Interleukin-6 potentiates endurance training adaptation and improves functional capacity in old mice

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Abstract

Background   Interventions to preserve functional capacities at advanced age are becoming increasingly important. So far, exercise provides the only means to counteract age-related decrements in physical performance and muscle function. Unfortunately, the effectiveness of exercise interventions in elderly populations is hampered by reduced acceptance and compliance as well as disuse complications. We therefore studied whether application of interleukin-6 (IL-6), a pleiotropic myokine that is induced by skeletal muscle activity and exerts broad systemic effects in response to exercise, affects physical performance and muscle function alone or in combination with training in aged mice.
Methods   Sedentary old male mice (Sed+Saline, $n = 15$) were compared with animals that received recombinant IL-6 (rIL-6) in an exercise-mimicking pulsatile manner (Sed+IL-6, $n = 16$), were trained with a moderate-intensity, low-volume endurance exercise regimen (Ex+Saline, $n = 13$), or were exposed to a combination of these two interventions (Ex+IL-6, $n = 16$) for 12 weeks. Before and at the end of the intervention, mice underwent a battery of tests to quantify endurance performance, muscle contractility *in situ*, motor coordination, and gait and metabolic parameters.
Results   Mice exposed to enhanced levels of IL-6 during endurance exercise bouts showed superior improvements in endurance performance (33% more work and 12% greater peak power compared with baseline), fatigue resistance *in situ* ($P = 0.0014$ *vs.* Sed+Saline; $P = 0.0199$ *vs.* Sed+IL-6; and $P = 0.0342$ *vs.* Ex+Saline), motor coordination (rotarod performance, $P = 0.0428$), and gait (gait speed, $P = 0.0053$) following training. Pulsatile rIL-6 treatment in sedentary mice had only marginal effects on glucose tolerance and some gait parameters. No increase in adverse events or mortality related to rIL-6 treatment was observed.
Conclusions   Administration of rIL-6 paired with treadmill running bouts potentiates the adaptive response to a moderate-intensity low-volume endurance exercise regimen in old mice, while being safe and well tolerated.

Keywords   Ageing; Muscle; Exercise; Myokine; Interleukin-6; Frailty; Fatigue

Introduction

Besides neurodegenerative events, functional deterioration of skeletal muscle is one of the major causes for loss of independence, admission to nursing homes, morbidity, and mortality in the elderly. Because of the high costs associated with care of these patients, sarcopenia and frailty put an escalating burden on healthcare systems.[1] In light of an expanding global geriatric population,[2] effective interventions to preserve or improve physical performance and functional capacities at advanced ages are thus becoming increasingly important, on both the individual and societal levels.

Endurance exercise improves cardiorespiratory, neuromuscular, and metabolic function, as it requires successful integration of these systems.[3] Endurance training could thus provide a powerful countermeasure for muscular decline, loss of motor coordination and mobility, and metabolic deteriorations with ageing.[4,5] Unfortunately, however, several factors hamper the effectiveness of exercise-based interventions, in particular in the elderly. First, at older age, muscle may show an attenuated immediate[6] and/or an abbreviated[7] response to mechanical loading and thus require more intense and/or more frequent workouts to significantly adapt.[8] Second, aged muscle may be more vulnerable to exercise-induced injury compared with young muscle[9,10] and display decreased regenerative capacity,[11] which can limit exercise intensity, the amount of training, or the potential for improvement. Third, many elderly people have accumulated disuse complications and suffer from morbidity, frailty, and coordinative deficits,[12] which may not allow to manage or tolerate the strenuous or sustained training required to induce significant adaptations. Taken together, novel and effective strategies to leverage the beneficial effects of exercise training on physical performance and neuromuscular function in ageing are highly desirable as effective and safe pharmacological therapies are still lacking.[13]

Myokines, signalling molecules produced and secreted by skeletal muscle, may play a pivotal role in meditating the positive muscular and systemic effects of exercise training, by their autocrine, paracrine, and/or endocrine action.[14] Interleukin-6 (IL-6), the founder member of the myokine family, increases up to 100-fold in the circulation in response to exercise, leading to a systemic spike towards the end of or shortly after a single bout of exercise, while returning back to baseline quickly thereafter.[15] During exercise, IL-6 acts as metabolic coordinator in the inter-organ crosstalk, by promoting hepatic glucose production[16] and lipolysis in adipose tissue.[17–19] IL-6 further facilitates the uptake and catabolism of energy substrates (i.e. glucose and fatty acids) in muscle fibres and thereby improves muscle function during single endurance exercise bouts.[20]

Even though IL-6 is a strong pro-inflammatory cytokine important for an adequate immune response to infection, for example, by activating immune cells, mounting the acute response in the liver, or initiating the fever response in the hypothalamus,[21] potent systemic anti-inflammatory effects have been attributed to IL-6 when released as a myokine.[22] This dichotomy in IL-6 function results in differential therapeutic avenues for pathological contexts. Anti-IL-6 agents are tested or used in the treatment of diseases characterized by a persistent, sterile inflammation, such as type 2 diabetes, multiple sclerosis, atherosclerosis, or rheumatoid arthritis. In comparison, the application of recombinant IL-6 (rIL-6) to mirror the anti-inflammatory and hence potentially beneficial effects of IL-6 as a myokine is still in its infancy for therapeutic use. For example, rIL-6-based therapy has been proposed to

mitigate neuropathies associated with cancer chemotherapy[23] and diabetes.[24,25] Surprisingly, the use of rIL-6 treatment mimicking the myokine function on age-associated decrements in physical performance and muscle function, and the combination with exercise to facilitate training and achieve synergistic effects, has not been studied so far.

Given the impaired exercise training capacity and/or blunted adaptive response of aged muscle to exercise stimuli, coupled with the proposed role of IL-6 in exercise adaptation, the aims of the current study were to investigate whether (i) a moderate-intensity, low-volume 12 week endurance exercise regimen is sufficient to improve functional capacities in old mice, (ii) the effect of the same intervention can be potentiated by exposing the animals to elevated IL-6 levels during training sessions, and (iii) long-term, pulsatile rIL-6 treatment can elicit endurance training-like effects in old sedentary mice in a safe and tolerable manner.
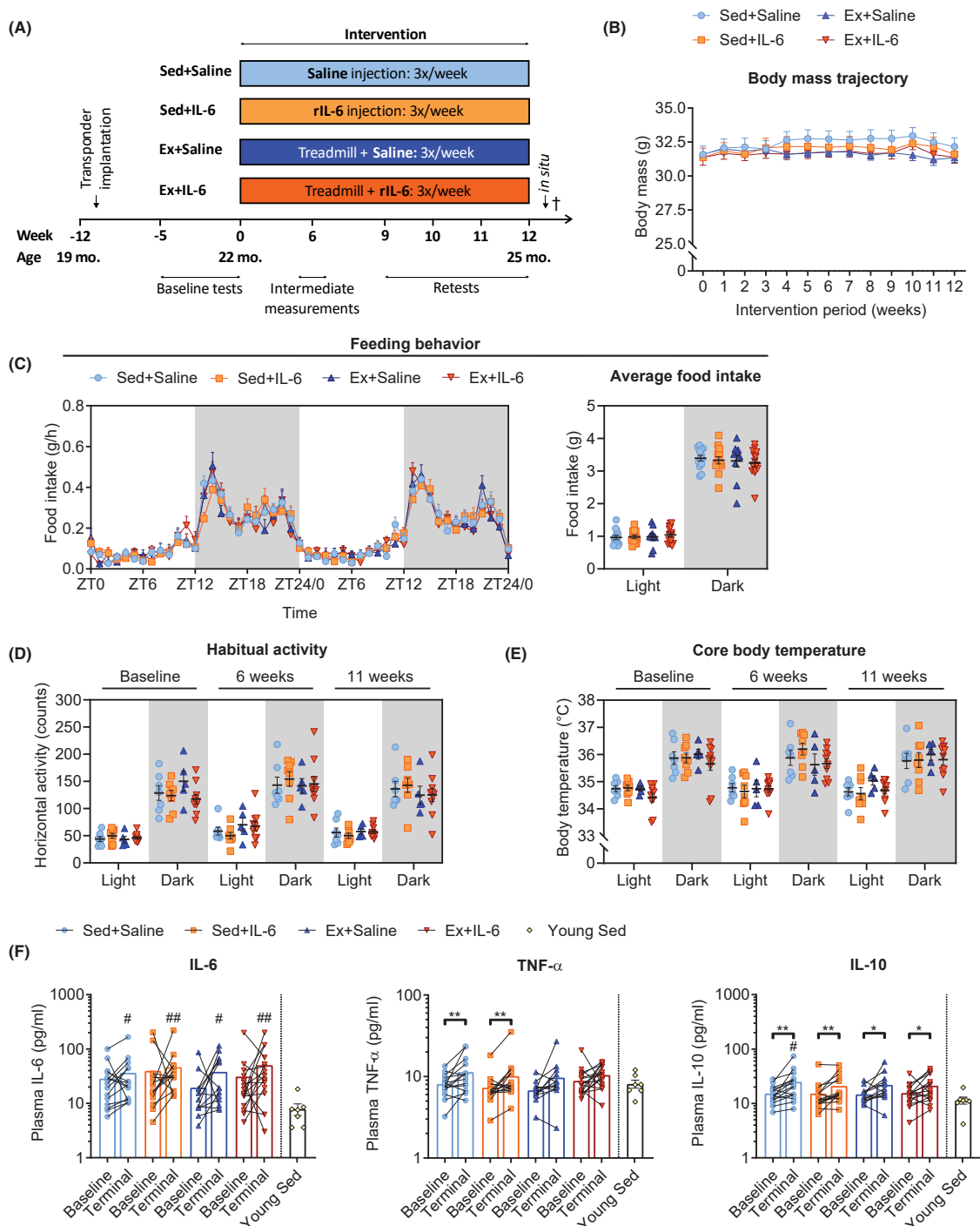
Methods

Animals

Aged and young male C57BL/6JRj mice were obtained from Janvier Labs (Le Genest-Saint-Isle, France) at an age of 19 and 5 months, respectively, and then kept under a 12 h light-to-dark cycle with light onset at 6 a.m. (Zeitgeber Time 0) at 23°C in the animal facility of the Biozentrum (Basel, Switzerland) until the end of the study. Mice were housed single caged with enrichment and received *ad libitum* access to regular chow and water. All experiments were approved by the veterinary office of the canton Basel-Stadt (Switzerland) and performed in accordance with the Swiss federal guidelines for animal experimentation under consideration of the well-being of the animals and the 3R (replace, reduce, and refine) principle.

Please see the Supporting Information for detailed experimental procedures.

Results

To study the adaptive response to endurance training in old mice and to test whether rIL-6 can act as a therapeutic agent to induce exercise-like effects or to potentiate training adaptation when combined with exercise bouts, we used aged male C57BL/6JRj mice as an experimental model. After a baseline characterization, mice were divided into four experimental groups at the age of 22 months (*Figure* 1A). Two groups were kept under sedentary conditions, one of which was subcutaneously injected with rIL-6 (10 μg/kg) three times per week for 12 weeks to mimic the transient increase of IL-6 plasma levels observed in response to single

Figure 1 Long-term rIL-6 treatment is safe and well tolerated. (A) Graphical illustration of the experimental approach of treadmill training and/or rIL-6 treatment. (B) Body mass trajectory for all four groups from start (0 weeks) to end (12 weeks) of the intervention (Sed+Saline, $n = 15$; Sed+IL-6, $n = 16$; Ex+Saline, $n = 13$; and Ex+IL-6, $n = 16$). (C) Feeding behaviour over 48 h (left panel; ZT, Zeitgeber time) and average food intake during light and dark phases (right panel) for this period assessed during Week 11 of the intervention (Sed+Saline, $n = 13$; Sed+IL-6, $n = 13$; Ex+Saline, $n = 10$; and Ex+IL-6, $n = 15$). (D) Habitual activity and (E) core body temperature during light and dark phases at baseline, in the 6th week and 11th week (Sed+Saline, $n = 7$; Sed+ IL-6, $n = 8$; Ex+Saline, $n = 5$; and Ex+IL-6, $n = 10$). (F) Plasma cytokine levels before (Baseline) and at the end (Terminal) of the study of interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and interleukin-10 (IL-10) (Sed+Saline, $n = 15$; Sed+IL-6, $n = 15$; Ex+Saline, $n = 13$; Ex+IL-6, $n = 16$; and young Sed, $n = 7$). (F) Y-axis has a logarithmic scale ($\log_{10}$). Data are presented as mean ± SEM (B, C) including individual values (right panel in C–E), mean, and individual paired values connected with a black line (F). (F) Paired Student's *t*-test and one-way ANOVA followed by Sidak's multiple comparisons of terminal measures of old groups and the young Sed group. *$P < 0.05$ and **$P < 0.01$. In (F): #$P < 0.05$ and ##$P < 0.01$ old groups *vs*. young Sed.

endurance exercise bouts (Sed+Saline, n = 15; Sed+IL-6, n = 16). Two additional groups were engaged in a moderate-intensity low-volume treadmill training regimen, in one group as a single intervention and in the other group combined with rIL-6 administration to enhance systemic IL-6 levels during and shortly after each exercise bout (Ex+Saline, n = 13; Ex+IL-6, n = 16). Maximum speed was 12 m/min in the first training session, which then was gradually increased every following session until 19 m/min (reached in the fifth week). Each training session lasted 45–50 min until all mice covered the same total distance.

Long-term recombinant interleukin-6 treatment alone or in combination with exercise training is safe and well tolerated

Epidemiological evidence suggests a link between IL-6 and age-related diseases associated with chronic systemic low-grade inflammation,[26] while there is a general lack of data regarding the safety of long-term recurrent rIL-6 administration. We therefore monitored general behaviour and well-being and assessed metabolic and inflammatory profiles under habitual non-exercise conditions. All four groups showed similar body mass trajectories throughout the study (Figure 1B). Comprehensive analysis of feeding behaviour (Figure 1C), habitual activity (Figure 1D), and several metabolic parameters [rate of oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$), respiratory exchange ratio (calculated as $\dot{V}CO_2/\dot{V}O_2$), and heat production; Figure S1A–S1D] revealed no significant differences between groups. In line with these observations, terminal plasma analyses of a suite of markers of blood biochemistry revealed no obvious metabolic changes or adverse effects of long-term rIL-6 treatment (Figure S2A). ALT and AST, two common markers to assess liver toxicity, were either unchanged or, in the case of ALT, even reduced in the Ex+IL-6 group. Moreover, LDH was lowered by rIL-6 and/or training, indicating reduced cellular damage in these mice.

Because IL-6 has been implicated in fever generation upon peripheral immune challenge and inflammation,[27] we implanted small transponders into the abdominal cavity of mice to tightly monitor core body temperature throughout the study. Animals treated with rIL-6 did not display higher body temperatures, suggesting that repeated rIL-6 injections did not elicit persistent pyrogenic effects (Figure 1E). To gain further insights into whether rIL-6 treatment and/or exercise affected the inflammatory status of the animals, we measured cytokines in the plasma at baseline and after 12 weeks of treatment. Resting IL-6 levels remained constant in all four groups during the intervention and thus were not affected by regular rIL-6 administration (Figure 1F). While interferon-γ and IL-1β levels also remained unchanged (Figure S2B), TNF-α plasma concentrations increased significantly during
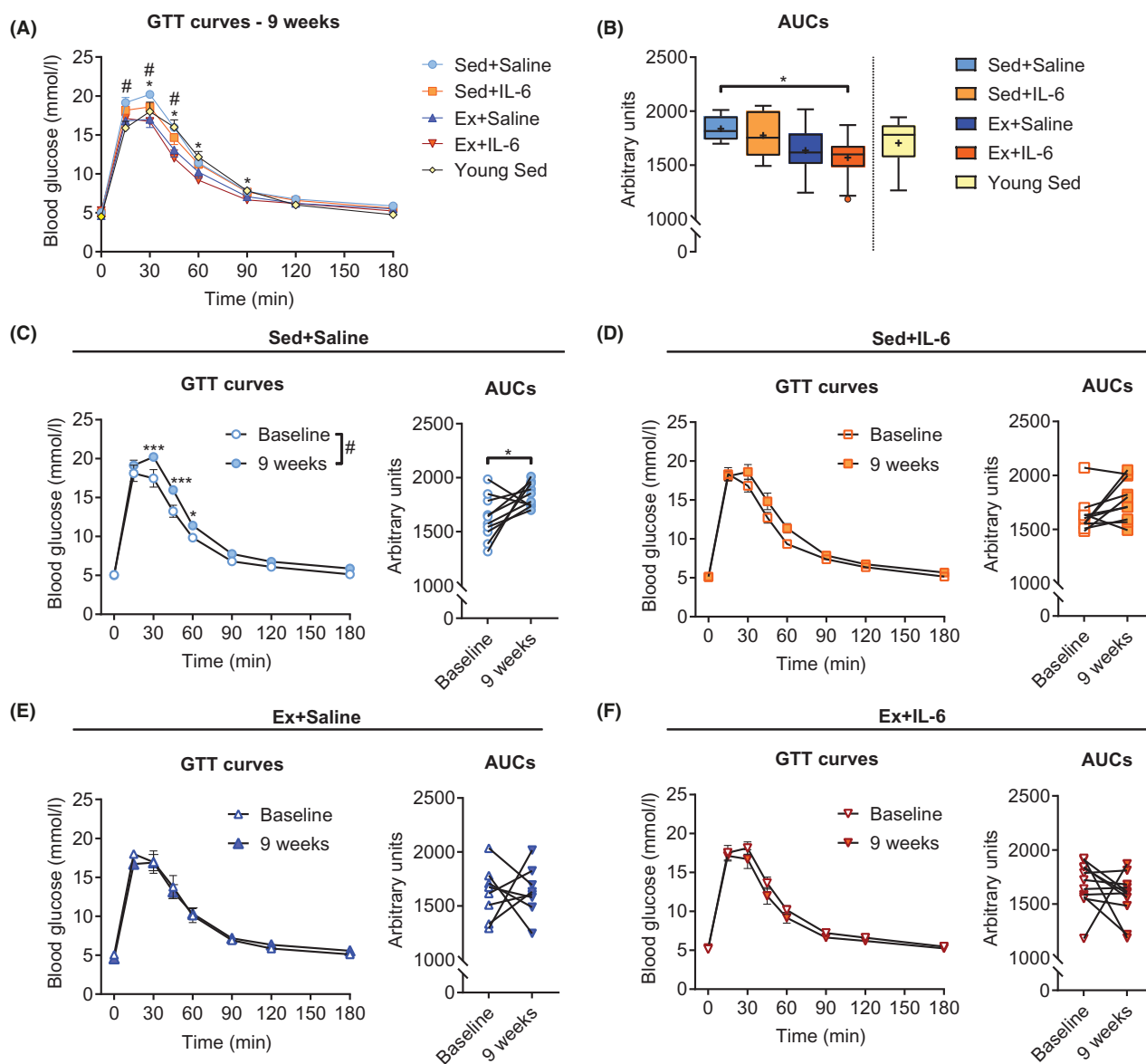
the intervention, but only in the sedentary groups, whereas IL-10 increased in all groups (Figure 1F). Comparison of terminal measures of the old groups with the values of an independent group of young sedentary mice revealed elevated levels of IL-6 and IL-1β in all old mice and IL-10 in Sed+Saline mice (Figures 1F and S2B). Finally, we did not observe an increase in adverse events or mortality related to rIL-6 treatment (Table S1). On the contrary, only one mouse in the rIL-6-treated groups (sedentary and exercised) died during the intervention, while seven animals died in the saline-treated groups (two in the sedentary and five in the exercise group).

Elevated IL-6 concentrations in response to exercise, as well as acute and short-term rIL-6 treatment, increased insulin secretion and glucose tolerance in young mice.[28] In the current study, exercise training resulted in significantly lower retest (in the ninth week of treatment) glucose tolerance test (GTT) curves compared with the Sed+Saline group, while rIL-6 treatment alone failed to do so (Figure 2A). The corresponding area under the curves (AUCs) were not different between the two exercise groups; however, only the AUC of IL-6 treated mice was significantly smaller compared with Sed+Saline mice (Figure 2B). Moreover, change from baseline AUC was negative only in the Ex+IL-6 group (Figure S3A). Figure 2C–2F shows the GTT curve and corresponding AUC comparisons between the baseline measurements (−2 weeks) and the retests for the individual groups, in which only the Sed+Saline group showed a significant decrease in glucose tolerance.

Body composition analysis revealed significantly reduced fat mass at 12 weeks compared with baseline in the two exercise groups (Figure S4A), whereas lean mass increased in all groups (Figure S4B), except for the Ex+IL-6 group. This resulted in a significant overall reduction of body fat percentage after 12 weeks in the two exercise groups as well as in the Sed+IL-6, but not the Sed+Saline group (Figure S4C). Interestingly, the Ex+IL-6 group had significantly less epididymal white adipose tissue (eWAT, a visceral fat depot in mice) compared with the Sed+Saline group, while no difference in subcutaneous white adipose tissue (sWAT) was detected at the end of the study (Figure S4D). In accordance with the lean body mass measurements, no differences in individual limb muscle masses were observed at the end of the study (Figure S4E).

Elevated levels of interleukin-6 during endurance training bouts improve treadmill running capacity and muscle fatigue resistance in situ

Endurance exercise capacity and its physiological determinants, such as peak oxygen uptake ($\dot{V}O_{2peak}$), are strong independent predictors of mortality[29] and decrease with age.[30] We therefore subjected mice to a short and intense ramp-sprint protocol (Figure S5A) to determine $\dot{V}O_{2peak}$ at baseline (−3 weeks) and in the 10th week of the intervention.
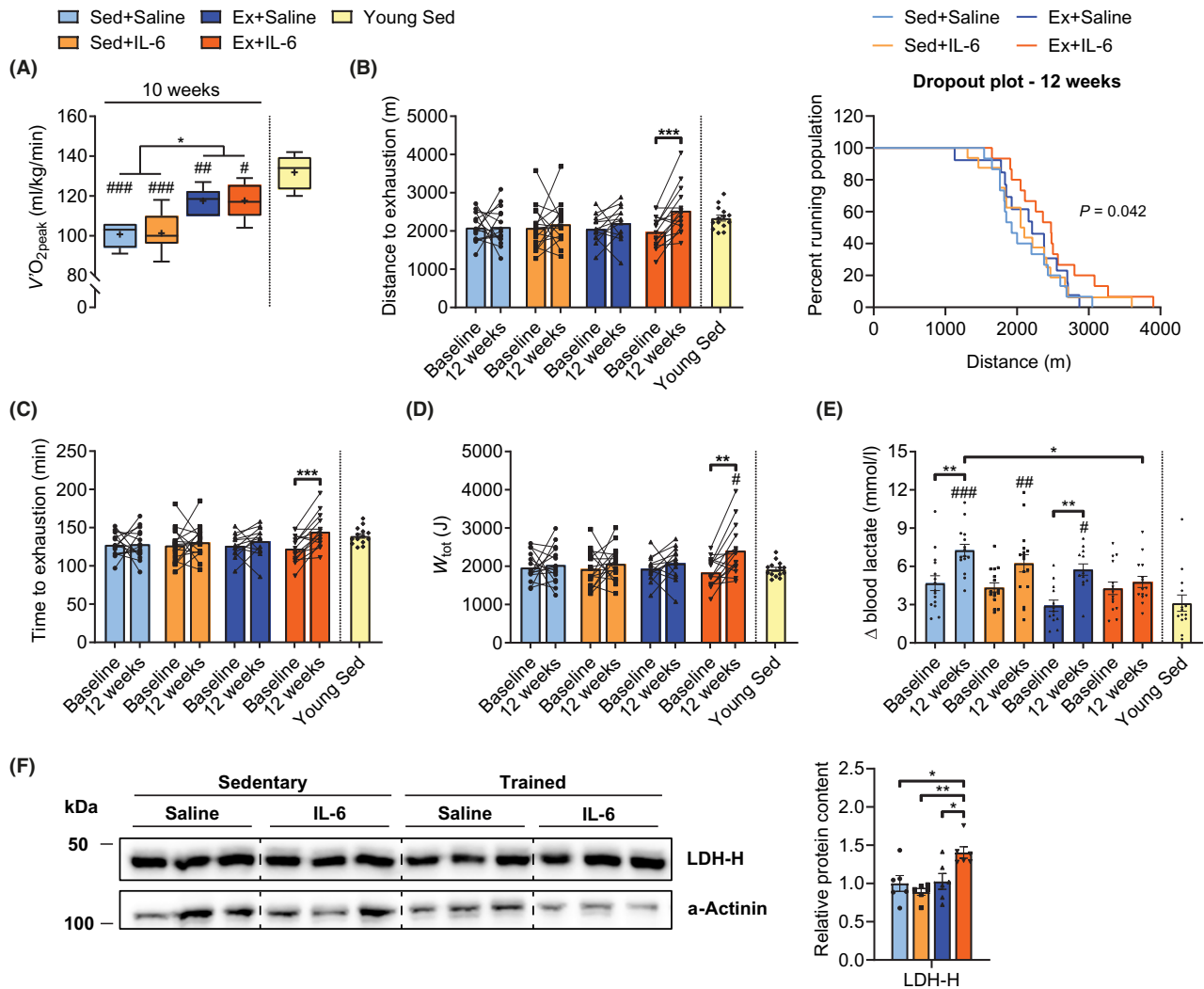
Figure 2 Effects of moderate-intensity low-volume endurance training on glucose tolerance. (A) Glucose tolerance test (GTT) curves and (B) corresponding area under the curves (AUCs) measured in the ninth week of treatment in the old groups and in young sedentary mice (Sed+Saline, $n = 10$; Sed+IL-6, $n = 10$; Ex+Saline, $n = 8$; Ex+IL-6, $n = 11$; and young Sed, $n = 12$). GTT curves and corresponding AUCs measured at baseline (-2 weeks) and after 9 weeks of treatment: (C) Sed+Saline group ($n = 10$), (D) Sed+IL-6 group ($n = 10$), (E) Ex+Saline group ($n = 8$), and (F) Ex+IL-6 group ($n = 11$). Data are presented as mean ± SEM (GTT curves), individual paired values connected with a black line (AUCs of C–F). Two-way ANOVA (repeated measures) followed by Sidak's multiple comparisons for GTT curves (A, C–F), one-way ANOVA followed by Sidak's multiple comparisons (B), and paired Student's *t*-test for AUCs (C–F). In (A): *$P < 0.05$ Sed+Saline *vs.* Ex+IL-6; #$P < 0.05$ Sed+Saline *vs.* Ex+Saline. In (C): #$P < 0.05$ result of the two-way ANOVA for the column factor. *$P < 0.05$ and ***$P < 0.001$.

Blood lactate levels of all four groups rose above 12 mmol/L when $\dot{V}O_{2peak}$ plateaued, indicating that the animals reached their peak performance (*Figure* S5B). Old endurance-trained mice showed higher retest $\dot{V}O_{2peak}$ values (*Figure* 3A) resulting from a pronounced decline in the two sedentary groups (*Figure* S5E). However, $\dot{V}O_{2peak}$ of the trained groups remained below the levels of young sedentary mice (*Figure* 3A), despite the absence of significant differences in speed reached and distance covered (*Figure* S5C and S5D). Of note,

compared with young sedentary mice, delta lactate was higher in all groups except for the Ex+IL-6 group (*Figure* S5F). While $\dot{V}O_{2peak}$ is largely determined by the ability of the cardiorespiratory system to deliver oxygen to exercising muscles,[31] muscle intrinsic aspects, such as diffusive oxygen transport and metabolic processes, strongly determine submaximal endurance performance and fatigue resistance. We therefore challenged the same mice with a long duration incremental step protocol (*Figure* S5G) at baseline and at the

Figure 3 rIL-6 treatment combined with moderate-intensity low-volume endurance training improves treadmill running capacity. (A) Peak oxygen up-take ($\dot{V}O_{2peak}$) in old groups in the 10th week and in an independent cohort of young sedentary mice reached during a short ramp-sprint protocol (Sed+Saline, $n = 6$; Sed+IL-6, $n = 7$; Ex+Saline, $n = 6$; Ex+IL-6, $n = 5$; and young Sed, $n = 14$). (B–E) Parameters assessed with a long duration incremental step protocol: (B) distance covered until exhaustion at baseline and after 12 weeks (retest) in old groups and young sedentary mice (left panel) and Kaplan–Meier plot for the retest showing the percentage of mice running at indicated distances (right panel). (C) Time to exhaustion and (D) total work (W_{tot}) performed at baseline and after 12 weeks in the older groups and in young sedentary mice. (E) Blood lactate levels expressed as the difference exhausted-basal (Δ) at baseline and after 12 weeks in older groups and in young sedentary mice. (B–E) Sed+Saline, $n = 15$; Sed+IL-6, $n = 16$; Ex+Saline, $n = 13$; Ex+IL-6, $n = 15$; and young Sed, $n = 14$. (F) Representative western blot of *M. quadriceps femoris* lactate dehydrogenase heart subunit (LDH-H) and corresponding band quantification. LDH-H values are normalized to α-actinin and expressed relative to Sed+Saline ($n = 6$ per group). Data are presented as box and whiskers in (A), as mean and individual paired values connected with a black line (B–D), or mean ± SEM including individual values (Young Sed group in B–F). One-way ANOVA followed by Sidak's multiple comparisons (A, E and F), paired Student's t-test (B–D), and log-rank test for trend (B). $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ as indicated. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ old groups *vs.* Young Sed group.

end of the intervention (12 weeks). In this test, the two sedentary groups showed no change from baseline (*Figures* 3B–3D and S5H), indicating that despite the marked reduction in $\dot{V}O_{2peak}$, fatigue resistance at submaximal performance may be unaffected. Surprisingly, performance of the two exercise groups was strongly diverging. Mice that were exposed to higher levels of rIL-6 during exercise training sessions clearly improved their running capacity, as running distance covered (*Figure* 3B) and time to exhaustion (*Figure* 3C)
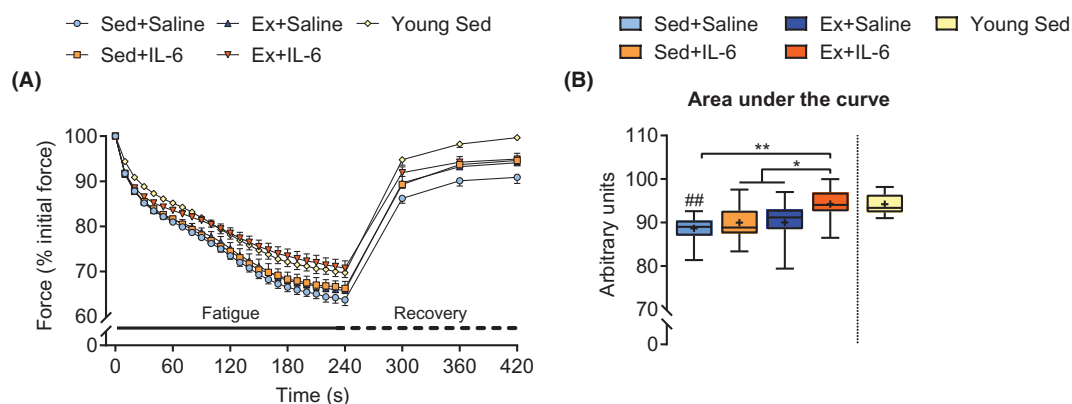
were both significantly longer compared with baseline. Moreover, these mice performed 33% more work (*Figure* 3D) and reached 12% greater peak power (*Figure* S5H) in the retest. In stark contrast, the Ex+Saline group did not improve in any of the assessed parameters and thus was more similar to the two sedentary control groups. In addition, measurements of blood lactate concentrations before (basal) and at the end (exhausted) of each test indicated a 'lactate threshold' decrease during the intervention, which was less

pronounced when treated with rIL-6 or endurance trained, and even absent when rIL-6 and training were combined (*Figures* 3E, 3F, and S5I). Moreover, even though they performed 19% more work and had a 10% higher power output at exhaustion, Ex+IL-6 mice accumulated significantly less lactate compared with Sed+Saline mice in the retest, and their delta lactate was not higher compared with young sedentary mice (*Figures* 3E and S5I). Western blot analysis for the lactate dehydrogenase heart subunit (LDH-H, transcribed from the *Ldhb* gene) that drives the conversion of lactate to pyruvate revealed a higher abundance of this form in Ex+IL-6 mice compared with the other old groups (*Figure* 3F), suggesting enhanced capacity for lactate clearance in these animals.
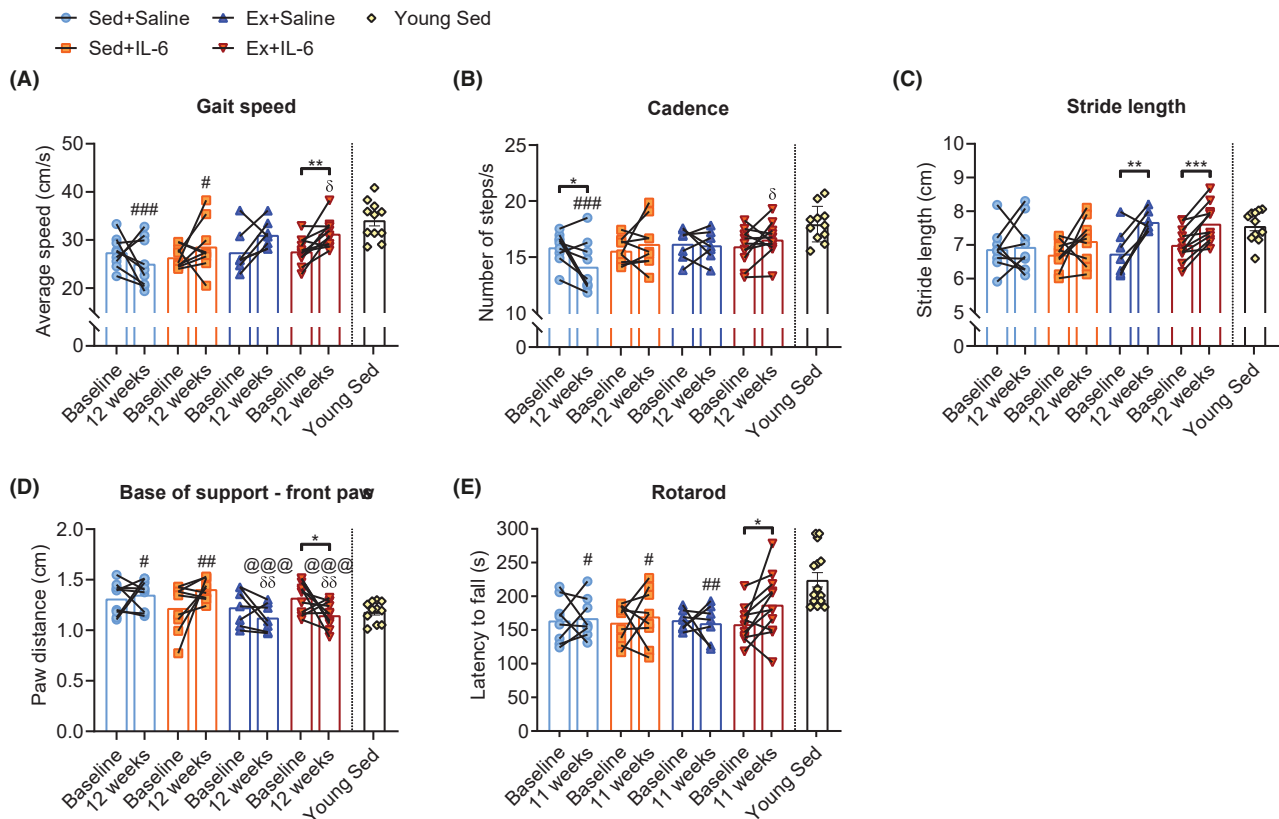
To further characterize muscle intrinsic aspects, we assessed *in situ* fatigability and contractile properties of the *M. tibialis anterior* by sciatic nerve stimulation at the end of the study. In response to a 4-min fatigue protocol, the Ex+IL-6 group showed the slowest drop in force production, leading to a clear separation of its fatigue curve from the curves of the other old groups, very reminiscent of young sedentary mice (*Figure* 4A). Intriguingly, the resulting AUC (*Figure* 4B) of the Ex+IL-6 group was not only significantly larger compared with the two sedentary groups but also compared with the Ex+Saline group, which suggests that additional IL-6 during training sessions was required to increase fatigue resistance in response to the 12 week endurance training programme in the *M. tibialis anterior*. There was no difference in area of the curve of the recovery period between the older groups (*Figures* 4A and S6A). Of note, the observed gain in muscular endurance was not at the expense of muscle force, as grip strength measures *in vivo* (*Figure* S6B) as well as single twitch and tetanic forces *in situ* (*Figure* S6C–S6F) were similar in all groups. Furthermore, contraction and relaxation velocities did not differ between groups (*Figure* S6G and S6H).

Administration of recombinant interleukin-6 during endurance training bouts improves gait and motor coordination in old mice

Walking speed is a strong independent predictor of life expectancy,[32] and unsteady gait and deficits in motor coordination largely contribute to the increased risk of falls and the ensuing devastating health consequences in elderly adults. We thus used the CatWalk XT voluntary gait analysis system and a rotarod-based test to examine gait and motor coordination. Compared with baseline, gait speed remained constant in both sedentary groups after 12 weeks (*Figure* 5A), but the number of steps per second (cadence) appeared to decrease significantly in the Sed+Saline group (*Figure* 5B). Moreover, compared with young sedentary mice, gait speed and cadence were reduced in Sed+Saline mice (*Figure* 5A and 5B). The decrease in cadence was mainly due to an increased duration of step cycle (sum of swing and stand phase, *Figure* S7A), which was driven by a longer duration of swing phase of both hindlimbs and forelimbs (*Figure* S7C and S7F). The slower limb movement in old sedentary mice at the end of the study was not observed in rIL-6-treated and/or endurance-trained mice. Moreover, rIL-6 administration during exercise bouts increased gait speed significantly after 12 weeks of training, whereas exercise alone did not (*Figure* 5A). While cadence remained unchanged, overall (i.e. hindlimbs and forelimbs combined) stride length increased significantly in both exercise groups (*Figure* 5B and 5C), suggesting that exercise induced an increase in step size, which was, however, not sufficient to improve gait speed. The additional increase in swing speed of forelimbs (*Figure* S7B) and decrease in stand phase of hindlimbs (*Figure* S7G) achieved by Ex+IL-6 mice, together with the increase in hindlimb swing phase in the Ex+Saline group (*Figure* S7F), might explain the observed discrepancy in gait speed. In addition, Ex+IL-6 mice reduced the base of

Figure 4 rIL-6 treatment combined with moderate-intensity low-volume endurance training improves muscle fatigue resistance *in situ*. (A) Average curves showing the force decline during a 4-min *in situ* muscle fatigue protocol on the *M. tibialis anterior* and of muscle force recovery up to 3 min after fatigue with (B) area under the curves corresponding to the force decline during muscle fatigue (Sed+Saline, *n* = 14; Sed+IL-6, *n* = 15; Ex+Saline, *n* = 12; Ex+IL-6, *n* = 14; and young Sed, *n* = 9). One-way ANOVA followed by Sidak's multiple comparisons (B). *$P < 0.05$ and **$P < 0.01$ as indicated. ##$P < 0.01$ old groups *vs.* Young Sed group.

Figure 5 rIL-6 treatment improves gait and motor coordination in endurance-trained mice. (A–E) Parameters of quantitative gait analysis at baseline and after 12 weeks of the intervention in old groups and in an independent cohort of young sedentary mice: (A) average body speed (cm/s), (B) cadence (number of steps/s), (C) average stride length (in cm) of hindlimbs and forelimbs, and (D) base of support, that is, distance (cm) between paws of forelimbs (Sed+Saline, $n = 9$; Sed+IL-6, $n = 9$; Ex+Saline, $n = 7$; Ex+IL-6, $n = 11$; and young Sed, $n = 11$). (E) Motor coordination assessed with an accelerated rotarod test at baseline and in the 11th week of the intervention in old groups and in young sedentary mice (Sed+Saline, $n = 8$; Sed+IL-6, $n = 9$; Ex+Saline, $n = 7$; Ex+IL-6, $n = 11$; and young Sed, $n = 14$). Data are presented as mean and individual paired values connected with a black line and mean ± SEM including individual values (Young Sed group in A–E). Paired Student's t-test and one-way ANOVA followed by Sidak's multiple comparisons between retests of old groups and young sedentary group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ as indicated. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ old groups vs. young Sed group. @@@$P < 0.001$ Sed+Saline vs. Ex+Saline or Ex+IL-6; δδ$P < 0.01$ Sed+IL-6 vs. Ex+Saline or Ex+IL-6.

support of their forepaws (*Figure* 5D), which is indicative of a more confident and secure gait. In accordance with the observed changes in voluntary gait, the Ex+IL-6 group was the only group that improved rotarod performance within the treatment period and even reached the level of young sedentary mice (*Figures* 5E and S7I).
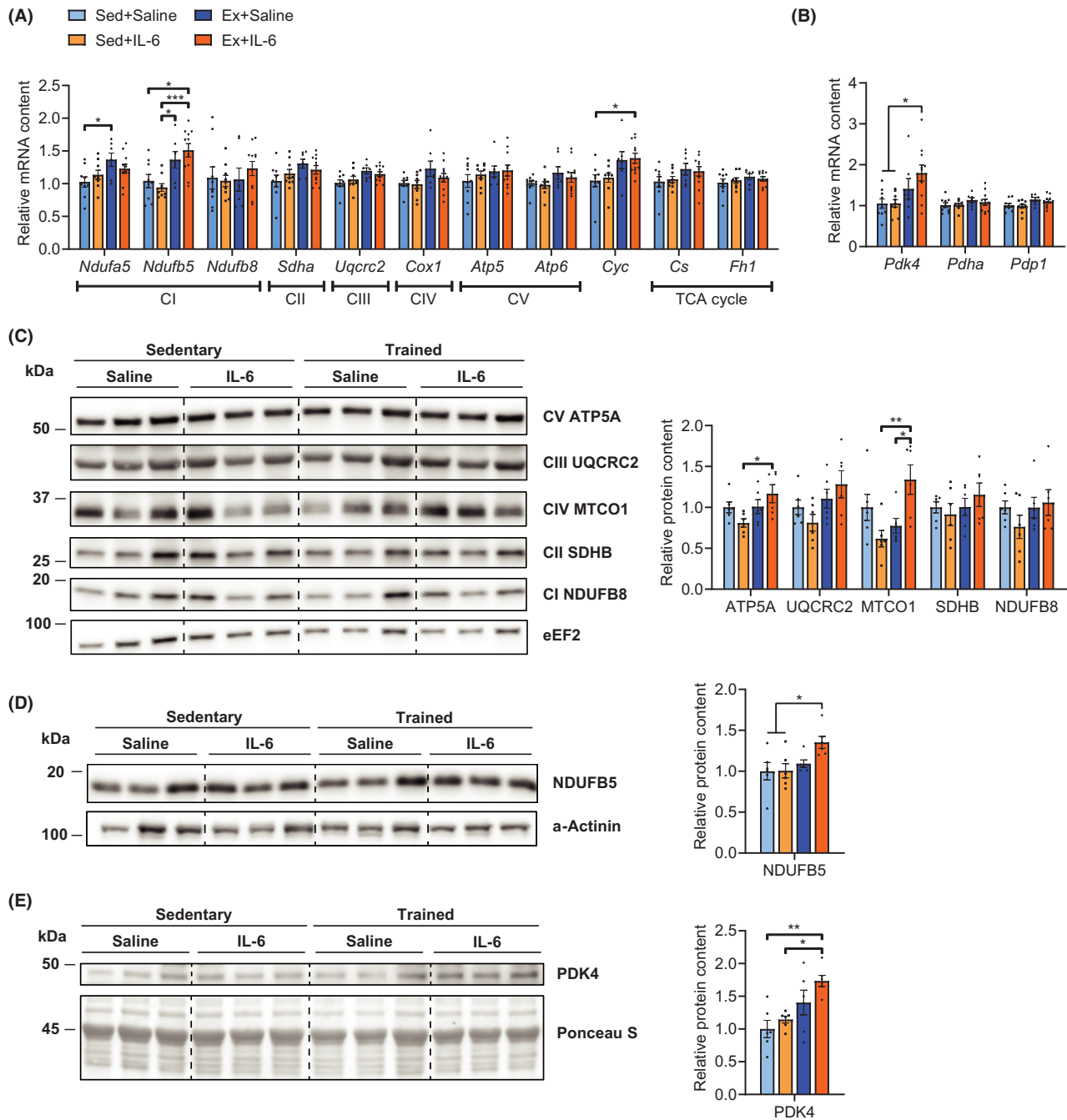
Exercised mice treated with recombinant interleukin-6 show increased expression of mitochondrial complex I components and pyruvate dehydrogenase kinase 4

The fact that only Ex+IL-6 mice improved fatigue resistance *in situ* together with the observation that rIL-6 increased running performance *in vivo* without affecting $\dot{V}O_{2peak}$ suggested that IL-6 potentiates skeletal muscle intrinsic adaptations to training. To get more insights on molecular changes, we assessed mRNA expression of metabolic genes

in the *M. quadriceps femoris*, a muscle heavily used during treadmill running and displaying mixed fibre-type composition. This analysis revealed no strong transcriptional differences between groups in key components of the electron transport chain except for complex I (*Figure* 6A). Moreover, genes encoding for proteins involved in fatty acid transport, synthesis, and oxidation, as well as components involved in glucose metabolism and storage, remained unaffected by either intervention (*Figure* S8A and S8B). In contrast, pyruvate dehydrogenase kinase 4 (*Pdk4*), a gene encoding for a kinase that inactivates pyruvate dehydrogenase (PDH), which in turn is a positive regulator of carbohydrate-derived energy substrate utilization in mitochondria,[33] was up-regulated in muscles of Ex+IL-6 mice (*Figure* 6B). Both the PDH component subunit E1 alpha (*Pdha1*) and pyruvate dehydrogenase phosphatase 1 (*Pdp1*, encoding a PDH activating phosphatase) showed similar expression in all groups.

To determine whether the transcriptional status is reflecting protein levels at the time of analysis, we performed

Figure 6 rIL-6-treated endurance-trained mice show increased expression of mitochondrial complex I components and PDK4. Relative *M. quadriceps femoris* mRNA levels of (A) mitochondrial genes of the electron transport chain [complex (C) I–V, Cyc] and tricarboxylic acid (TCA) cycle and (B) of pyruvate dehydrogenase kinase 4 (*Pdk4*), pyruvate dehydrogenase E1 component subunit alpha (*Pdha1*), and pyruvate dehydrogenase phosphatase 1 (*Pdp1*). Sed+Saline, $n = 9$; Sed+IL-6, $n = 9$; Ex+Saline, $n = 7$; and Ex+IL-6, $n = 11$. Expression values were determined by qPCR and normalized to *Tbp*. Data are shown as the mean fold change ± SEM including individual values relative to the expression of the control (Sed+Saline) set to 1. Relative *M. quadriceps femoris* protein levels of OXPHOS (C), NDUFB5 (D), and PDK4 (E) assessed by western blot ($n = 6$ per group). Target band intensities were normalized to the loading control (eEF2, α-actinin, or Ponceau S stain), and data are shown as the mean fold change ± SEM including individual values relative to the control (Sed+Saline) set to 1. One-way ANOVA followed by Sidak's multiple comparisons (A–E). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

western blots for OXPHOS proteins and PDK4 with tissue from the same muscles. Ex+IL-6 mice showed higher complex IV and V protein abundance in the OXPHOS blot (*Figure* 6C). An additional western blot for the complex I component

NDUFB5, which showed clearest difference in gene expression, revealed a slight up-regulation in the Ex+IL-6 group compared with the two sedentary groups (*Figure* 6D). Also, in accordance with gene expression, PDK4 protein levels were

highest in IL-6-treated exercised mice (*Figure* 6E). The increase in OXPHOS proteins and enhanced levels of PDK4 in skeletal muscle, together with elevated LDH-H, is indicative of a boosted oxidative capacity and lactate as well as fatty acid-derived substrate flux, which may provide performance advantage during prolonged exercise at the submaximal level. Of note, total resting levels of the downstream effector protein of IL-6 signalling, signal transducer and activator of transcription 3 (STAT3), were increased in skeletal muscle by both rIL-6 treatment and endurance training (*Figure* S8C).

Discussion

The age-associated decline in skeletal muscle function is one of the main drivers of loss of independence, admission to nursing homes, increased risk for chronic diseases, morbidity, and mortality in the elderly. The only efficacious method to prevent and mitigate sarcopenia, frailty, and other pathologies associated with this functional decline is exercise based, using both resistance training to address loss in muscle mass and strength, and endurance training to improve cardiovascular function, fatigue, and frailty. Unfortunately, training interventions are notoriously difficult to implement and adhere to, in the general population, but due to pre-existing frailty, impaired gait as well as reduced balance and motor coordination, co-morbidities, and other events, even more so in older individuals.[34] We therefore assessed how a low-volume, moderate-intensity endurance training could be combined with the application of rIL-6 to achieve synergy in improving the functional capacity of skeletal muscle in old mice.

Notably, even when only performed at old age, and despite the low volume, endurance training conferred potent beneficial effects on various parameters, most of which could not be recapitulated by the pulsatile administration of rIL-6 as performed in this study. Importantly, however, when combined with training, rIL-6 powerfully enhanced a broad spectrum of biological programmes that are impaired in ageing. Indeed, following 12 weeks of training, Ex+IL-6 mice displayed higher endurance performance and prolonged contractions at higher relative force *in situ*, which excelled the effects of training. The combined treatment thereby most efficiently improved running capacity and fatigability in old mice. Second, the Ex+IL-6 group showed ameliorated gait and motor coordination, none of which could be improved to a similar extent in mice trained without rIL-6 administration. Even though increased muscular endurance is important for motor coordination, neuronal changes (e.g. vestibular and proprioceptive input, nerve conduction velocity, or neuromuscular junction functionality) and motor planning are additional crucial components that could potentially be affected by IL-6. Intriguingly, rIL-6 treatment

has been shown to be protective in chemotherapy-induced and diabetes-induced neuropathies,[23–25] alluding to a direct effect of rIL-6 on neuronal integrity and potentially function. This potential therapeutic effect of rIL-6 in combination with exercise on skeletal muscle-autonomous as well as systemic functions is underlined by the third outcome, the moderate beneficial effect on glucose tolerance and body composition. The sedentary group showed a decrease in glucose tolerance during the intervention, which was not observed when treated with rIL-6 or endurance trained. Even though there was no difference in retest GTT AUCs between the two sedentary or exercise groups, the combination of rIL-6 and training significantly lowered GTT AUC compared with the Sed+Saline group. Glucose tolerance is indicative of metabolic flexibility, and its impairments are a hallmark of the age-related metabolic syndrome. Often, insulin sensitivity and glucose tolerance are closely linked to body composition. In our cohorts, both training groups exhibited a reduction in fat mass, but only in the combination group (Ex+IL-6) a significant decrease in the mass of eWAT was observed. In contrast to sWAT, excessive eWAT has been linked to pro-inflammatory events and the development of insulin resistance and type 2 diabetes.[35]

Despite the equal values of $\dot{V}O_{2peak}$, submaximal treadmill running capacity increased remarkably in animals that were exposed to higher levels of IL-6 during single training bouts, whereas endurance training alone was not sufficient to elevate any of the measured parameters. Therefore, the two exercise groups may differ in the proportion of $\dot{V}O_{2peak}$ (i.e. the rate of 'fractional utilization') sustained during prolonged exercise. The fact that Ex+IL-6 mice had less lactate accumulation at exhaustion despite the higher power output supports the notion that adaptations within skeletal muscle are responsible for the improved performance, as higher lactate thresholds are often associated with training-induced increases in skeletal muscle oxidative capacity and optimized production, disposal, and clearance of lactate.[36]

In skeletal muscle, rIL-6 treatment elevated basal levels of its downstream target STAT3, similar to what was observed in endurance-trained mice. Endurance exercise-induced STAT3 activation is necessary for the beneficial effects of training on exercise performance and glucose homeostasis.[37] While STAT3 exerts many effects in different cells, a direct regulatory link between this transcription factor and metabolic genes might exist. For example, low *Stat3* expression was associated with low *Pdk4* expression in human prostate tumours, and subsequent analyses of several chromatin immunoprecipitation DNA-sequencing (ChIP-Seq) data sets showed binding of STAT3 to the promoter region of *Pdk4*.[38] In addition, ChIP assays on control and shSTAT3 cells with or without prior IL-6 stimulation showed highest STAT3 levels and *Pdk4* promoter region binding with IL-6 stimulation and a reduction with *Stat3* knockdown.[38] In line with these

reports, exercise combined with rIL-6 up-regulated resting levels of PDK4 gene and protein expression in skeletal muscle in our study. PDK4 is an inhibitor of PDH, which in turn is a positive regulator of carbohydrate-derived energy substrate utilization in mitochondria. Higher PDK4 levels may thus allow a rapid or more efficient up-regulation of fatty acid oxidation during exercise and thereby providing performance advantage by sparing glycogen. It has previously been shown that a single injection of rIL-6 decreases skeletal muscle PDH activity in mice in the fed state[39] and that skeletal muscle PDH activity is higher in muscle-specific IL-6 knockouts compared with controls at rest and at 60 min of exercise.[40] Concomitant with elevated PDK4, the combination of training and rIL-6 increased the transcript and protein levels of OXPHOS components and those of LDH-H in our study. Collectively, this metabolic remodelling could favour more efficient oxidation of lipids and lactate and thereby contribute to the higher endurance and fatigue resistance in this group.

Many therapeutic approaches aim at neutralizing the pro-inflammatory effects of IL-6, and hence, application of exogenous rIL-6 could theoretically be regarded as an unsafe approach. To mitigate potential deleterious effects of persistently elevated IL-6 as observed in many chronic diseases, we therefore aimed at a pulsatile, low-dose application replicating the regulation of systemic IL-6 as a myokine during and after acute exercise bouts. Because IL-6 levels return to baseline within ~2 h after injection,[28] IL-6 was indeed not chronically elevated in terminal plasma measurements in our mice. Second, the absence of any specific change in other pro-inflammatory cytokines, for example, TNF-α, IL-1β, or interferon-γ, in the rIL-6-treated groups compared with the levels in the control groups indicates that the application of rIL-6 did not induce a pro-inflammatory environment. This conclusion is supported by the absence of any pyrogenic activity, modulation of spontaneous locomotion, body mass, or food intake by rIL-6. Furthermore, inconspicuous ALT and AST levels in the rIL-6 groups indicate normal liver health, and the reduction in plasma LDH by rIL-6 and/ or training could imply an even reduced cellular damage in these mice. Collectively, these data demonstrate that a pulsatile, low-dose administration of rIL-6 over 12 weeks, with or without concomitant endurance training, is safe and well tolerated and lacks any discernable adverse effects in old mice.

adverse effects of this treatment were observed, implying that such an intervention is safe and well tolerated. Even though future studies will aim at a careful investigation of sarcopenia, healthspan, and lifespan, the present data demonstrate functional improvements in endurance performance, fatigue resistance, gait and motor coordination, and potential benefits on glucose tolerance. Thus, if this intervention can be translated to elderly individuals, rIL-6 could not only facilitate training interventions, and hence increase adherence and compliance, but also directly result in a massive improvement of quality of life by affecting gait and motor coordination. Importantly, fatigability, a key hallmark of human frailty,[41] could likewise be significantly mitigated. Collectively, these improvements could alleviate insecurity, avoidance of physical activity and exercise, falls and the ensuing fractures and hospitalizations, and the loss of independence as well as admission to nursing homes delayed. Clinical trials and safety studies with rIL-6 have already been performed in non-geriatric individuals. Therefore, following a careful evaluation of safety, tolerability, and adverse effects in the elderly, the use of combined rIL-6 training interventions in the prevention and treatment of age-associated functional decline could be initiated in a relatively short amount of time and help to overcome the reduced training response or even exercise intolerance often observed in this population.[41]

Acknowledgements

Conflict of interest

Y.S. is a consultant for Sonnet Biotherapeutics CH SA, a company promoting the development of low-dose IL-6 treatments for peripheral neuropathies. The other authors declare no competing interests.

Conclusions

Our results demonstrate that the application of pulsatile, low-dose rIL-6 can potentiate the beneficial effects of a low-volume endurance training on muscle intrinsic and systemic parameters in old mice. Of note, no detectable

Ethics statement

All procedures involving animals were approved by the veterinary office of the canton Basel-Stadt (Switzerland) and performed in accordance with the Swiss federal guidelines for animal experimentation under consideration of the

well-being of the animals and the 3R (replace, reduce, and refine) principle. The manuscript does not contain clinical studies or patient data.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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