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Supplemental information

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Regulatory T cells require IL-6 receptor alpha signaling to control skeletal muscle function and regeneration

Maike Becker[1,2,10], Sini S. Joseph[2,3,4,10], Francisco Garcia-Carrizo[2,5,11], Robby Zachariah-Tom[2,6,7,11], Daria Opaleva[1,2], Isabelle Serr[1,2], Matthias H. Tschöp[2,3,4], Tim J. Schulz[2,5,8,12], Susanna M. Hofmann[2,6,7,12], and Carolin Daniel[1,2,9]*

[10,11,12] These authors contributed equally.
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Supplemental Information titles and legends

Figure S1

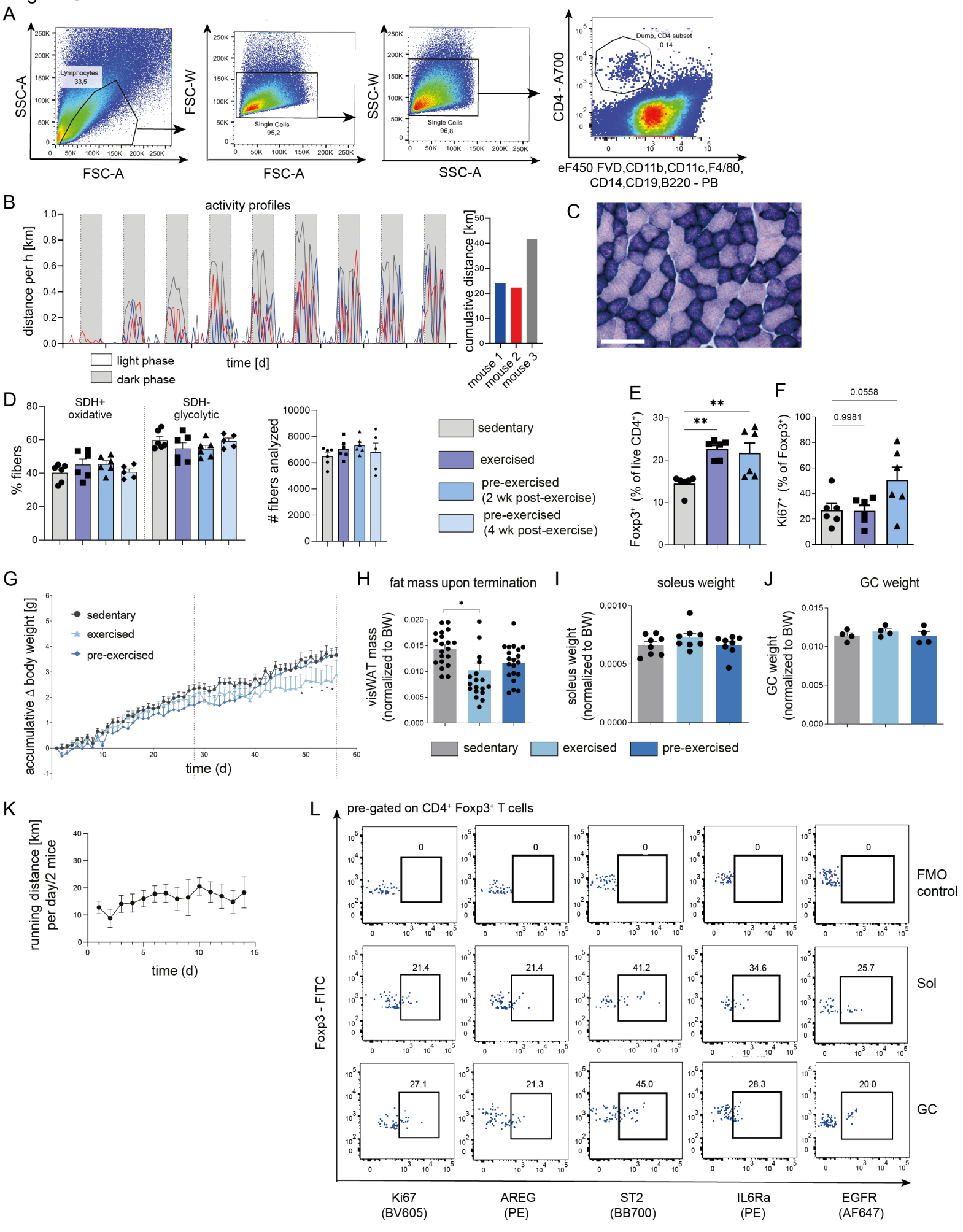

A
SSC-A
FSC-A
Lymphocytes
33,5
FSC-W
FSC-A
Single Cells
95,2
SSC-W
SSC-A
Single Cells
96,8
CD4 - A700
eF450 FVD,CD11b,CD11c,F4/80,
CD14,CD19,B220 - PB
Dump, CD4 subset
0.14
B
activity profiles
distance per h [km]
time [d]
light phase
dark phase
cumulative distance [km]
mouse 1
mouse 2
mouse 3
C
D
SDH+
oxidative
SDH-
glycolytic
% fibers
# fibers analyzed
sedentary
exercised
pre-exercised
(2 wk post-exercise)
pre-exercised
(4 wk post-exercise)
E
Foxp3⁺ (% of live CD4⁺)
**
**
F
Ki67⁺ (% of Foxp3⁺)
0.0558
0.9981
G
accumulative Δ body weight [g]
sedentary
exercised
pre-exercised
time (d)
H
fat mass upon termination
visWAT mass
(normalized to BW)
*
I
soleus weight
soleus weight
(normalized to BW)
J
GC weight
GC weight
(normalized to BW)
sedentary
exercised
pre-exercised
K
running distance [km]
per day/2 mice
time (d)
L
pre-gated on CD4⁺ Foxp3⁺ T cells
Foxp3 - FITC
FMO
control
Sol
GC
0
21.4
21.4
41.2
34.6
25.7
27.1
21.3
45.0
28.3
20.0
Ki67
(BV605)
AREG
(PE)
ST2
(BB700)
IL6Ra
(PE)
EGFR
(AF647)

Figure S1: Physiological parameters assessed during voluntary wheel running.

(A) Muscle-residing *ex vivo* Tregs were analyzed based on lymphocyte gating using forward and side scatter, followed by doublet exclusion based on FSC-A vs. FSC-W and SSC-A vs. SSC-W. $CD4^+$T cells were then gated using a set of exclusion markers (CD11b, CD11c, CD14, F4/80, B220, CD8a) and dead cell stains (sytox blue or fixable viability dye eFluor450) followed by the transcription factor Foxp3 for Tregs (see Figure 1A for Foxp3 staining example).

(B) Example of activity profiles of mice subjected to voluntary wheel running using low-profile Wi-Fi wheels. Shown is the distance run per hour in the dark vs light phase over a period of 10 days and the corresponding cumulative distance.

(C-D) Representative image obtained by enzymatic staining of cryosectioned GC muscles for SDH activity and the corresponding summary graph. Medium and dark blue muscle fibers are SDH+ and are referred to as being oxidative. Light blue muscle fibers are SDH- and considered being glycolytic. The scale bar (white) is 100 µm. The groups were defined as: sedentary (eight weeks rest), exercised (four weeks rest, four weeks exercise), pre-exercised (two weeks rest, four weeks exercise, two weeks rest) and pre-exercised (four weeks exercise, four weeks rest). Each symbol refers to a biological replicate.

(E-F) Pilot study analyzing E) $Foxp3^+$ Treg and F) $Ki67^+$ proliferating Treg frequencies in Soleus of sedentary, exercised (four weeks of voluntary wheel running), and pre-exercised mice (analyzed two weeks after the four weeks voluntary wheel running period).

(G) Accumulative delta body weight of sedentary, exercised and pre-exercised WT mice (n= 14 mice per group).

(H) Visceral adipose tissue mass normalized to body weight upon termination (n= 20 mice per group).

(I-J) Weight of I) Soleus and J) Gastrocnemius normalized to body weight in sedentary, exercised and pre-exercised mice.

(K) Voluntary wheel running of WT mice represented as average running distance per day in km for two mice (from three independent experiments).

(L) Representative FACS plots showing the presence of Ki67, AREG, ST2, IL6Rα and EGFR on muscle-residing $Foxp3^+$Tregs (in Soleus and Gastrocnemius) and the corresponding fluorescence minus one (FMO) staining controls.

Data are represented as mean±SEM. Each point refers to a biological replicate. Data were analyzed by one-way ANOVA followed by Tukey's or Šidák's post hoc test for multiple comparisons (D-F, H-J) or two-way ANOVA followed by Dunnett's post hoc test (G). *$p< 0.05$, **$p< 0.01$. Related to Figure 1 and 2.

Figure S2

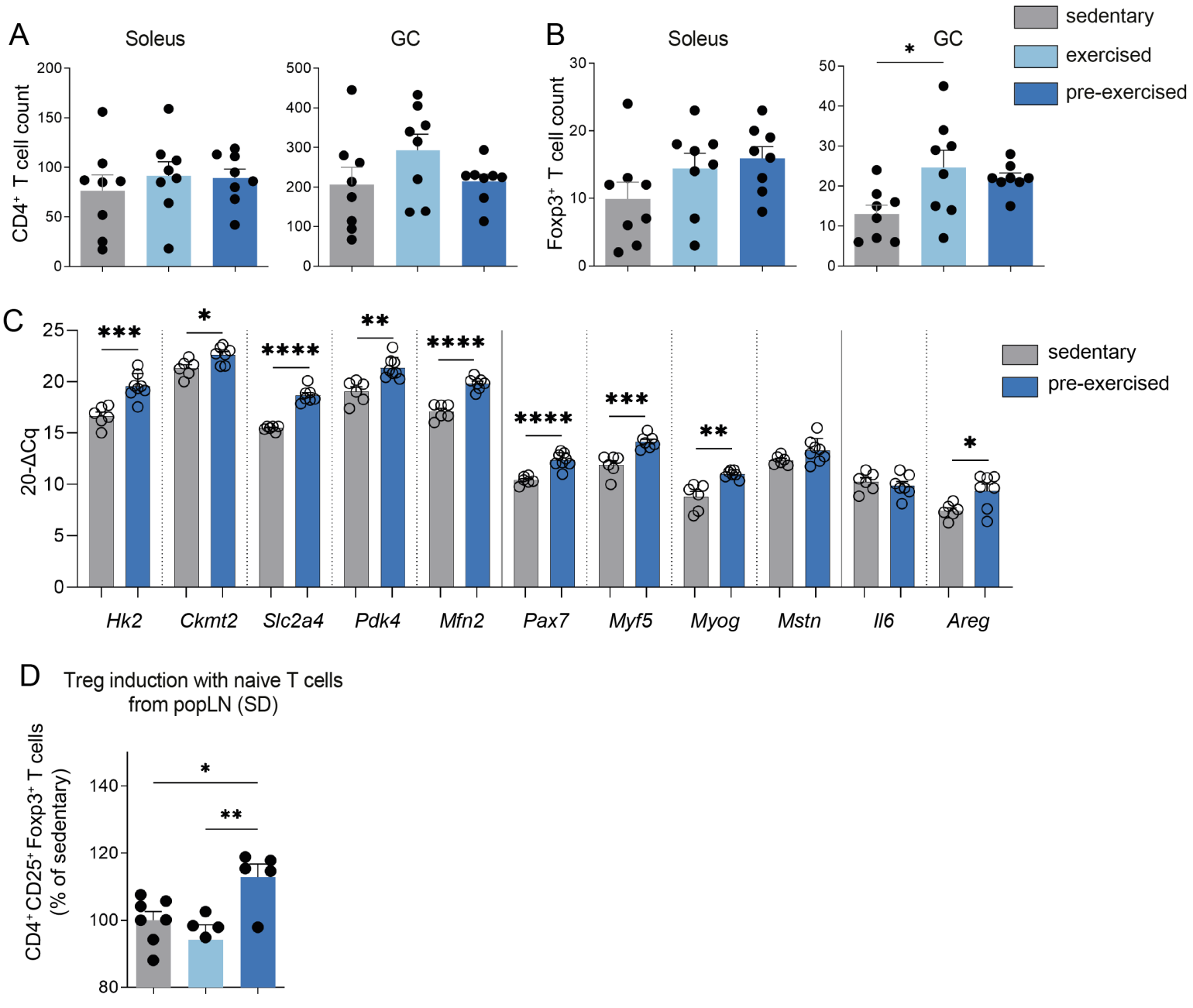

A
Soleus
GC
CD4⁺ T cell count
B
Soleus
GC
Foxp3⁺ T cell count
sedentary
exercised
pre-exercised
C
20-ΔCq
sedentary
pre-exercised
Hk2
Ckmt2
Slc2a4
Pdk4
Mfn2
Pax7
Myf5
Myog
Mstn
Il6
Areg
D
Treg induction with naive T cells from popLN (SD)
CD4⁺ CD25⁺ Foxp3⁺ T cells (% of sedentary)

Figure S2: Impact of exercise on muscle-residing $CD4^+$T cells.

(A-B) A) $CD4^+$ T cell and B) $Foxp3^+$ Treg counts from Soleus and Gastrocnemius (GC) muscle per isolation.

(C) Gene expression analysis of Soleus of sedentary (n=6) and pre-exercised (n=7) WT mice. Gene expression was normalized to *Histone H3.* Data of sedentary mice as shown in Figure 1.

(D) Bar graph showing induced $CD4^+CD25^+Foxp3^+$ Tregs (represented as % of sedentary) from the *in vitro* Treg induction assays using limited TCR stimulation of naïve $CD4^+$T cells sorted from popliteal lymph nodes of sedentary, exercised and pre-exercised mice. Shown are biological replicates from 2-3 independent experiments.

Data are expressed as mean±SEM. Data were analyzed by Student's unpaired two-tailed *t*-test (C) or one-way ANOVA followed by Tukey's post hoc test for multiple comparison (A, B, D) with *p< 0.05, **p< 0.01, ***p< 0.001 and ****p< 0.0001. Related to Figure 2.

Figure S3

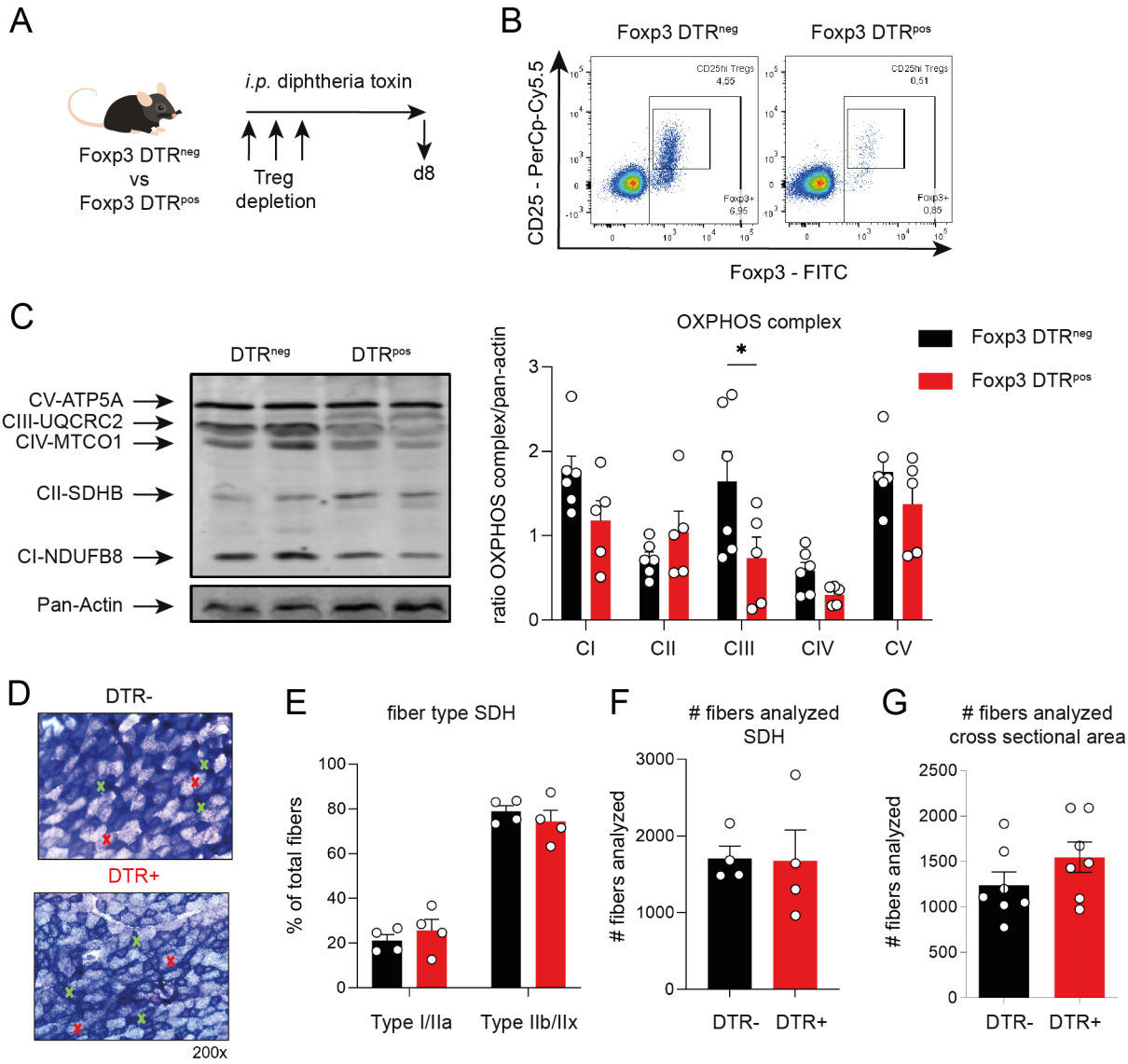

A
Foxp3 DTRneg
vs
Foxp3 DTRpos
i.p. diphtheria toxin
Treg
depletion
d8
B
Foxp3 DTRneg
Foxp3 DTRpos
CD25 - PerCp-Cy5.5
Foxp3 - FITC
CD25hi Tregs
4,55
Foxp3+
6,95
CD25hi Tregs
0,51
Foxp3+
0,85
C
DTRneg
DTRpos
CV-ATP5A
CIII-UQCRC2
CIV-MTCO1
CII-SDHB
CI-NDUFB8
Pan-Actin
OXPHOS complex
ratio OXPHOS complex/pan-actin
CI
CII
CIII
CIV
CV
Foxp3 DTRneg
Foxp3 DTRpos
D
DTR-
DTR+
200x
E
fiber type SDH
% of total fibers
Type I/IIa
Type IIb/IIx
F
fibers analyzed
SDH
fibers analyzed
DTR-
DTR+
G
fibers analyzed
cross sectional area
fibers analyzed
DTR-
DTR+

Figure S3: Treg depletion in Foxp3 DTR mice affects muscle homeostasis.

(A) Experimental scheme to deplete Foxp3^{+}Tregs using *i.p*. diphtheria toxin (DT) in Foxp3DTR mice.

(B) Representative FACS plot showing the Treg depletion efficacy in popliteal LN of Foxp3DTR- and Foxp3DTR+ mice on day 8.

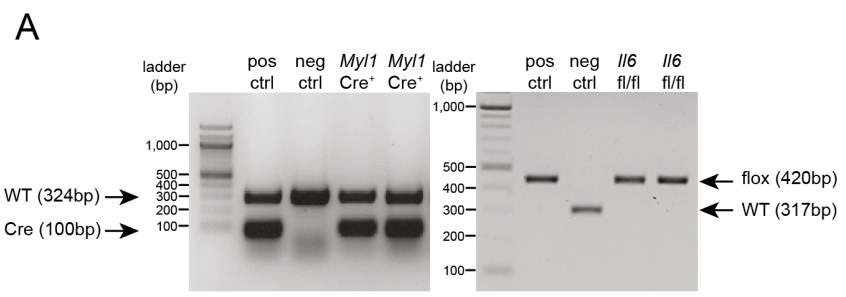
(C) Western Blotting results showing the analysis of OXPHOS complexes in Gastrocnemius upon Treg depletion in Foxp3DTR- and Foxp3DTR+ mice. In the western blot, two biological replicates per experimental group are shown. In the summary plot analyzing CI-CV, each dot represents a biological replicate.

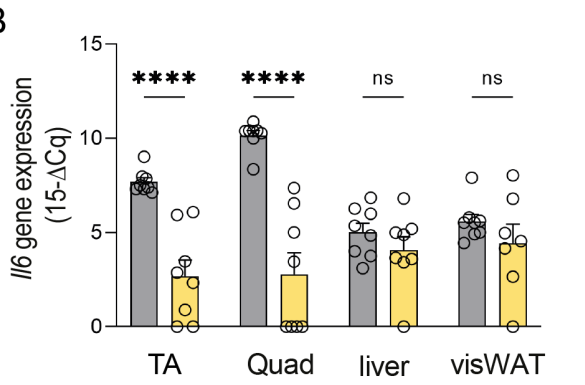
(D-F) Representative pictures and quantification of succinate dehydrogenase (SDH) staining of Gastrocnemius upon Treg depletion.

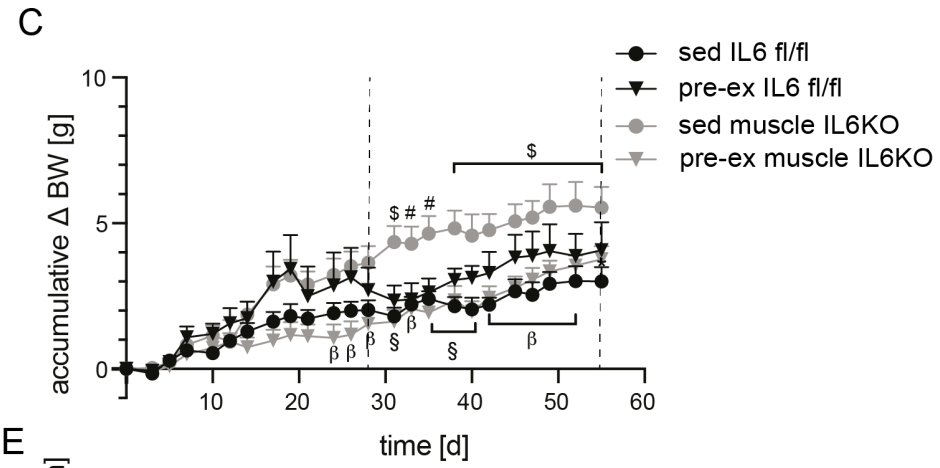
(G) Quantification of the cross-sectional area of Gastrocnemius fibers upon Treg depletion in Foxp3 DTR- and Foxp3 DTR+ mice.

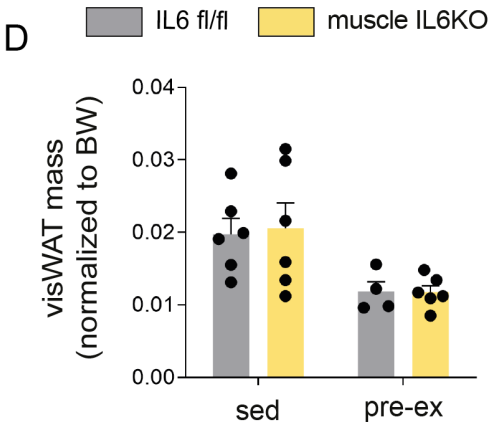
Data are represented as mean±SEM. Each dot refers to a biological replicate. Student's unpaired two-tailed *t*-test (C, E-G) with *p< 0.05. Related to Figure 3.

Figure S4


A
ladder (bp)
pos ctrl
neg ctrl
Myl1 Cre+
Myl1 Cre+
ladder (bp)
pos ctrl
neg ctrl
Il6 fl/fl
Il6 fl/fl
1,000
500
400
300
200
100
WT (324bp)
Cre (100bp)
flox (420bp)
WT (317bp)


B
Il6 gene expression (15-ΔCq)
****
****
ns
ns
TA
Quad
liver
visWAT


C
sed IL6 fl/fl
pre-ex IL6 fl/fl
sed muscle IL6KO
pre-ex muscle IL6KO
accumulative Δ BW [g]
time [d]


D
IL6 fl/fl
muscle IL6KO
visWAT mass (normalized to BW)
sed
pre-ex

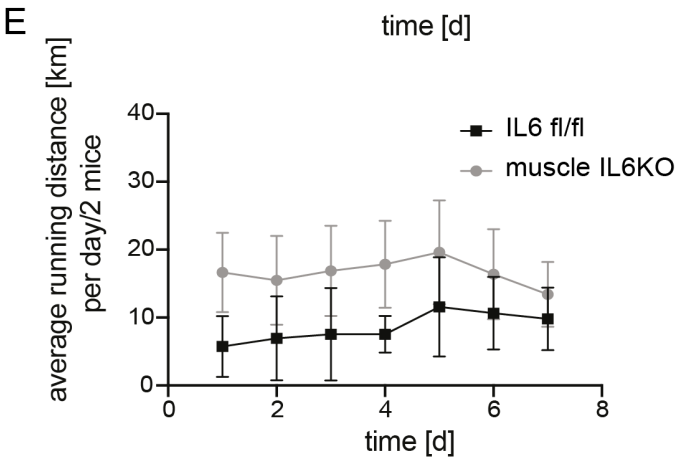

E
IL6 fl/fl
muscle IL6KO
average running distance [km] per day/2 mice
time [d]

Figure S4: Impact of exercise on muscle fiber IL6KO mice.

(A) Genotypic characterization of muscle fiber-specific IL6KO mice.

(B) Graph showing the *Il6* mRNA levels in muscles (Quadriceps, Tibialis anterior), liver and visceral adipose tissue in muscle fiber IL6KO mice and floxed controls by qPCR (n= 8 mice per group). Not detected as 0 value.

(C) Accumulative delta body weight of sedentary and pre-exercised muscle fiber IL6KO mice and floxed control mice upon standard diet (n= 6 mice per group). # ($p< 0.01$), $ ($p< 0.001$) comparing sedentary muscle fiber IL6KO mice to sedentary floxed control mice; β ($p< 0.01$), § ($p< 0.001$) comparing sedentary muscle fiber IL6KO mice to pre-exercised muscle fiber IL6KO mice.

(D) Visceral adipose tissue mass normalized to body weight of sedentary and pre-exercised muscle fiber IL6KO mice and floxed controls.

(E) Voluntary wheel running profile of muscle fiber IL6KO mice and floxed controls represented as average running distance per day in km for two mice (from three independent experiments).

Data are expressed as mean±SEM. Each point represents a biological replicate. Data were analyzed by two-way ANOVA followed by Tukey's post hoc test for multiple comparison (C, E, D) or Student's unpaired two-tailed *t*-test (B). *$p< 0.05$, **$p< 0.01$, ***$p< 0.001$. Related to Figure 4.

Figure S5

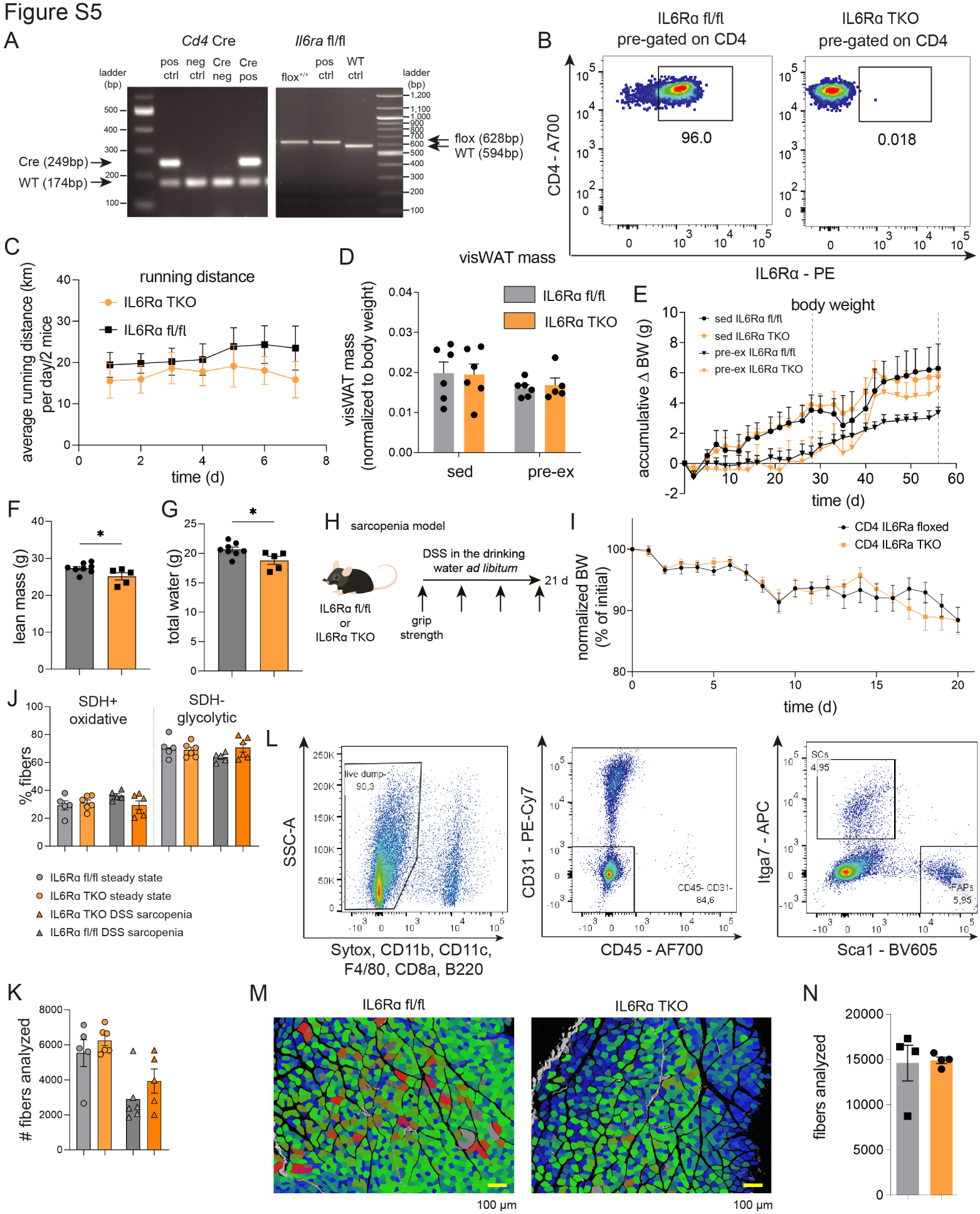

A
Cd4 Cre
Il6ra fl/fl
ladder (bp)
pos ctrl
neg ctrl
Cre neg
Cre pos
flox+/+
WT ctrl
Cre (249bp)
WT (174bp)
flox (628bp)
WT (594bp)
B
IL6Rα fl/fl pre-gated on CD4
IL6Rα TKO pre-gated on CD4
CD4 - A700
96.0
0.018
IL6Rα - PE
C
running distance
IL6Rα TKO
IL6Rα fl/fl
average running distance (km) per day/2 mice
time (d)
D
visWAT mass
IL6Rα fl/fl
IL6Rα TKO
visWAT mass (normalized to body weight)
sed
pre-ex
E
body weight
sed IL6Rα fl/fl
sed IL6Rα TKO
pre-ex IL6Rα fl/fl
pre-ex IL6Rα TKO
accumulative Δ BW (g)
time (d)
F
lean mass (g)
G
total water (g)
H
sarcopenia model
IL6Rα fl/fl or IL6Rα TKO
DSS in the drinking water ad libitum
21 d
grip strength
I
CD4 IL6Rα floxed
CD4 IL6Rα TKO
normalized BW (% of initial)
time (d)
J
SDH+ oxidative
SDH- glycolytic
% fibers
IL6Rα fl/fl steady state
IL6Rα TKO steady state
IL6Rα TKO DSS sarcopenia
IL6Rα fl/fl DSS sarcopenia
L
live dump-
90,3
SSC-A
Sytox, CD11b, CD11c, F4/80, CD8a, B220
CD31 - PE-Cy7
CD45- CD31-
84,6
CD45 - AF700
SCs
4,95
FAPs
5,95
Itga7 - APC
Sca1 - BV605
K
fibers analyzed
M
IL6Rα fl/fl
IL6Rα TKO
100 µm
100 µm
N
fibers analyzed

Figure S5: Impact of exercise on mice with a T cell-specific IL6Rα KO.

(A) Genotypic characterization of IL6Rα TKO mice by CD4 Cre and IL6Ra floxed PCR. PCR products were separated on a 2% agarose gel (w/v, in 1X TAE buffer) and stained using Midori Green.

(B) FACS staining of *ex vivo* IL6Rα on $CD4^+$T cells from inguinal lymph nodes in IL6Rα TKO mice and floxed controls.

(C) Voluntary wheel running profile of IL6Rα TKO mice and floxed control mice represented as average running distance per day in km for two mice (from three independent experiments).

(D) Visceral adipose tissue mass normalized to body weight of sedentary and pre-exercised IL6Rα TKO mice and floxed controls (n= 6).

(E) Accumulative delta body weight of sedentary and pre-exercised IL6Rα TKO mice and floxed controls fed the standard diet (n= 4-6 mice per group).

(F-G) Lean mass and total water measured by EchoMRI body composition analysis of IL6Rα TKO mice and floxed control mice after pre-exercise.

(H) Scheme of the sarcopenia model using IL6Rα TKO mice and floxed control mice that received DSS *ad libitum* in the drinking water. Grip strength was measured once a week.

(I) Normalized body weight change of (H) of IL6Rα TKO mice and floxed control mice.

(J-K) Analyses of enzymatic SDH stainings in Gastrocnemius muscles of either steady state IL6Rα fl/fl vs IL6Rα TKO mice, or mice subjected to DSS-induced sarcopenia as of (H). not significant.

(L) Full gating scheme of the analysis of satellite cells (SCs; gated as $Itga7^+Sca1^-CD45^-CD31^-dump^-$) and fibro-adipogenic progenitor cells (FAPs; gated as $Sca1^+Itga7^-CD45^-CD31^-dump^-$).

(M-N) Analysis of the cross-sectional area of Gastrocnemius muscle of pre-exercised IL6Rα TKO and floxed control mice at the end of the experiment. Scale bar (yellow) is 100 µm.

Data are expressed as mean±SEM. Data were analyzed by Student's unpaired two-tailed *t*-test (F, G, N), one-way ANOVA followed by Tukey's post hoc test for multiple comparison (J), or two-way ANOVA followed by Tukey's post hoc test for multiple comparison (C, E, I) with *p< 0.05. Related to Figure 4.

Figure S6

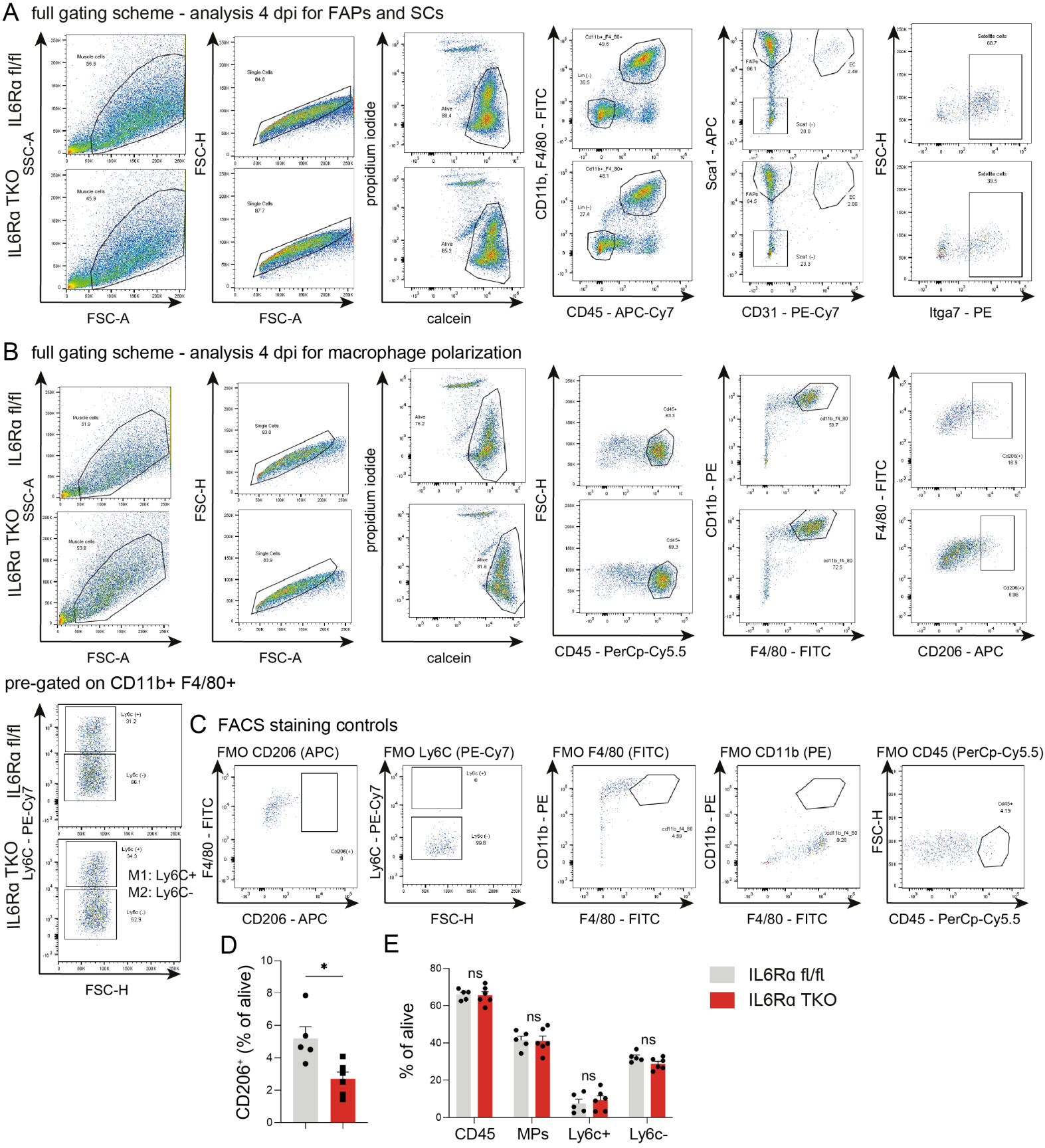

A full gating scheme - analysis 4 dpi for FAPs and SCs
IL6Rα fl/fl
IL6Rα TKO
SSC-A
FSC-A
FSC-H
propidium iodide
calcein
CD11b, F4/80 - FITC
CD45 - APC-Cy7
Sca1 - APC
CD31 - PE-Cy7
Itga7 - PE
B full gating scheme - analysis 4 dpi for macrophage polarization
CD45 - PerCp-Cy5.5
CD11b - PE
F4/80 - FITC
CD206 - APC
pre-gated on CD11b+ F4/80+
Ly6C - PE-Cy7
M1: Ly6C+
M2: Ly6C-
C FACS staining controls
FMO CD206 (APC)
FMO Ly6C (PE-Cy7)
FMO F4/80 (FITC)
FMO CD11b (PE)
FMO CD45 (PerCp-Cy5.5)
D
CD206+ (% of alive)
E
% of alive
ns
CD45
MPs
Ly6c+
Ly6c-
IL6Rα fl/fl
IL6Rα TKO

Figure S6: Muscle injury in mice with a T cell-specific IL6Rα loss.

(A) Full gating scheme for the analysis of endothelial cells (ECs; gated as $CD45^{-}CD11b^{-}F4/80^{-}Sca1^{+}CD31^{+}$), macrophages (MPs; gated as live $CD45^{+}CD11b^{+}F4/80^{+}$) satellite cells (SCs; gated as $Itga7^{+}Sca1^{-}CD45^{-}CD31^{-}dump^{-}$) and fibro-adipogenic progenitor cells (FAPs; gated as $Sca1^{+}Itga7^{-}CD45^{-}CD31^{-}dump^{-}$).

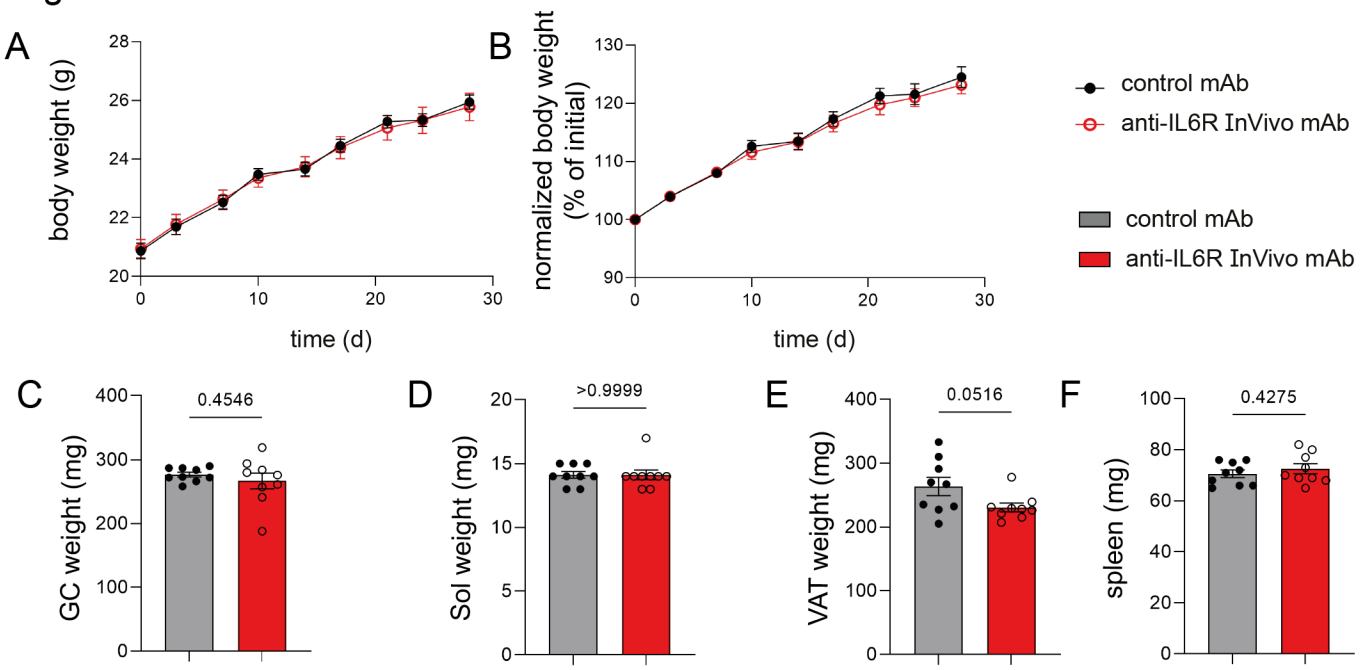
(B) Full gating scheme for the analysis of pro-inflammatory $Ly6C^{+}$/anti-inflammatory $Ly6C^{-}$ cells or $CD206^{+}$ macrophages.

(C) Corresponding FACS FMO staining controls for (A-B).

(D-E) Quantification for *ex vivo* phenotypic analysis of macrophages upon muscle injury in IL6Rα TKO and floxed control mice 4 dpi.

Data are expressed as mean±SEM. Data were analyzed by Student's unpaired two-tailed *t*-test (D, E) with *$p< 0.05$. Related to Figure 5.

Figure S7


A
body weight (g)
time (d)
B
normalized body weight (% of initial)
time (d)
control mAb
anti-IL6R InVivo mAb
control mAb
anti-IL6R InVivo mAb

C
GC weight (mg)
0.4546
D
Sol weight (mg)
>0.9999
E
VAT weight (mg)
0.0516
F
spleen (mg)
0.4275

Figure S7: Treg modulation affects muscle function.

(A-B) Body weight change of C57Bl/6J mice treated with anti-IL6R mAb or control mAb for four weeks twice weekly. 2way ANOVA with Šidák's post hoc test. ns.

(C-F) Organ weights after four weeks of anti-IL6R mAb or control mAb treatment. Student's unpaired two-tailed *t*-test. Data represent mean±SEM. ns = not significant. Related to Figure 7.