.01$). However, the positive area in HFF exercise mice was significantly lower than in HFF control mice ($p < 0.01$, Fig. 2B). Although the hepatic triglyceride contents varied according to diet ($F_{1,30} = 44.7$, $p < 0.01$) and exercise ($F_{1,30} = 4.08$, $p < 0.05$), the interaction was not significantly different (Fig. 2C).

3.3. Effect of HFF diet and exercise training on hepatic inflammation

The TNF-α, which is a marker of hepatic inflammation, varied in the livers as a diet × exercise interaction ($F_{1,30} = 14.64$, $p < 0.01$). Post-hoc comparisons revealed that the TNF-α levels in both HFF-control and -exercise groups were higher than in both the ND control and exercise groups ($p < 0.01$). In addition, TNF-α was significantly lower in HFF exercise mice than in HFF control mice ($p < 0.01$, Fig. 3A).

The changes in several cytokine mRNA expression levels are shown in Fig. 3B. The TNF-α mRNA level was not significantly affected by exercise and diet × exercise interactions. However, diet had a statistically significant effect ($F_{1,30} = 13.29$, $p < 0.01$), and exercise showed a tendency toward having a significant effect ($F_{1,30} = 3.56$, $p = 0.07$) on the TNF-α mRNA level. Although diet and/or exercise did not affect IL-1β and IL-6 mRNA levels, the mRNA level of IL-10 varied as a diet × exercise interaction ($F_{1,30} = 4.82$, $p < 0.05$). Post-hoc comparisons revealed that the IL-10 mRNA level was significantly lower in the HFF control mice than in the ND control mice ($p < 0.05$). The IL-10 mRNA level was not significantly altered by exercise training (Fig. 3B).

3.4. Effect of HFF diet and exercise training on hepatic injury

We scored NAFLD activity using H&E staining for hepatic injury assessment. Post-hoc comparisons revealed that the NAFLD activity score, as determined by the degree of steatosis, lobular inflammation, and hepatocellular ballooning (Kleiner et al., 2005), was significantly increased in the HFF control mice compared to the ND control mice ($p < 0.01$). Compared with HFF control mice, HFF exercise mice had significantly decreased NAFLD activity score ($p < 0.01$, Fig. 4A and B). In TUNEL-positive cells, a marker of cell apoptotic death, we found a significant diet × exercise interaction ($F_{1,28} = 21.77$, $p < 0.01$). Post-hoc comparisons revealed that the number of TUNEL-positive cells was significantly greater in HFF control mice compared with ND control mice ($p < 0.01$). However, the number of positive cells in HFF exercise mice was significantly lower than that in HFF control mice ($p < 0.01$, Fig. 4A and C). Furthermore, a significant diet × exercise interaction in the plasma ALT level was indicated by ANOVA ($F_{1,30} = 4.91$, $p < 0.01$). The plasma ALT level was significantly higher in the HFF control mice compared with the ND control group ($p < 0.01$). However, the plasma ALT level in HFF exercise mice was significantly lower than that in HFF control mice ($p < 0.05$, Fig. 4D).

3.5. Effect of HFF diet and exercise training on hepatic fibrosis

To investigate the effect of exercise training on HFF diet-induced hepatic fibrosis, we examined Sirius red, Masson trichrome,

Table 2
The comparison of body mass, fat tissue mass, liver mass, food intake, water intake and calorie intake between normal (ND) and high fat diets and high fructose water (HFF) in control and exercise trained mice.

	ND		HFF		Two-way ANOVA		
	Control	Exercise	Control	Exercise	Diet	Exercise	Interaction
Body mass (g)	33.2 ± 1.4	32.3 ± 1.2	50.5 ± 0.7	47.8 ± 1.1	$p < 0.01$	NS	NS
Liver mass (g)	1.46 ± 0.05	1.38 ± 0.06	3.24 ± 0.19[a]	2.26 ± 0.09[b,c]	$p < 0.01$	$p < 0.01$	$p < 0.05$
Epididymal fat mass (g)	1.22 ± 0.13	1.21 ± 0.15	1.76 ± 0.06	1.92 ± 0.11	NS	NS	NS
Fat mass/body mass (%)	3.61 ± 0.30	3.70 ± 0.39	3.50 ± 0.13	4.09 ± 0.31	NS	NS	NS
Food intake (mg/weeks)	20.7 ± 1.0	20.7 ± 0.1	19.00 ± 0.4	19.6 ± 0.6	$p < 0.01$	NS	NS
Water intake (ml/days)	3.19 ± 0.18	3.33 ± 0.33	3.39 ± 0.22	3.33 ± 0.15	NS	NS	NS
Calorie intake (kcal/weeks)	78.8 ± 4.0	79.0 ± 0.2	118.1 ± 2.1	121.8 ± 4.2	$p < 0.01$	NS	NS

Data are means ± SEM. NS, not significant.
[a] $p < 0.05$, different from ND control.
[b] $p < 0.05$, different from ND exercise.
[c] $p < 0.05$, different from HFF control.

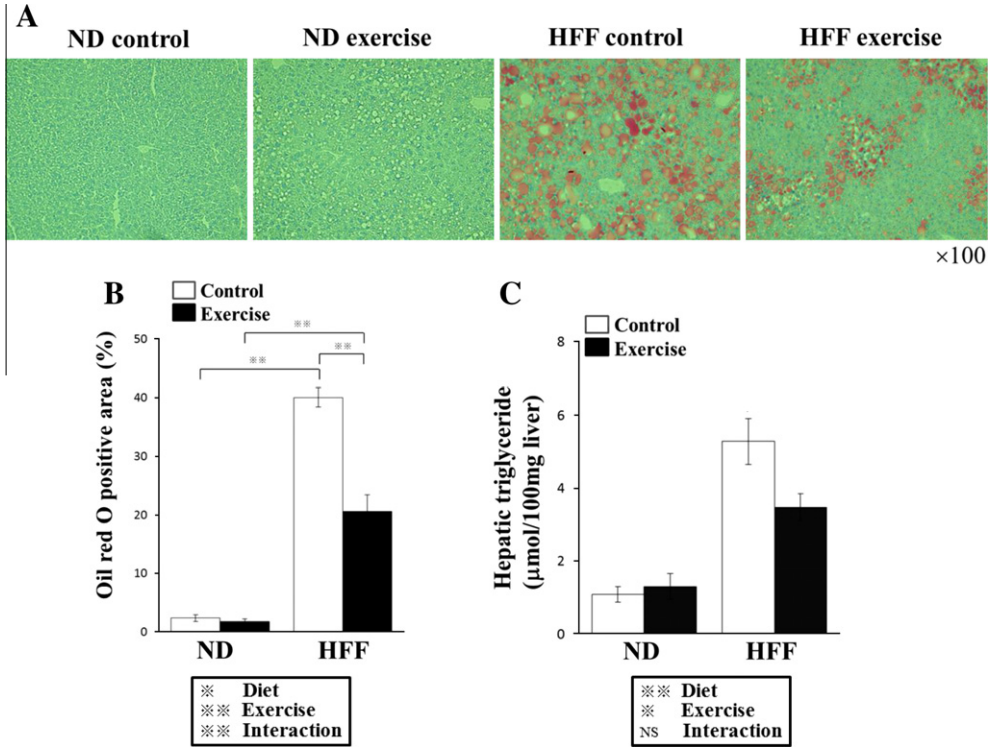

Fig. 2. Effect of exercise training on hepatic steatosis in ND- and HFF-fed mice. (A) Oil Red O staining (red; neutral lipids) of liver sections ($n = 32$). (B) Oil Red O-positive areas, and (C) hepatic triglyceride contents. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). NS; not significant, $^{**}p < 0.01$, $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

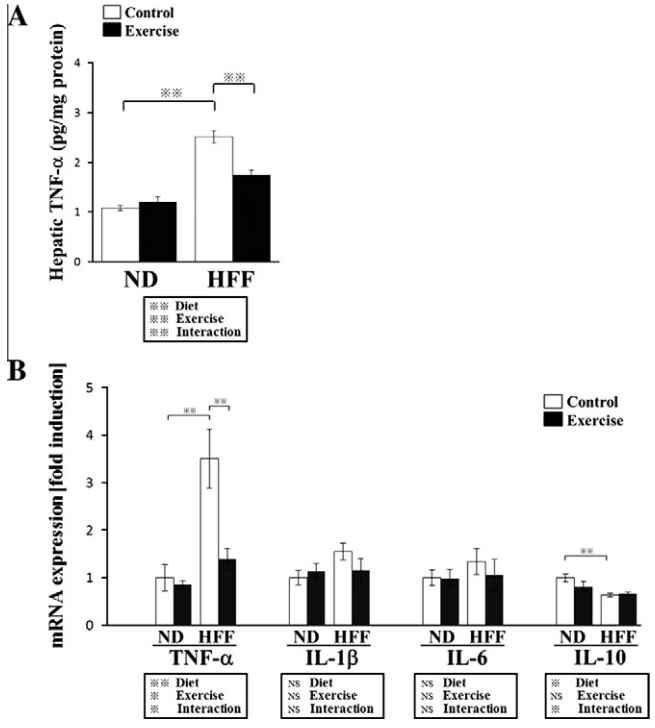

Fig. 3. Effect of exercise training on hepatic inflammation in ND- and HFF-fed mice. (A) Hepatic TNF-α protein levels and (B) hepatic mRNA levels of TNF-α, IL-1β, IL-6, and IL-10. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). NS; not significant, $^{**}p < 0.01$, $^{*}p < 0.05$.

and α-SMA immunohistochemistry strains (Fig. 5A and B), and measured the expression of fibrogenic marker genes in the liver (Fig. 5C). Sirius red and Masson trichrome, which were used to stain tissue collagen fiber, revealed that the HFF diet caused deposition of collagen fiber. However, HFF exercise trained mice showed a marked improvement in liver histology, as seen by the reduction in the amount of detected collagen fibers. α-SMA immunohistochemistry, which was used to stain activated hepatic stellate cells, revealed the presence of greater numbers of α-SMA-positive cells in HFF control mice throughout the lobule in a pericellular distribution. However, the numbers of α-SMA-positive cells were attenuated by exercise training in HFF mice. In the Sirius red-positive area, we found a significant diet × exercise interaction ($F_{1,28} = 20.98$, $p < 0.01$). Post-hoc comparisons revealed that the Sirius red-positive area was significantly greater in HFF control mice than in ND control mice ($p < 0.01$). However, the Sirius red area in HFF exercise mice was significantly lower than that in HFF control mice ($p < 0.01$, Fig. 5B). With respect to mRNA levels of collagen 1α and TIMP1, which are markers of hepatic fibrosis, we found a significant diet × exercise interaction (collagen 1α mRNA; $F_{1,30} = 15.40$, $p < 0.01$, TIMP1 mRNA; $F_{1,30} = 16.24$, $p < 0.01$). Post-hoc comparisons revealed that collagen 1α and TIMP1 mRNA levels were higher in HFF control mice than in ND control mice (collagen 1α and TIMP1 mRNA; $p < 0.01$, respectively), but these mRNA levels were significantly lower in HFF exercise mice than in HFF control mice (collagen 1α and TIMP1 mRNA; $p < 0.01$, respectively, Fig. 5C). The mRNA levels of α-SMA and TGF-β were also significantly affected by both diet (α-SMA mRNA; $F_{1,30} = 30.86$, $p < 0.01$, TGF-β mRNA; $F_{1,30} = 6.74$, $p < 0.05$) and exercise (α-SMA mRNA ; $F_{1,30} = 7.46$, $p < 0.05$, TGF-β mRNA; $F_{1,30} = 4.85$, $p < 0.05$), but a diet × exercise interaction was not observed.

3.6. Effect of HFF diet and exercise training on macrophage infiltration into the liver

Tissue inflammation, which is thought to play a very active role in hepatic injury and fibrosis, was investigated in this study by the

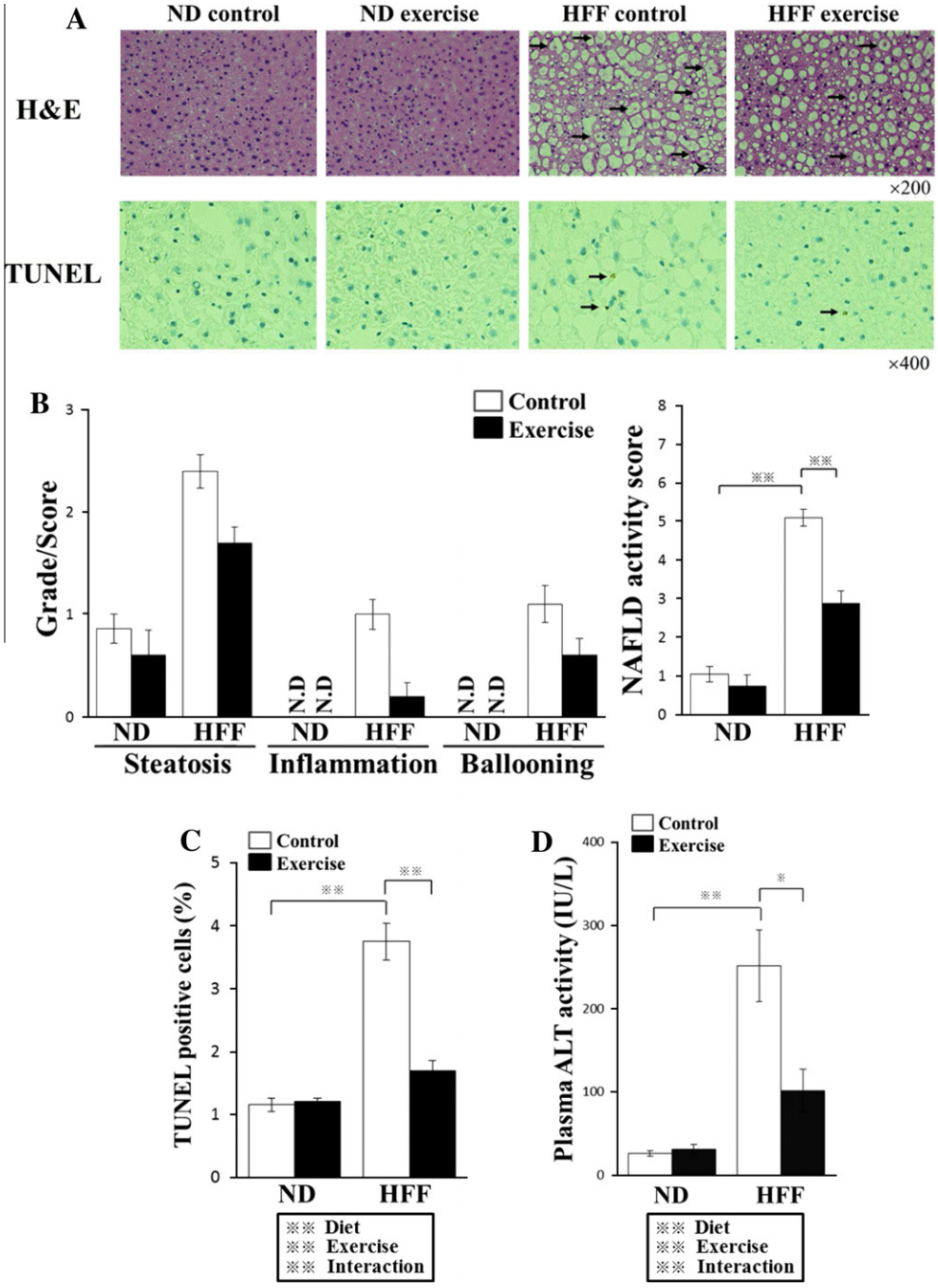

Fig. 4. Effect of exercise training on hepatic injury in ND- and HFF-fed mice. (A) H&E (arrowheads; lobular inflammation, and arrows; hepatocyte ballooning) and TUNEL staining (arrows; TUNEL-positive cells) of liver sections, (B) NAFLD activity scores, (C) number of TUNEL-positive cells, and (D) plasma ALT activity. Values represent means ± SEM. Analyses of differences in TUNEL-positive cells and plasma ALT activity between groups was performed using 2-way ANOVA for multiple groups (boxed text). $^{**}p < 0.01$, $^{*}p < 0.05$.

assessment of macrophages in the liver by F4/80 immunohistochemistry staining. While administration of the HFF augmented the number of macrophage clusters preferentially distributed to cell pericentral region, exercise training reduced this infiltration (Fig. 6A). In fact, the number of F4/80-positive cell varied as a significant diet × exercise interaction ($F_{1,28} = 12.67$, $p < 0.01$). Post-hoc comparisons revealed that the number of F4/80-positive cells was significantly higher in HFF control mice compared with ND control mice ($p < 0.01$). However, the positive cells in HFF exercise mice was significantly lower than in HFF control mice ($p < 0.01$, Fig. 6B). Similarly, we also found a significant diet × exercise interaction respect to the mRNA levels of CD11c and TLR4 (CD11c mRNA; $F_{1,30} = 14.78$, $p < 0.01$, TLR4 mRNA; $F_{1,30} = 8.26$, $p < 0.01$). Post-hoc comparisons revealed that the mRNA levels of CD11c and TLR4 were significantly increased in the HFF control mice compared to the ND control mice (CD11c mRNA; $p < 0.01$, TLR4 mRNA; $p < 0.05$). However, these mRNA were significantly lower in HFF exercise mice than in HFF control mice (CD11c and TLR4 mRNA; $p < 0.01$, respectively, Fig. 6C).

3.7. Effect of HFF diet and exercise training on infiltration of T lymphocytes

To determine T lymphocyte infiltration, we examined the populations of cytotoxic and helper T cells. For populations of $CD8^+$ $CD3^+$ and $CD4^+$ $CD3^+$ cells, which are markers of cytotoxic T cells

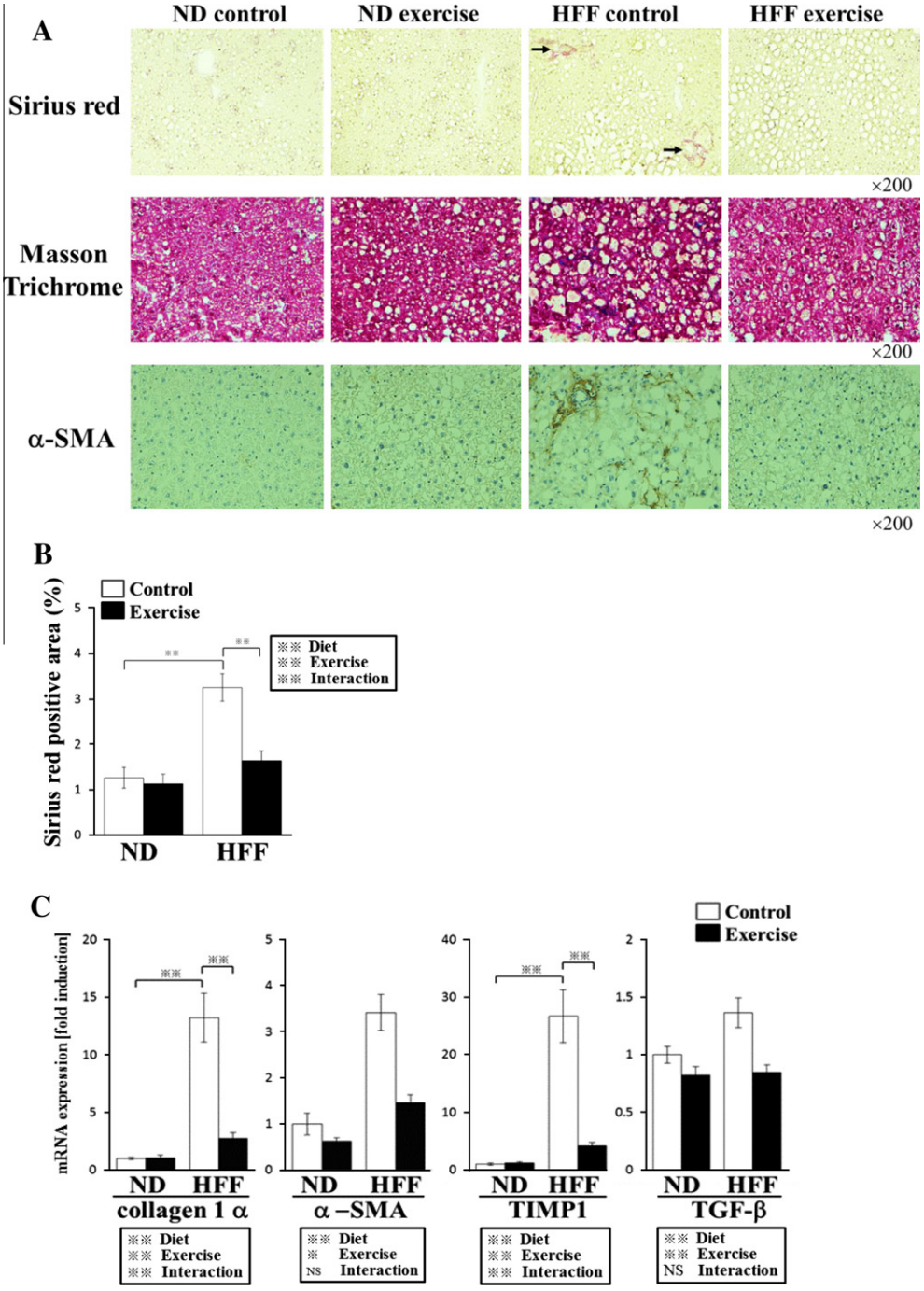

Fig. 5. Effect of exercise training on hepatic fibrosis in ND- and HFF-fed mice. (A) Sirius red (arrows; collagen fiber), Masson Trichrome (blue; collagen fiber), and α-SMA immunohistochemistry (brown; α-SMA-positive cells) staining of liver sections. (B) The Sirius red-positive areas, and (C) mRNA expression levels of hepatic fibrogenic markers. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). NS; not significant, $^{**}p < 0.01$, $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and helper T cells, 2-way ANOVA did not reveal significant effects of diet and exercise and those interactions (Fig. 7).

3.8. Effect of HFF diet and exercise training on macrophage chemoattractants

We found a significant diet × exercise interaction for hepatic MCP-1, which is a major macrophage chemokine ($F_{1,30} = 6.00$, $p < 0.05$). Post-hoc comparisons revealed that MCP-1 was significantly higher in the HFF control mice than in the ND control mice ($p < 0.01$). However, MCP-1 was significantly lower in HFF exercise mice than in HFF control mice ($p < 0.05$, Fig. 8A). Furthermore, we observed a significant effect of diet ($F_{1,30} = 17.82$, $p < 0.01$), but not a diet × exercise interaction with respect to the MCP-1 mRNA level (Fig. 8B). 2-way ANOVA revealed a statistically significant diet × exercise interaction for the CXCL14 mRNA expression level ($F_{1,30} = 19.13$, $p < 0.01$). Post-hoc comparisons revealed a significant difference between the HFF control mice and the HFF exercise mice ($p < 0.01$). In contrast, diet, exercise and the interaction of diet and exercise did not affect RANTES mRNA expression (Fig. 8B).

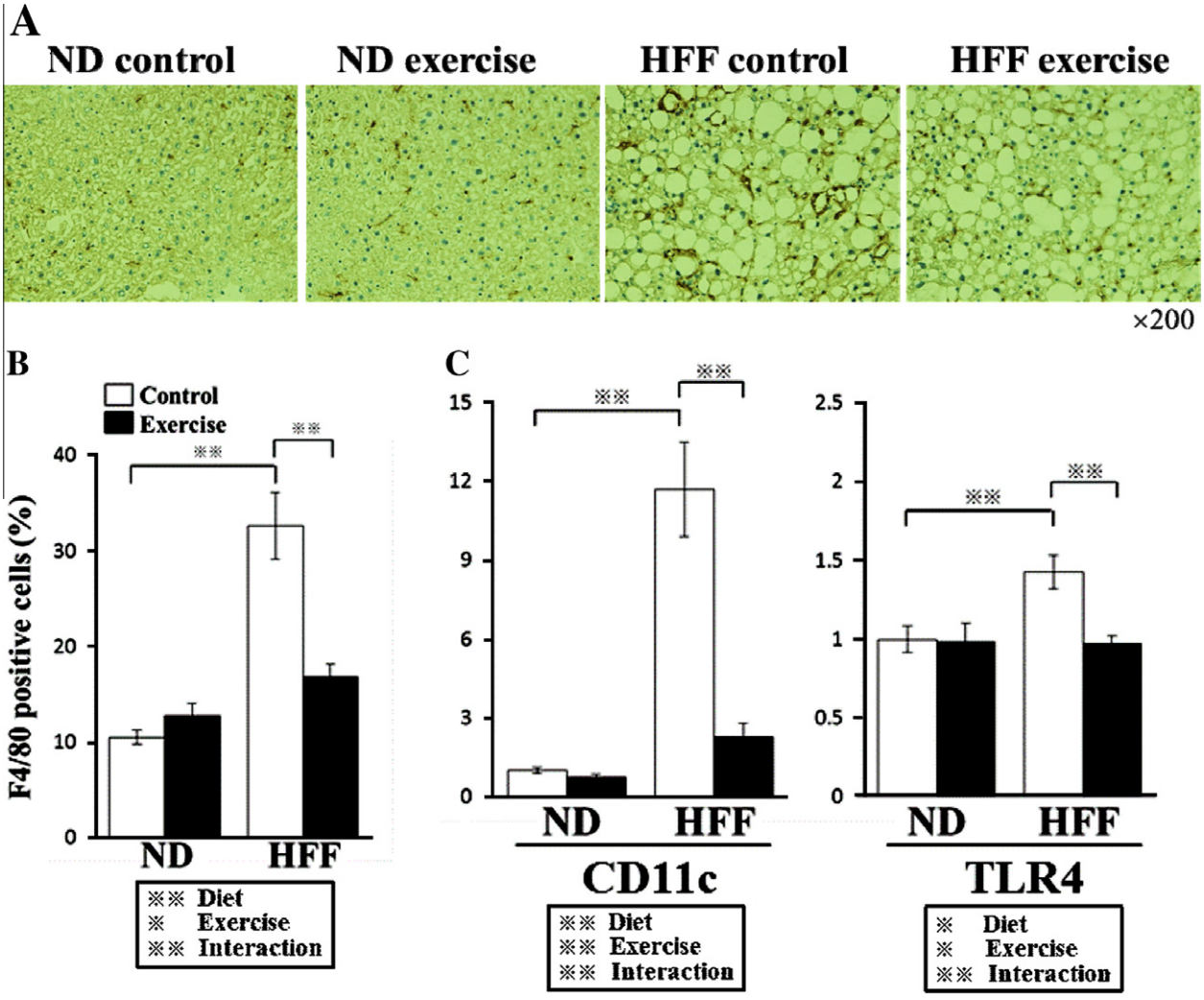

Fig. 6. Effect of exercise training on infiltration of macrophages into the liver of ND- and HFF-fed mice. (A) F4/80 immunohistochemistry (brown; F480-positive cells) staining of liver sections, and (B) number of F4/80-positive cells. (C) Hepatic mRNA levels of CD11c and TLR4. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). $^{**}p < 0.01$, $^{*}p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Although recent studies have shown that forced treadmill and swimming exercise training decreases serum ALT activity and hepatic steatosis in obese mice (Marques et al., 2010; Schultz et al., 2010), it remains unclear whether exercise training attenuates NASH development. In this study, we evaluated the effect of exercise training on HFF induced steatosis, hepatic injury, inflammation, and fibrosis (Kohli et al., 2010) with changes in macrophage infiltration. Our results indicated that these parameters are attenuated by exercise training in HFF-induced obese mice.

It has been reported that accumulation of hepatic triglycerides is causally related to hepatic inflammation and fibrosis (Day and James, 1998). Exercise has the potential to prevent the development of hepatic steatosis, depending on the amount of weight loss (Promrat et al., 2010). In this study, exercise training induced the loss of liver mass and hepatic triglyceride accumulation in HFF mice, whereas body mass did not change. Therefore, our results suggest that exercise training may directly attenuate hepatic steatosis regardless of whether exercise results in weight loss. In addition, our results agree with the findings of recent studies, which have reported that exercise training attenuated hepatic steatosis in obese mice (Marques et al., 2010; Schultz et al., 2010). Recent studies have shown that hepatic ceramide plays an important role in NASH development (Chocian et al., 2010; Alkhouri et al., 2009; Malhi and Gores, 2008). It will be necessary to examine whether exercise training reduces ceramide content in the liver during diet-induced obesity in mice.

The anti-inflammatory properties of exercise have been demonstrated (Gleeson et al., 2011; Pedersen, 2011). Recently, we reported that exercise training attenuates TNF-α mRNA expression in adipose tissue of high fat-induced obese mice (Kawanishi et al., 2010). In this study, exercise training decreased hepatic TNF-α in diet-induced obese mice. Additionally, TNF-α mRNA expression in the liver was also attenuated by exercise in HFF-fed mice. It is well known that the HFF diet leads to the production of TNF-α and induces hepatic inflammation with high expression of TNF-α mRNA in the liver (Roth et al., 2011; Sohet et al., 2009; Tetri et al., 2008). Therefore, exercise training may improve hepatic inflammation by inhibiting the HFF diet-induced TNF-α mRNA expression in obese mice.

TNF-α plays a critical role in the development of NASH by inducing hepatic injury and fibrosis. Li et al. (2003) reported that anti-TNF antibody treatment improves fatty liver and hepatic

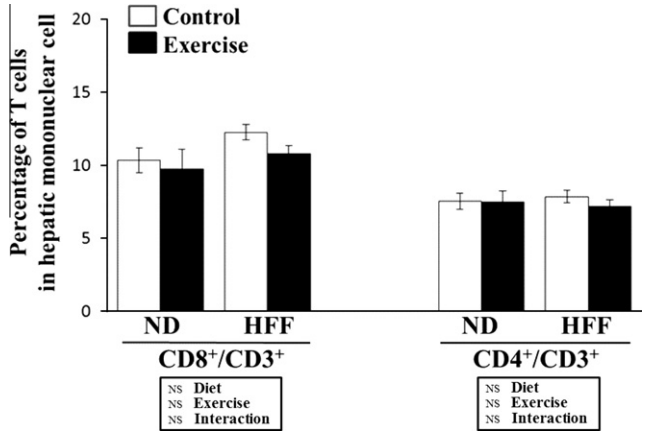

Fig. 7. Effect of exercise training on infiltration of T lymphocytes in ND- and HFF-fed mice. Populations of $CD8^+$ $CD3^+$ and $CD4^+$ $CD3^+$ cells in hepatic mononuclear cells were determined using flow cytometry. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). NS; not significant.

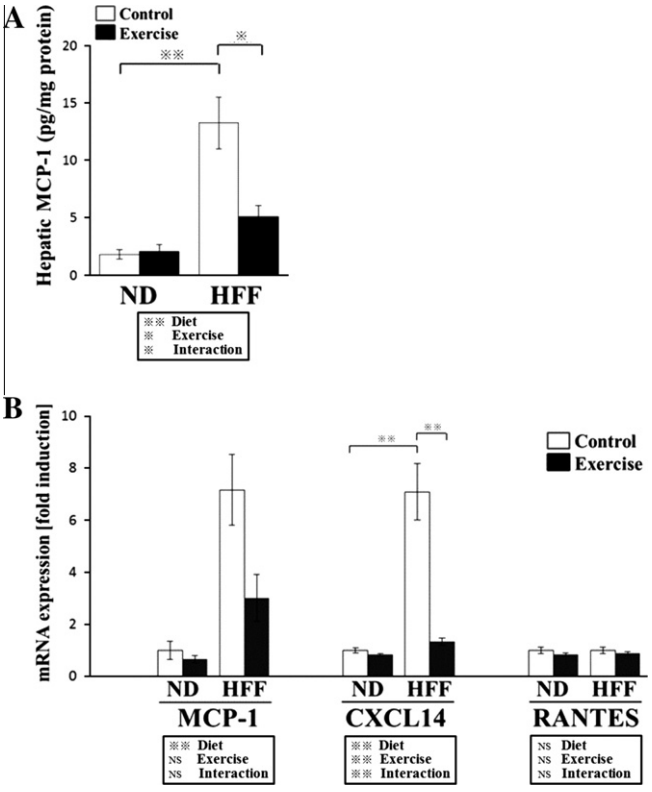

Fig. 8. Effect of exercise training on macrophage chemoattractants in ND- and HFF-fed mice. (A) Hepatic MCP-1, and (B) mRNA expression levels of hepatic macrophage-specific chemokines. Values represent means ± SEM. Analyses were performed using 2-way ANOVA for multiple groups (boxed text). NS; not significant, $^{**}p < 0.01$, $^{*}p < 0.05$.

injury in NASH model mice. Furthermore, Tomita et al. (2006) reported that TNF-α receptor deficient mice show lower hepatocyte apoptosis than wild-type mice. We also observed that exercise training in obese mice attenuated TUNEL-positive cells in the liver, and reduced ALT activity in plasma, and reduced the TNF-α level in the liver. Several studies reported that inflammatory cytokines like TNF-α and IL-1β in the liver induce cell apoptosis via activation of pro-apoptotic proteins in NASH model mice (Zhang et al., 2010; Miura et al., 2010; Kudo et al., 2009). Furthermore, exercise training decreased collagen deposition along with reduction of the TGF-β and TIMP1 mRNA expression in the liver, and attenuated activated hepatic stellate cells in the present study. Tomita et al. (2006) reported that TNF-α induces up-regulation of TGF-β and TIMP1 via activation of hepatic stellate cells, which enhances collagen accumulation. Therefore, exercise training may suppress TNF-α, which is an important factor for NASH due to HFF-induced obesity.

Interestingly, it has been shown that Kupffer cells are associated with NASH development (Zhang et al., 2010). Recent studies showed enhanced infiltration of $CD11b^+$ and $F4/80^+$ macrophages in HFF diet-induced obese mice (Kohli et al., 2010; Karlmark et al., 2009). Kupffer cells produce inflammatory cytokines such as TNF-α, which induce TGF-β and TIMP-1 production in activated hepatic stellate cells (Tomita et al., 2006). Imamura et al. (2005) suggested that reducing macrophage infiltration into the liver suppresses activation of hepatic stellate cells and hepatic fibrosis. Therefore, the induction of TNF-α production by hepatic resident macrophages may play a key role in the pathogenesis of hepatic inflammation and fibrosis. Indeed, this study showed that the alteration in F4/80 positive cells in the liver was similar to the altered pattern of hepatic TNF-α and collagen 1α mRNA levels in the liver. Therefore, suppression of macrophage infiltration may be associated with an improvement of hepatic inflammation and fibrosis in obese mice. Furthermore, exercise training both decreased TNF-α levels and F4/80 positive cells in the liver and decreased the $CD11b^+$ and $F4/80^+$ cell population in the hepatic mononuclear cells of obese mice (data not shown). Therefore, the ability of exercise to reduce TNF-α and hepatic fibrosis might be caused by depression of macrophage infiltration.

Macrophage activation has been operationally classified into two separate polarization states: M1 and M2 (Gordon and Taylor, 2005). M1 macrophages strongly express TLR4, which produces inflammatory cytokines such as TNF-α. In contrast, M2 macrophages produce IL-10, which suppresses inflammation and oxidative stress. Interestingly, mice with high-fat diet-induced obesity show a phenotypic switching from M2 macrophages to M1 macrophages in adipose tissue, resulting in an induced inflammatory state (Lumeng et al., 2007). Interestingly, we previously demonstrated that exercise training reduced the expression of inflammatory cytokines and M1 macrophage markers in the adipose tissue of obese mice (Kawanishi et al., 2010). Accordingly, it is possible that exercise training attenuates inflammation in the liver by both suppressing macrophage infiltration and accelerating phenotypic switching from M1 to M2 macrophages in obese mice. M1 macrophages strongly express CCR2, which modulates macrophage infiltration to the several tissues (Murray and Wynn, 2011). In various liver disease models, CCR2 deficient mice exhibited reduced macrophage infiltration and fibrosis compared to wild-type mice. (Seki et al., 2009; Dambach et al., 2002). Furthermore, Tamura et al. (2010) reported that high expression of TNF-α mRNA after the administration of a high fat and sucrose diet was attenuated by CCR2 inhibitor treatment in obese mice. Obesity-induced M1 macrophage infiltration into the liver is an important factor for TNF-α production and hepatic fibrosis. Exercise training attenuated both mRNA expression and cell surface expression of CD11c, which is a marker of M1 macrophages, and did not change CD206 mRNA expression, which is a marker of M2 macrophages in the liver (data not shown).

A recent study proposed that TLR4 plays a pathogenic role in NASH, because TLR4-knockout mice showed both lower hepatic inflammation and fibrosis than wild-type mice (Seki et al., 2007). In this study, we observed that exercise training reduced the mRNA expression of both TLR4 and hepatic TNF-α. Therefore, the exercise training-induced reduction of TNF-α in the liver might be regulated by the down-regulation of TLR4. Collectively, our findings indicate that exercise training suppresses the infiltration of inflammatory

macrophages in obese mice and that the reduction in hepatic inflammation, fibrosis, and injury by exercise training is associated with suppression of inflammatory macrophage infiltration.

The factors affecting the inhibition of macrophage infiltration into the liver by exercise training are presently unknown. A recent study reported that cytotoxic T cells produce RANTES, which modulates the activation and infiltration of macrophages in adipose tissue, and CD8a deficient mice also show lower number of infiltrating inflammatory macrophages than control mice following administration of a high-fat diet (Nishimura et al., 2009). On the other hand, helper T cells produce cytokines such as IL-4 and IL-10, which suppress macrophage infiltration (Odegaard et al., 2007). In this study, exercise training and HFF did not change the population of cytotoxic T ($CD3^+$ $CD8^+$) cells and helper T ($CD3^+$ $CD4^+$) cells in the liver. Furthermore, exercise training and the HFF diet did not alter the gene expression of RANTES. Therefore, cytotoxic T cells and helper T cells might not be associated with suppression of macrophage infiltration. However, helper T cell activation has been operationally defined as several separate polarization states such as regulatory T and Th17 cells, which are associated with the induction of inflammation (Winer et al., 2009). Regulatory T cells have also been shown to inhibit macrophage activation (Tiemessen et al., 2007). In contrast, Th17 cells produce IL-17, which induces chemokines such as MCP-1 (Park et al., 2005). Therefore, it will be necessary to examine whether exercise training modulates Th17 and regulatory T cell populations in the near future.

Macrophage-specific chemokines such as MCP-1 and CXCL14 are associated with adipose tissue inflammation, and mice deficient in these chemokine did not exhibit macrophage infiltration despite their obesity (Kanda et al., 2006; Nara et al., 2007). Interestingly, Baeck et al. (2012) also reported that MCP-1 deficient mice exhibited reduced macrophage infiltration, and hepatic inflammation, injury compared to control mice following administration of a methionine and choline deficient diet. In the present study, exercise training reduced MCP-1 and CXCL14 mRNA levels. Therefore, exercise training-induced depression of macrophage infiltration results from the suppression of chemokine production such as MCP-1 and CXCL14.

A limitation of this study was that citrate synthase activity was not evaluated in relation to lipid metabolism. In addition, exercise intensity was not based on the aerobic capacity of the animal. However, this type of exercise training has been demonstrated to increase maximal oxygen uptake max and citrate synthase activity in skeletal muscle of mice (Röckl et al., 2007). Because of increased citrate synthase activity to promote lipolysis, it is considered that this increase might have effects on the development of fatty liver. However, Machado et al. (2012) have reported that citrate synthase activity in skeletal muscle does not correlate to fatty liver, hepatic inflammation and fibrosis. On the other hand, it is unclear whether citrate synthase activity in the liver is associated with the development of NASH. Therefore, future study is necessary to investigate the effect of exercise training on citrate synthase activity in the liver and skeletal muscle. Moreover, future study is necessary to investigate the relationship between aerobic capacity and improvement of inflammation and fibrosis.

Another limitation was that we did not examine the reproducibility of plasma ALT activity, hepatic TNF-α and MCP-1 levels. In this study, the liver was used in the analysis for a variety of histological, flow cytometric and PCR analyses, etc., and the amount of liver that could be used for protein extraction was small. In terms of hepatic MCP-1 and TNF levels observed in this study, it was necessary to measure in multiple.

In conclusion, we have demonstrated that exercise training markedly reduces the hepatic TNF-α level and hepatic fibrosis markers in the liver. Furthermore, exercise training attenuates F4/80-positive cells and chemokine expression in the liver. Taken together, our results suggest that the development of hepatic inflammation, fibrosis and macrophage infiltration caused by ingestion of high-fat diet and high-fructose water is attenuated by exercise training.

Information about the contributions of each author

Noriaki Kawanishi designed research, analyzed data (all analysis) or performed statistical analysis, wrote manuscript. Hiromi Yano analyzed data (histological analysis). Tsubasa Mizokami analyzed data (flow cytometry analysis and histological analysis). Masaki Takahashi analyzed data (real-time PCR analysis and histological analysis). Eri Oyanagi analyzed data (histological analysis). Katsuhiko Suzuki had primary responsibility for final content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2012.04.006.

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