Role of gut-liver axis in circadian exercise and dietary interventions to improve metabolic health

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Chapter 3

Early but not late exercise training in mice exacerbates hepatic inflammation in developing non-alcoholic fatty liver disease

Early but not late exercise training in mice exacerbates hepatic inflammation in developing non-alcoholic fatty liver disease

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Article type: Research letter

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Author's contribution: Study concept and design (AK, ZY, PCNR, MS), acquisition of data (AK, ZY, JML, HJPZ, MS), analysis and interpretation of data (AK, ZY, JML, HJPZ, BG, PCNR, MS), drafting of the manuscript (AK, ZY, MS), critical revision of the manuscript for important intellectual content (JML, BG, PCNR), and study supervision (BG, PCNR, MS). All authors have made a significant contribution to this study and have approved the final manuscript.

Acknowledgements: We thank Trea Streefland and Reshma Lalai (Div. of Endocrinology, Dept. of Medicine, LUMC, Leiden, the Netherlands) for their excellent technical assistance. We furthermore thank Lars Hoeve, Sjahnaaz Bholai and Jack Brouwer for their technical contributions. This study was financed by a grant from the Novo Nordisk Foundation to M.S. (NNF18OC0032394). Z.Y. was supported by a full-time PhD scholarship from the China Scholarship Council.

Over two billion people worldwide are estimated to have non-alcoholic fatty liver disease (NAFLD), defined by excess hepatic fat. Commonly, progression into non-alcoholic steatohepatitis (NASH) is characterized by the onset of liver inflammation following exacerbated steatosis [1]. This suggests that reducing liver inflammation – e.g. through lifestyle interventions involving exercise training – may come secondary to a reduction of steatosis. However, both the metabolic and inflammatory processes involved in NAFLD are under circadian control and could respond differently to exercise at different times of day [2]. To investigate the time-of-day dependent effect of exercise training on NAFLD amelioration in the early disease stages we trained high-fat high-cholesterol (HFHC)-fed APOE*3-Leiden.CETP mice during their early or late active period. This mouse model was chosen due to its humanized lipid metabolism and its ability to develop all hallmarks of human NAFLD upon HFHC feeding [3]. The animals were treadmill trained five times per week for eight weeks at either *Zeitgeber* time (ZT)13 (E-RUN) or ZT22 (L-RUN). Corresponding sedentary animals (E-SED and L-SED) were put into empty cages at the same time to control for experiment-induced stress. After eight weeks, all mice were killed at the same circadian time (ZT17) on the day after the last exercise training bout to allow for comparisons between all four groups and to reduce the confounding effect of the acute exercise.

At the end of the study, all mice had a similar body weight (Fig. 1A) and lean body mass (Fig. S1A), but trained mice had gained less fat mass than sedentary mice (Fig. 1B), indicating a measurable exercise effect. Fasting plasma glucose, that when elevated is independently positively associated with the risk to develop NAFLD [4], was unchanged among the groups (Fig. 1C). Furthermore, no differences in hepatic steatosis, NAFLD activity score, plasma ALT levels, portal inflammation and liver weight were observed between the groups (Fig. 1D-F and Fig. S1D-E), likely due to an overall limited potential to improve these parameters in early stages of steatosis without signs of NASH. Accordingly, liver lipid levels (triglycerides (TG), total cholesterol (TC), and phospholipids (PL)) (Fig.1G-I) as well as plasma levels of TG and TC (S1B-C) remained unchanged between the exercising and sedentary groups regardless of the time of training.

Surprisingly, exercise training had a time-of-day specific impact on liver inflammation, challenging the notion that hepatic inflammation merely follows the level of steatosis. In livers collected at the same circadian timepoint one day after the last training, flow cytometry analyses of isolated hepatic immune cells revealed

an unexpected hepatic increase in the total number of leukocytes, neutrophils and monocytes in response to early training that did not reflect increased blood immunocyte levels (Fig. 2A-C and Fig. S2C-F). Notably, blood leukocytes were even signifanctly decreased in E-RUN compared ot E-SED at the same time liver leukocytes were elevated (Fig. S2C), consistent with migration of these cells to the liver. Late training, however, had no effect on these immune cell populations in blood or liver. This increase of specific cell populations following early training may indicate disease acceleration, as infiltrating neutrophils are associated with NAFLD development and disease progression [5, 6]. In line, infiltrating monocytes, which are recruited to the liver partly through hepatocyte-derived stress signals (such as IL-1β and TNFα), differentiate into pro-inflammatory macrophages that contribute to tissue damage and the loss of resident macrophages [7]. Interestingly, early training also increased the number of natural killer (NK) cells in the liver (Fig. 2D). Although the contribution of these cells to NAFLD development and progression to NASH remains controversial, they can produce large quantities of pro-inflammatory cytokines such as IFNγ [8]. Taken together, early training leads to an inflammatory response in the liver characterized by an increase of pro-inflammatory and tissue damage-associated cell populations.

An increase in hepatic inflammation following early training was also confirmed by gene expression analyses in isolated hepatic immune cells. The gene expression of the pro-inflammatory secreted factors IL-1β (*Il1b*) and Tnf-α (*Tnf*) was increased after early training but not after late training (Fig. 2E-F). Similarly, the expression of the macrophage marker F4/80 (*Adgre1*) tended to be increased following early training (Fig. 2G). In line, early training also increased the expression of *Tnf, Il1b* and *Adgre1* in whole liver tissue (Fig. S1D-F).

It is unclear whether the observed increase of liver inflammation with early training is beneficial or detrimental in NAFLD development. As the number of circulating immune cells as well as their activity exhibits a circadian rhythm in mice and humans, exercise in the early active phase may stimulate cell migration into the liver at the time these cells are most prone to migrate into peripheral organs [9]. Consequently, one could speculate that by stimulating liver inflammation in developing steatosis, early training activates a rapid alert system that supports disease resolution. Conversely, it has been shown that early exercise can acutely worsen metabolic diseases as seen in people with obesity and type 2 diabetes where early high intensity cycling elicited unfavorable blood glucose spikes that did

not occur with late exercise [10]. Accordingly, our findings could indicate that early training accelerates disease progression while late exercise training potentially targets liver steatosis and inflammation at a later disease stage. However, while not affecting liver lipid levels, the hepatic gene expression of Srebp1c (*Srebf1*), the mediator of insulin-induced fatty acid synthesis, was similary downregulated with early and late training (Fig. S1G), suggesting that the regulation of metabolic and inflammatory disease drivers may not be synchronized. Future studies need to assess the translatability of our findings to advanced disease stages and to human NAFLD. Notably, we observe distinct inflammatory modulation already at an early disease stage with a low NAFLD score, low grade hepatic steatosis and before the disease becomes inflammation-driven. This may present a previously underappreciated inflammation-targeted treatment opportunity in a large part of the population at risk for NASH.

In summary, we demonstrate that early and late exercise training in a mouse model of NAFLD differently influenced liver inflammation in developing steatosis. Here, an unexpected increase in liver inflammation was observed with early exercise training.

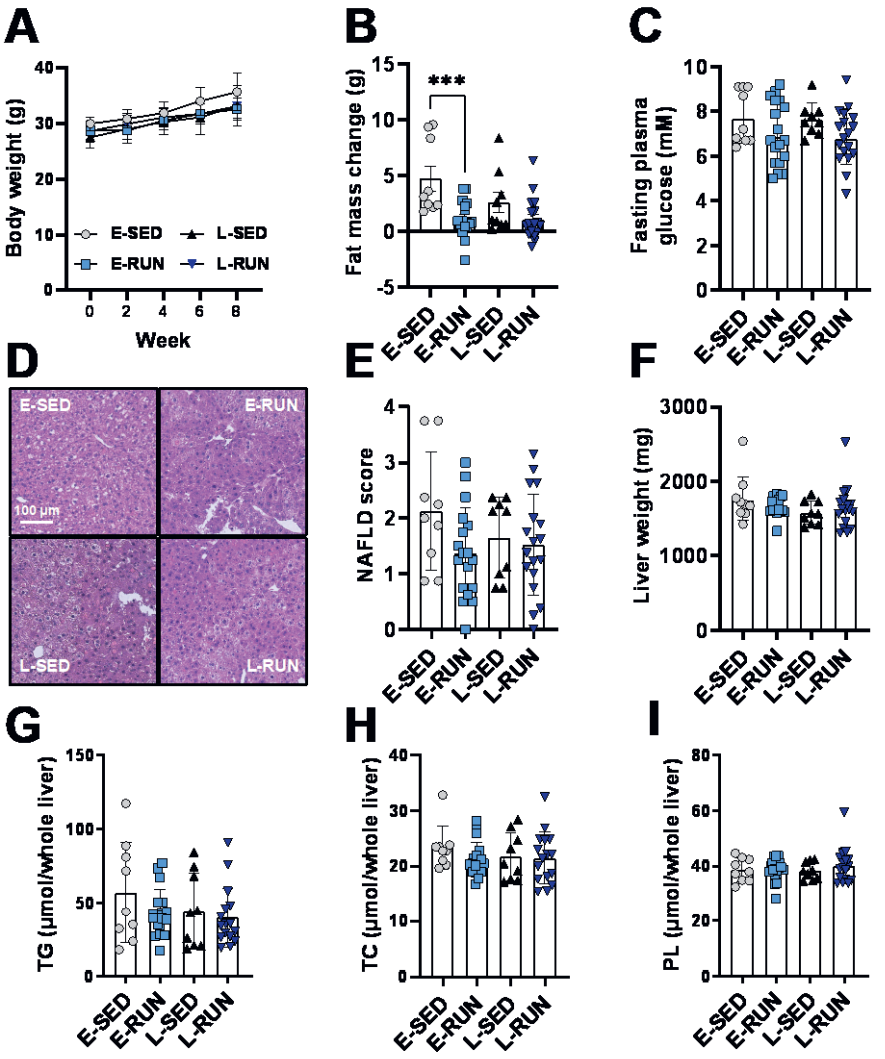

Figure 1. Early and late exercise training both tend to improve body composition but not developing liver steatosis in APOE*3-Leiden.CETP mice. Over 8 weeks of training, body weight (A) and changes of fat mass (B) were monitored and fasting plasma glucose was measured after 8 weeks (C). Representative liver images are shown (D) that were used to assess the NAFLD score (E). Liver weight (F), liver triglyceride (G), total cholesterol (H), and phospholipid (PL) content (I) were assessed. ***P<0.001 in one-way ANOVA, n=9-18.

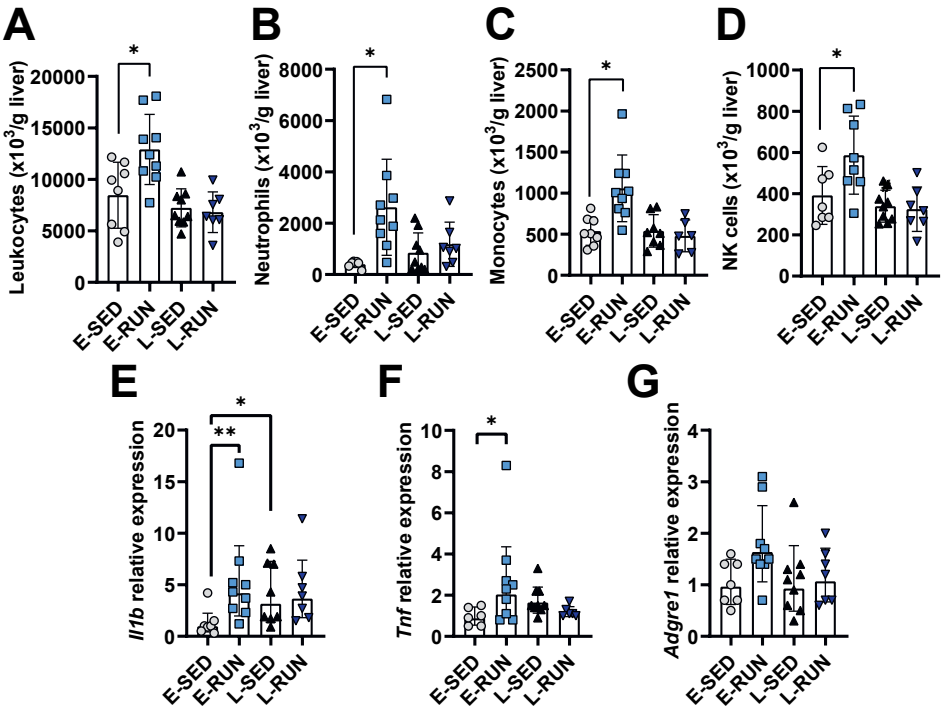

Figure 2. Early exercise promotes distinct changes to liver immune cell populations and inflammatory markers in developing steatosis. The total number of liver leukocytes (A), neutrophils (B), monocytes (C) and NK cells (D) was determined after 8 weeks of treadmill training. Gene expression of *Il1b* (E), *Tnf* (F) and *Adgre1* (G) was assessed in isolated liver immune cells and shown relative to E-SED. *P<0.05, **P<0.01 in one-way ANOVA, n=7-9.

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Conflict of interest

The authors have no conflict of interest.

Supplementary information:

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-hour 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 high fat high cholesterol (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 based on body weight, fat mass, lean mass (EchoMRI 100-Analyzer; EchoMRI, Houston, Texas), plasma triglycerides (TG), and total plasma cholesterol (TC). Group sizes were based on previous studies with this model. Early dark phase running ("Early runners", E-RUN, n=18) took place one hour after lights off at *Zeitgeber* time (ZT) 13-14; late dark phase running ("Late runners", L-RUN, n=18) one hour 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 hours after the last exercise bout via CO_2 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, Illinois), 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 three days of treadmill acclimatization with increasing speed and running duration the mice were trained five times per week for one hour (15 min warm-up at 6-15 m/min, 15 min at 15 m/min and 30 min at 17 m/min; 899 m per bout per mouse) for a total of eight 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 measurements

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 the Phospholipid Reagent kit (Instruchemie, Delfzijl, The Netherlands).

Plasma ALT measurement

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

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, St. Louis, MO, USA) and 1 mM $CaCl_2$ as previously described [5]. The digested tissues were passed through 100 µm cell strainers and washed with PBS with 0.5% BSA and 2 mM EDTA (PBS/BSA/EDTA). The samples were spun down, washed

again and the hepatocytes pelleted. The leukocytes in the supernatant were pelleted and treated with erythrocyte lysis buffer (0.15 M NH_4Cl; 1 mM $KHCO_3$; 0.1 mM Na_2EDTA). After washing with PBS/BSA/EDTA, the leukocytes were isolated with magnetic-activated cell sorting (MACS) 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, St. Louis, MO, USA) after which the fixed leukocytes were further processed for flow cytometry. For this, the isolated CD45+ cells were incubated with a cocktail of antibodies:

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, Fremont, CA, USA). Gating of flow cytometry data was performed using FlowJoTM v10.8 Software (BD Biosciences, Franklin Lakes, NJ, USA) as shown in Figure S2.

Histological analyses

Fresh liver tissue was fixed in 4% paraformaldehyde, embedded in paraffin and sectioned into 5 µm thick sections for H&E 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 a score 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

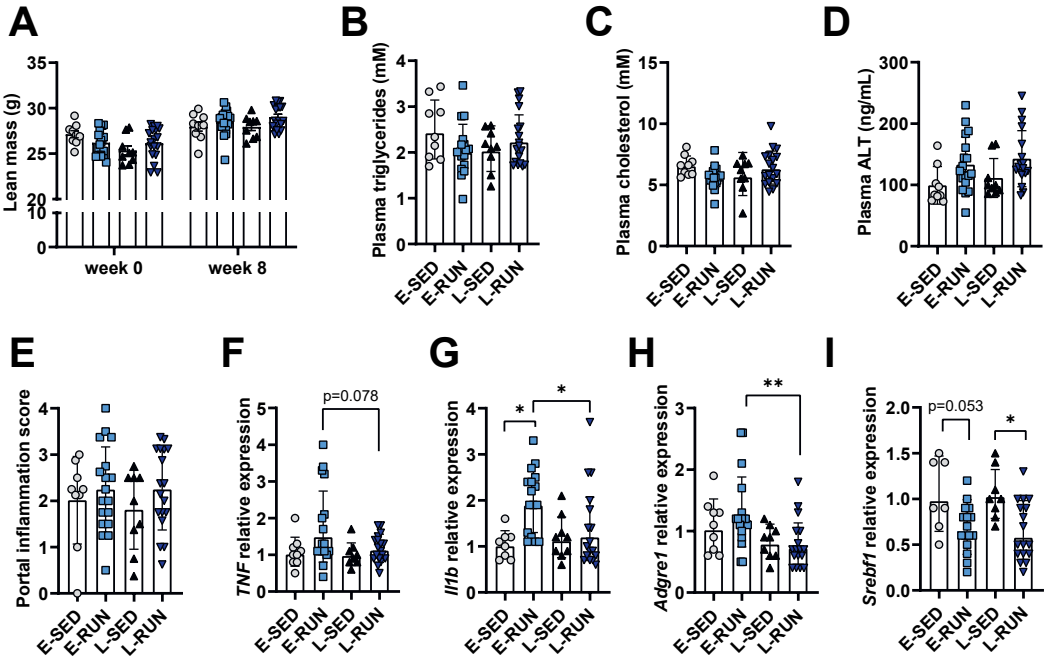
RNA was isolated from isolated immune cells as well as freshly frozen liver tissue using TRIzol (Thermo Fisher, Waltham, Massachusetts). Following reverse transcription with M-MLV Reverse Transcriptase (Promega, Madison, Wisconsin), qRT-PCR was performed using SYBR Green (Promega). The primers used were:

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

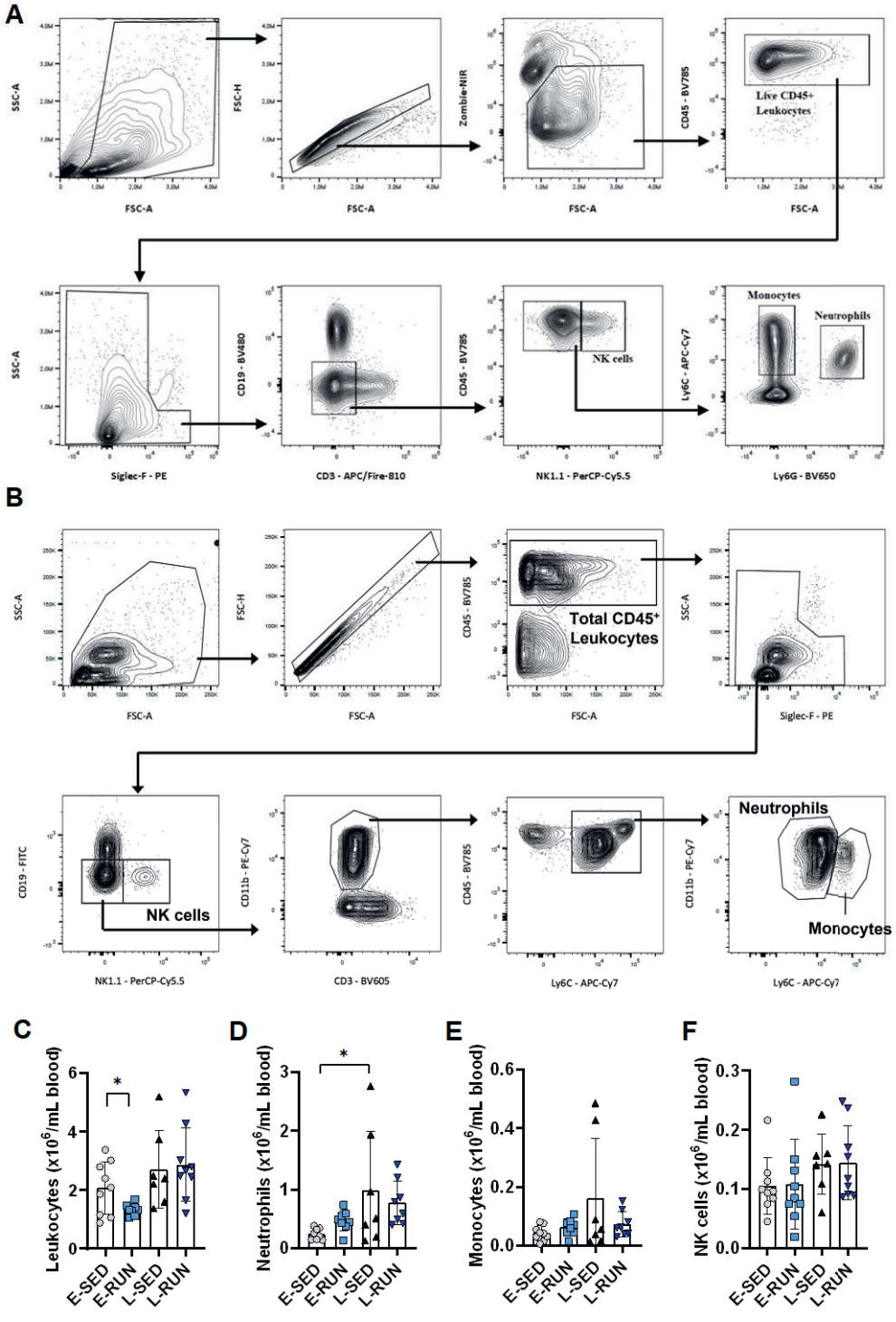
Expression of genes of interest was normalized to expression of the housekeeping gene *Rplp0* and is shown relative to E-SED.

Statistical analyses

All individual data points are shown or data are otherwise expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism 9.01 (GraphPad, La Jolla, California) and one-way or two-way ANOVAs 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 $P<0.05$.



Supplementary Figure 1. Lean mass changes, plasma lipids, plasma ALT levels, portal inflammation and liver gene expression after 8 weeks of early or late exercise training. Lean mass was measured before and after 8 weeks of exercise training (A). Plasma triglycerides (B), total plasma cholesterol (C), plasma alanine aminotransferase (ALT) levels (D), portal inflammation after Knodell et al. [7] (E) and liver gene expression of *Tnf* (D), *Il1b* (E), *Adgre1* (F) and *Srebf1* (G) were assessed after 8 weeks. Gene expression is shown relative to E-SED. *P<0.05, **P<0.01 in one-way ANOVA, n=9-18.


A
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FSC-A
Live CD45+
Leukocytes
SSC-A
Siglec-F - PE
CD19 - BV480
CD3 - APC/Fire-810
CD45 - BV785
NK1.1 - PerCP-Cy5.5
NK cells
Ly6C - APC-Cy7
Ly6G - BV650
Monocytes
Neutrophils
B
SSC-A
FSC-A
FSC-H
FSC-A
CD45 - BV785
FSC-A
Total CD45+
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CD19 - FITC
NK1.1 - PerCP-Cy5.5
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Supplementary Figure 2. Gating strategy for the analysis of liver and blood immune cells and circulating immune cell levels after 8 weeks of early or late exercise training. Isolated hepatic immune cells from livers collected at ZT17 were sorted using spectral flow cytometry and gated as shown (A). Blood immune cells isolated from heart puncture blood collected at the same time were sorted and gated as shown (B). Circulating leukocytes (C), neutrophils (D), monocytes (E) and NK cells (F) were quantified and are shown per mL blood. *P<0.05, in one-way ANOVA, n=7-9.

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