**Supplementary Materials and Methods**

***Animal study***

NAFLD-prone male APOE\*3-Leiden.CETP mice were obtained as previously described.1,2 At 8–14 weeks of age, mice were group-housed in light-tight cabinets at 21°C under 12/12-h light-dark conditions. The cabinets were illuminated with white fluorescent light (200–250 lux). All training bouts and experiments took place under dim red light illumination during the active period of the mice. Mice were fed a NAFLD-inducing HFHC diet containing 60% fat and 1% cholesterol (Ssniff, Soest, Germany) *ad libitum*. After a 1 week of dietary habituation, animals were block-randomized into three groups by body weight, fat mass, lean mass (EchoMRI 100-Analyzer; EchoMRI, Houston, TX, USA), plasma triglycerides (TG), and TC. Group sizes were based on previous studies using this model. Early dark phase running (early runners, E-RUN, *n* = 18) took place 1 h after lights off at *Zeitgeber* time (ZT) 13–14; late dark phase running (late runners, L-RUN, *n* = 18) 1 h before lights on at ZT 22-23. Sedentary mice (SED, *n* = 18) did not train. The sedentary control group was split up, *n* = 9 was housed in the same light cabinet as E-RUN and half *n* = 9 in the same light cabinet as L-RUN and are shown as E-SED and L-SED, respectively. Body weight was assessed weekly and body composition, unfasted TG, and TC again after 8 weeks. All animals were killed at ZT17 17–26 h after the last exercise bout via CO2 inhalation and perfused with ice-cold PBS for before tissues were collected. The sacrifice timepoint was chosen to allow for comparisons between all four groups and to reduce the confounding effect of the acute exercise. All animal experiments were performed in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the National Committee for Animal experiments by the Ethics Committee on Animal Care and Experimentation of the Leiden University Medical Center.

***Exercise training***

Mice were trained on a rat treadmill with five lanes (MazeEngineers, Skokie, IL, USA), allowing 3–4 cage mates to run on one lane together. No electric shocks were used, instead mice were gently nudged when they stopped running. After 3 days of treadmill acclimatization with increasing speed and running duration the mice were trained five times per week for 1 h (15 min warm-up at 6–15 m/min, 15 m at 15 m/min and 30 min at 17 m/min; 899 m per bout per mouse) for a total of 8 weeks. To account for experimental stress the training mice experienced, all SED mice were moved in groups into empty cages without bedding for the duration of the running bout.

***Plasma and liver lipid assays***

Plasma TG and TC were measured after the dietary habituation directly before the start of the training and after 8 weeks. Liver lipids were extracted from snap-frozen liver tissue using the Bligh and Dyer protocol.3 TG and TC concentrations where measured using the Total Triglyceride and Total Cholesterol assay kits (both Roche Diagnostics, Almere, The Netherlands) as described previously,4 and PL was measured using a phospholipid reagent kit (Instruchemie, Delfzijl, The Netherlands).

***Plasma ALT assay***

Plasma alanine aminotransferase (ALT) levels were measured in the samples collected at sacrifice using the Mouse ALT ELISA Kit ab282882 (Abcam, Cambridge, UK).

***Liver and blood immune cell isolation and flow cytometry***

Liver tissue was collected in ice-cold RPMI 1640+Glutamax (Thermo Fisher Scientific, Waltham, MA, USA), minced and digested for 45 min at 37°C using collagenase type IV from *Clostridium histolyticum* (1 mg/mL; Sigma-Aldrich, St. Louis, MO, USA), 2000 U/mL DNase (Sigma-Aldrich) and 1 mM CaCl2 as previously described.5 The digested tissues were passed through 100 µm cell strainers and washed with phosphate buffered saline with 0.5% bovine serum albumen and 2 mM ethylenediaminetetraacetic acid (PBS/BSA/EDTA). The samples were spun down, washed again, and the hepatocytes were pelleted. The leukocytes in the supernatant were pelleted and treated with erythrocyte lysis buffer (0.15 M NH4Cl; 1 mM KHCO3; 0.1 mM Na2EDTA). After washing with PBS/BSA/EDTA, the leukocytes were isolated with magnetic-activated cell sorting using LS columns and CD45 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Blood leukocytes were isolated in parallel from cardiac puncture blood. Isolated CD45+ cells were counted and stained with Zombie NIR (Biolegend, San Diego, CA, USA) followed by fixation with 1.9% paraformaldehyde (Sigma-Aldrich) after which the fixed leukocytes were further processed for flow cytometry. For this, the isolated CD45+ cells were incubated with a cocktail of antibodies:

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| --- | --- | --- | --- | --- | --- |
| **Target**  | **Clone** | **Conjugate** | **Source** | **Catalog no.** | **RRID** |
| CD3 | 17A2 | APC/Fire-810 | Biolegend | 100267 | AB\_2876392 |
| CD11b | M1/70 | PE-Cy7 | eBioscience | 25-0112-82 | AB\_469588 |
| CD19 | 1D3 | BV480 | BD Biosciences | 566107 | AB\_2739509 |
| CD45 | 30-F11 | BV785 | Biolegend | 103149 | AB\_2564590 |
| Ly6C | HK1.4 | APC-Cy7 | Biolegend | 128025 | AB\_10643867 |
| Ly6G | 1A8 | BV650 | Biolegend | 127641 | AB\_2565881 |
| NK1.1 | PK136 | PerCP-Cy5.5 | Biolegend | 108727 | AB\_2132706 |
| Siglec-F | E50-2440 | PE | BD Biosciences | 552126 | AB\_394341 |
| **Other reagents** |  |  |  |  |  |
| True-Stain monocyte blocker | Biolegend | 426103 | n.a. |
| Brilliant stain buffer plus | BD Biosciences | 566385 | n.a. |
|  |  |  |  |

The stained samples were measured by spectral flow cytometry using a 3-laser Cytek Aurora spectral flow cytometer (Cytek Biosciences, Fremont, CA, USA). Spectral unmixing of the flow cytometry data was performed using SpectroFlo v3.0 (Cytek Biosciences). Gating of flow cytometry data was performed using FlowJoTM v10.8 Software (BD Biosciences, Franklin Lakes, NJ, USA) as shown in **Supplementary Figure 2**.

***Histological analysis***

Fresh liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin and 5 µm thick sections were prepared for hematoxylin and eosin staining. The NAFLD activity score was determined using an established general NAFLD scoring system for rodent models, where microsteatosis, macrosteatosis, and hypertrophy are all evaluated, with scores from 0 (less than 5% of the tissue) to 3 (more than 66% of the tissue), with a maximum total NAFLD score of 9.6

***Gene expression analysis***

RNA was isolated from isolated immune cells as well as freshly frozen liver tissue using TRIzol (Thermo Fisher Scientific). Following reverse transcription with M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), qRT-PCR was performed using SYBR Green (Promega). The primers used were:

|  |  |  |
| --- | --- | --- |
| **Gene** | **Forward primer (5' – 3')** | **Reverse primer (5' – 3')** |
| *Adgre1* | CTTTGGCTATGGGCTTCCAGTC | GCAAGGAGGACAGAGTTTATCGTG |
| *Il1b* | GCAACTGTTCCTGAACTCAACT | ATCTTTTGGGGTCCGTCAACT |
| *Rplp0* | GGACCCGAGAAGACCTCCTT | GCACATCACTCAGAATTTCAATGG |
| *Srebf1* | AGCCGTGGTGAGAAGCGCAC | ACACCAGGTCCTTCAGTGATTTGCT |
| *Tnf* | AGCCCACGTCGTAGCAAACCAC | TCGGGGCAGCCTTGTCCCTT |

Expression of genes of interest was normalized to expression of the housekeeping gene *Rplp0* and is shown relative to E-SED.

***Statistical analyses***

Results were reported as means ± standard error of the mean. The statistical analysis was performed using GraphPad Prism 9.01 (GraphPad, La Jolla, CA, USA) and one-way or two-way analysis of variance followed by Tukey's multiple comparisons test, where appropriate. In case of missing values, mixed-effects analyses were performed instead. Statistical outliers were removed after identification by Grubb’s test. Differences between groups were considered statistically significant if *p* < 0.05.

**References**

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