Muscle-specific deletion of SOCS3 does not reduce the anabolic response to leucine in a mouse model of acute inflammation

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Abstract

Excessive inflammation reduces skeletal muscle protein synthesis leading to wasting and weakness. The janus kinase/signal transducers and activators of transcription-3 (JAK/STAT3) pathway is important for the regulation of inflammatory signaling. As such, suppressor of cytokine signaling-3 (SOCS3), the negative regulator of JAK/STAT signaling, is thought to be important in the control of muscle homeostasis. We hypothesized that muscle-specific deletion of SOCS3 would impair the anabolic response to leucine during an inflammatory insult. Twelve week old (n=8 per group) SOCS3 muscle-specific knockout mice (SOCS3-MKO) and littermate controls (WT) were injected with lipopolysaccharide (LPS, 1 mg/kg) or saline and were studied during fasted conditions or after receiving 0.5 g/kg leucine 3 h after the injection of LPS. Markers of inflammation, anabolic signaling, and protein synthesis were measured 4 h after LPS injection. LPS injection robustly increased mRNA expression of inflammatory molecules (*Socs3, Socs1*, *Il-6*, *Ccl2*, *Tnfα* and *Cd68*). In muscles from SOCS3-MKO mice, the *Socs3* mRNA response to LPS was significantly blunted (~6-fold) while STAT3 Tyr705 phosphorylation was exacerbated (18-fold). Leucine administration increased protein synthesis in both WT (~1.6-fold) and SOCS3-MKO mice (~1.5-fold) compared to basal levels. LPS administration blunted this effect, but there were no differences between WT and SOCS3-MKO mice. Muscle-specific SOCS3 deletion did not alter the response of AKT, mTOR, S6 or 4EBP1 under any treatment conditions. Therefore, SOCS3 does not appear to mediate the early inflammatory or leucine-induced changes in protein synthesis in skeletal muscle.

Highlights

- SOCS3 deletion in skeletal muscle does not alter LPS-induced inflammation.
- SOCS3 does not mediate the anabolic response to leucine in skeletal muscle.
- Leucine augments the LPS-induced expression of IL-6 and STAT3 phosphorylation.

Key words

SOCS3, inflammation, anabolic signaling, protein synthesis

Abbreviations

SOCS3-MKO, SOCS3 muscle-specific knockout; LPS, lipopolysaccharide

1. Introduction

Food intake, particularly protein, stimulates protein synthesis and attenuates protein breakdown in healthy skeletal muscle. This anabolic response is crucial for the normal regulation of muscle mass and homeostasis. During conditions of muscle wasting such as cancer cachexia, sepsis, immobilization and ageing, the amino acid-induced anabolic response is impaired and is commonly referred to as 'anabolic resistance'. The mechanisms that contribute to the development of anabolic resistance are not completely understood during these conditions, however, the excessive production of reactive oxygen species (ROS) and pro-inflammatory cytokines play a central role as they inhibit the mechanistic target of rapamycin complex 1 (mTORC1). Indeed, we have demonstrated that the injection of lipopolysaccharide (LPS, an established model of acute inflammation) reduced the anabolic response (signaling and protein synthesis) to leucine in mice [10].

The janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway is activated by a plethora of inflammatory ligands. In skeletal muscle the JAK/STAT3 pathway is important for the regulation of inflammatory signaling, as well as successful myogenesis [14] and myofibre regeneration [9]. Inflammatory signaling via the JAK/STAT pathway is carefully controlled by several negative feedback mechanisms, including the suppressors of cytokine signaling (SOCS) proteins, of which SOCS3 is of particular importance in skeletal muscle. Although SOCS3 is expressed at low levels in the absence of inflammation, SOCS3 mediates the extent and duration of cytokine-induced signaling thereby modulating cellular homeostasis. Muscle-specific overexpression of SOCS3 impairs insulin sensitivity in overweight mice [11], yet improves muscle morphology [4, 13]. In contrast, muscle-specific SOCS3 deletion improved glucose homeostasis [8], but increased the early inflammatory response after myotoxic injury [15]. Whether SOCS3 is also implicated in the regulation of anabolic signaling in skeletal muscle has yet to be established.

The aim of this study was to identify the role of SOCS3 on amino acid induced changes in protein synthesis in skeletal muscle. We tested the impact of muscle-specific deletion of SOCS3 on leucine-induced increases in protein synthesis during an inflammatory insult. Basal and leucine stimulated protein synthesis rates were measured 4 hours following LPS exposure, as were mTOR and inflammatory signaling after a 1 hour exposure to 0.5 g/kg leucine. We hypothesized that (a) deletion of SOCS3 in skeletal muscle would exacerbate the inflammatory response following exposure to an inflammatory insult; and (b) this would result in reduced leucine-stimulated protein synthesis due to increased anabolic resistance.

2. Methods

2.1 Animals

The SOCS3 muscle-specific knockout (SOCS3-MKO) mice were generated as described previously [8, 15]. The muscle specific deletion of *Socs3* in these mice has been confirmed in our previous studies [15]. All procedures were approved by the Animal Ethics Committee of The University of Melbourne and conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes as described by the National Health and Medical Research Council of Australia. All mice were housed in the Biological Research Facility at The University of Melbourne under a 12-hour light/dark cycle with drinking water available *ad libitum*. At 12 weeks of age, male and female SOCS3-MKO (KO) or wild-type (WT) littermates were randomly assigned to two groups: a group of control mice receiving saline (CON; n=16); or a group of mice receiving LPS (1 mg/kg intraperitoneal [*i.p*], n=16). LPS or saline was administered after a 2 hour fast. Each group was separated into 'Basal' (saline injection, n=8) and 'Leu' (n=8) groups. The 'Leu' group received 0.5 g/kg leucine dissolved in saline via *i.p*. injection 3 hours after LPS injection and 1 hour before the mice were killed [7].

2.2 Muscle protein synthesis

We utilized SUnSET methodology to establish the relative rate of protein synthesis, as described [7]. Briefly, puromycin (0.4 µmol/kg; Sigma-Aldrich) was injected (*i.p*) 30 minutes after the injection with leucine or saline. Thirty minutes after puromycin administration mice were killed via cervical dislocation. The quadriceps (QUAD) muscles were rapidly dissected, frozen and stored at −80°C for subsequent analyses.

2.3 RNA extraction and qPCR

Total RNA was extracted from the QUAD muscles. The Bio-Rad CFX384 PCR system (Bio-Rad Laboratories) was used to perform qPCR as described previously [3, 7]. Gene sequences were obtained from GenBank to design primers which are listed here (*Socs1* F: CTTAACCCGGTACTCCGTGA, *Socs1* R: GAGGTCTCCAGCCAGAAGTG [NM_001271603]; *Cd68* F: TCCAAGCCCAAATTCAAATC, *Cd68* R: ATTGTATTCCACCGCCATGT [NM_001291058]) or described elsewhere [3, 7]. Data were analyzed using a comparative quantification cycle (Cq) method where the amount of target relative to *β-actin* is given by $2^{-\Delta\Delta Cq}$. The PCR instrument generated a melting point dissociation curve for all PCR products to confirm the presence of a single amplified product.

2.4 Protein extraction and immunoblotting

Protein was extracted from the QUAD muscles and immunoblotting performed as described previously [3] using Criterion™ TGX Stain-Free™ Precast Gels (Bio-Rad Laboratories) and Trans-Blot® Turbo™ Transfer System (Bio-Rad Laboratories). To allow maximum probing efficiency, the membrane was cut according to size. Primary antibodies were diluted in blocking buffer and membranes were incubated overnight at 4°C; puromycin (clone 12D10, Millipore, Kilsyth, Victoria, Australia), phospho-STAT3 (Tyr705), STAT3, phospho-Akt (Ser473), Akt, phospho-mTOR (Ser2448), mTOR, pS6 (Ser235/236), S6, p4EBP1 (Thr37/46) and 4EBP1 (Cell Signaling Technology Inc., Danvers, MA). Membranes were incubated with secondary antibodies diluted in blocking buffer for 1 hour at room temperature [anti-mouse IgG Fc 2a (1:50000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies (1:3000, GE Healthcare, NSW, Australia)]. Data for phosphorylated and total proteins were normalized to total protein volume density (determined by imaging the Stain-Free Precast Gels). For STAT3 analysis, phosphorylated and total proteins were detected on the same membrane following antibody stripping and re-probing protocols. For Akt/mTOR/S6/4EBP1, phosphorylated and total proteins were run on separate gels to avoid issues arising from ineffective antibody stripping. For each puromycin membrane, a standard curve for serial dilutions of a puromycin-positive sample with a puromycin-negative muscle sample (100, 75, 50, 25, and 0%) was used to normalize changes in staining density and determine relative muscle protein synthesis as described in detail previously [7].

2.5 Statistical analysis

Values were expressed as means ± SEM unless stated otherwise. For ease of visualization, data were normalized to the appropriate control group (WT-CON basal), unless stated otherwise. To compare between groups for the CON and LPS treatment groups, two-way ANOVAs (genotype X basal/leucine) were used. Tukey's post hoc test was used to determine significant main effects. Significant differences ($P<0.05$) and trends ($P<0.1$) are reported where appropriate.

3. Results

Muscle-specific deletion of SOCS3 increases STAT3 phosphorylation but does not alter the early inflammatory response to LPS.

Firstly, muscle-specific deletion of SOCS3 resulted in a reduction in *Socs3* mRNA under basal/control conditions, compared to WT mice (main effect for genotype, P<0.05; Figure 1A). We next examined the effect of muscle-specific SOCS3 deletion on the early inflammatory response to acute LPS exposure. Administration of LPS to WT mice robustly increased the mRNA expression of *Socs3* (~37-fold), *Socs1* (~6-fold), *Il-6* (~20-fold), *Ccl2* (~18-fold), *Tnfα* (~33-fold) and *Cd68* mRNA (~1.3-fold). In SOCS3-MKO mice, a similar magnitude response was observed for *Socs1* (~16-fold), *Il-6* (~20-fold), *Ccl2* (~19-fold), *Tnfα* (~46-fold) and *Cd68* mRNA (~1.1-fold) (Figure 1D). In muscles from SOCS3-MKO mice, the *Socs3* mRNA response to LPS was significantly blunted (~6-fold, P<0.001; Figure 1D). Correspondingly, we found that acute exposure to LPS increased pSTAT3 Tyr705 (10-fold), which was exacerbated in the SOCS3-MKO mice (18-fold, P<0.05; Figure 1E-F). Interestingly, administration of leucine following LPS augmented the inflammatory response in both WT and SOCS3-MKO mice as evidenced by increased *Il-6* (~43 and 77-fold respectively, P<0.05; Figure 1D) and *Ccl2* mRNA expression (~24 and 136-fold respectively, NS; Figure 1D), yet returned *Cd68* mRNA expression back to basal levels (P<0.01; Figure 1D). We observed an increase in STAT3 phosphorylation in the SOCS3-MKO mice with the addition of leucine (~32-fold, P<0.05; Figure 1E-F) which was significantly higher than that observed in SOCS3-MKO treated with LPS only (~19-fold higher, P<0.001; Figure 1E-F).

LPS-induced reductions in the anabolic response to leucine are not affected by skeletal muscle-specific SOCS3 deletion.

In the basal state, protein synthesis (as measured by puromycin incorporation) was not different between WT and SOCS3-MKO mice (Figure 2A-D). Leucine administration increased protein synthesis in both WT (~1.6-fold, P<0.05) and SOCS3-MKO mice (~1.5-fold, P<0.05) compared to WT-CON (Basal/saline injected), but not following LPS injection (Figure 2C-D). Muscle-specific SOCS3 deletion did not alter phosphorylation of AKT, mTOR, S6 or 4EBP1 under any treatment conditions (Figure 2E-H). Phosphorylated mTOR (S2448) was increased by leucine administration in both genotypes (main effect, P<0.001) under control conditions (Figure 2E-F). Following LPS exposure, administration of leucine enhanced 4EBP1 phosphorylation (Figure 2G-H; main effect, P<0.05), whereas neither AKT nor S6 phosphorylation were altered by LPS or leucine (Figure 2E-H).

4. Discussion

SOCS3 is a key negative regulator of STAT3 and a critical regulator of inflammatory signaling in several tissue types, including skeletal muscle [15]. Due to its known role in the regulation of inflammation we hypothesized that SOCS3 deletion in skeletal muscle would enhance the inflammatory response to LPS injection and reduce the anabolic response to leucine. Using mice specifically lacking SOCS3 in MCK-expressing mature muscle fibers we showed that although reduced SOCS3 expression increases the phosphorylation of STAT3 it does not modulate the anabolic response to leucine.

The increase in STAT3 phosphorylation following LPS administration in the SOCS3-MKO mice was not associated with parallel changes in early inflammatory signaling, such as increased *Il-6* mRNA expression. These observations are in contrast with our previous observations that SOCS3 deletion in skeletal muscle increased the inflammatory profile (*Tnfα*, *F4/80* and *Cd68*) within 3 days of regeneration following myotoxic injury [15]. However, the extent and timing of the inflammatory response between these conditions is vastly different and *Il-6* mRNA has previously been shown to peak (106-fold) as early as two hours after LPS administration *in vivo* [6]. In addition, the magnitude of the inflammatory response under basal conditions (changes in *Il-6* and *Ccl2* mRNA expression; ~20-fold) observed in the present study was much smaller compared to previous studies using this LPS protocol and timing of tissue collection in C57BL/6 mice (*Il-6:* ~80-fold; *Ccl2*: ~65-fold) [7]. Although the SOCS3-MKO mice were generated on a C57BL/6 background, these mice retain a somewhat mixed genetic background due to the injection of BALB/c blastocysts into C57BL/6 mice to generate the original SOCS3$^{fl/fl}$ strain [5]. This difference in genetic background may account for the observed differences in the magnitude of the LPS response between the current study and our previously published results in C57BL/6 mice [7].

Administration of 0.5 g/kg leucine 3 hours after LPS injection augmented LPS-induced *Il-6* mRNA expression and STAT3 Tyr705 phosphorylation. The observation that leucine increases the acute inflammatory response in LPS treated mice seems consistent with our previous report showing increased *Tnfα*, *Socs3,* and *Ccl2* mRNA expression after leucine administration following LPS treatment [7]. These observations and other lines of research support a specific role for leucine in the production of cytokines within skeletal muscle. Firstly, in food restricted rodents, the inflammatory response to LPS is markedly reduced [12], suggesting that overall nutrition or specific nutrients contribute to the activation of inflammatory cells. Secondly, increases in serum IL-6 levels and STAT3 phosphorylation (and abundance) following leucine or whey protein administration are thought to promote cytokine-mediated cell proliferation as evidenced by improved wound closure in

diabetic rodents [1, 2]. Although our observations suggest leucine can augment the inflammatory response to LPS independently of other nutrients, the role that increased inflammatory signalling plays in the regulation of the anabolic response remains unclear.

Lipopolysaccharide administration did not reduce *basal* protein synthesis in WT or SOCS3-MKO mice to the same extent as we reported previously in LPS-treated C57BL/6 mice (-22%) [7]. However, LPS attenuated the leucine induced anabolic response in WT and SOCS3-MKO mice, consistent with our previous observations. Generally, the anabolic response, i.e. protein synthesis and phosphorylation status of key signaling proteins, was not different between genotypes, suggesting that SOCS3 does not play a key role in the regulation of protein turnover in response to inflammatory insults and leucine availability in skeletal muscle.

5. Conclusion

Our data demonstrate that deletion of muscle specific SOCS3 enhances STAT3 phosphorylation but does not further impair leucine-stimulated protein synthesis or anabolic signaling following exposure to LPS. Further work is required to elucidate the mechanism of leucine stimulated increases in IL-6 and STAT3 phosphorylation, and the implications for skeletal muscle protein turnover during inflammatory conditions.

Conflict of interest

The authors declare there are no conflicts of interest.

Authorship

MKC, DJH, RK designed and carried out experiments, analyzed and interpreted the results and wrote the manuscript. AC, JT and TN conducted the animal experiments including genotyping, injections and dissections. MKC, DJH, KS, RK, DIS and GSL edited and revised the manuscript. RK and GSL provided financial support. All authors read and approved the final manuscript.

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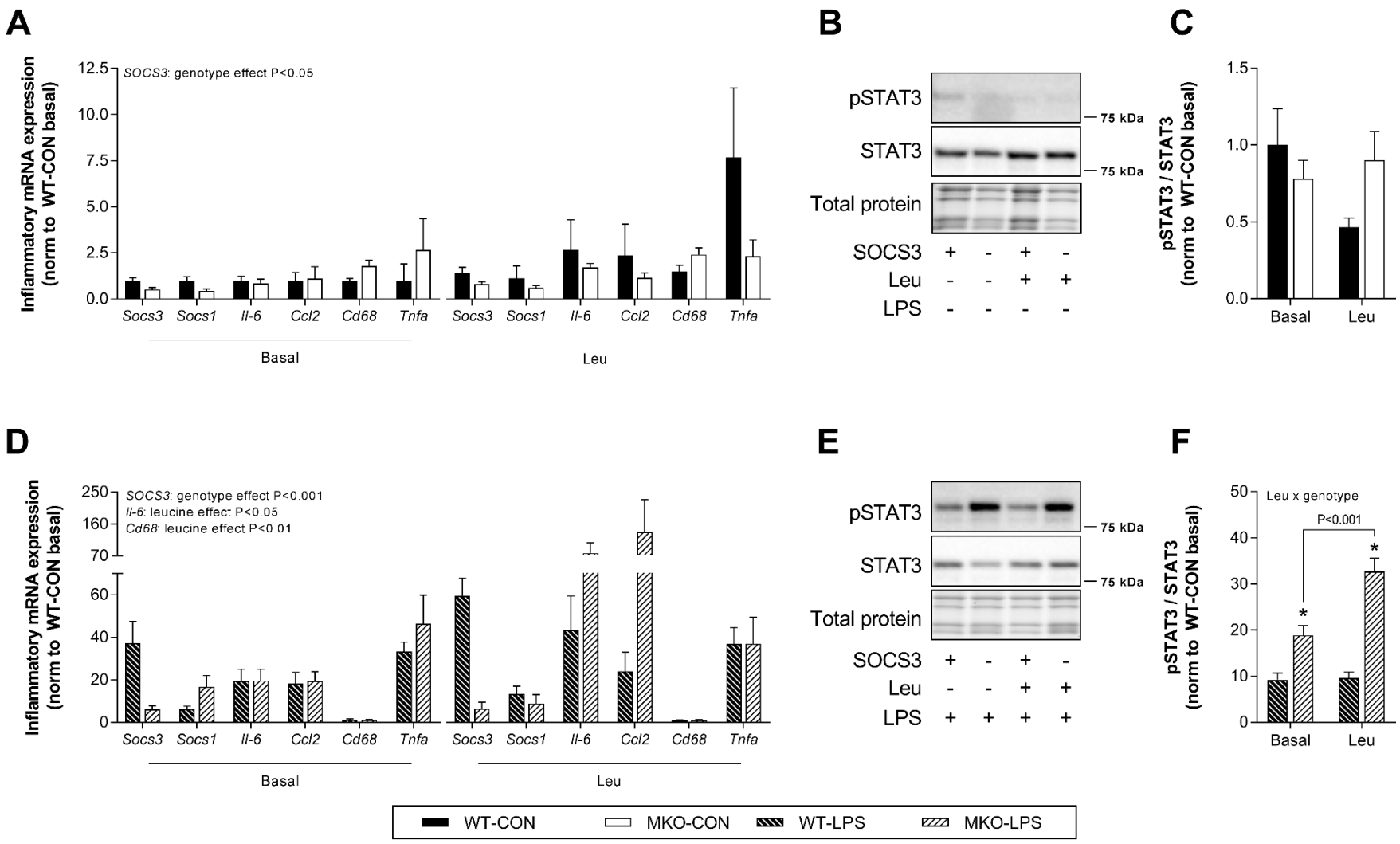
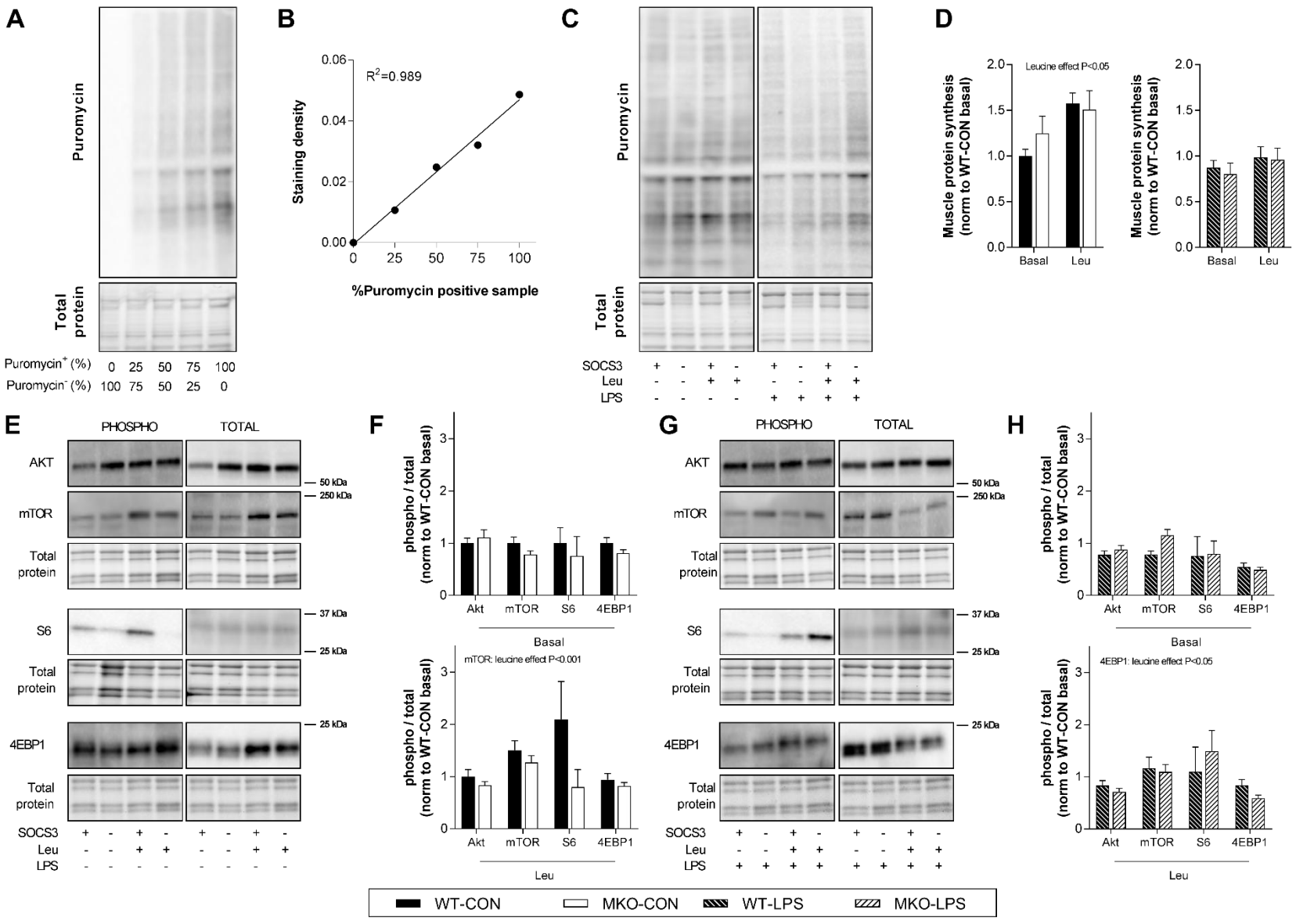

A
Inflammatory mRNA expression (norm to WT-CON basal)
SOCS3: genotype effect P<0.05
Socs3 Socs1 Il-6 Ccl2 Cd68 Tnfa Socs3 Socs1 Il-6 Ccl2 Cd68 Tnfa
Basal
Leu
B
pSTAT3
STAT3
Total protein
75 kDa
75 kDa
SOCS3 + - + -
Leu - - + +
LPS - - - -
C
pSTAT3 / STAT3 (norm to WT-CON basal)
Basal Leu
D
Inflammatory mRNA expression (norm to WT-CON basal)
SOCS3: genotype effect P<0.001
Il-6: leucine effect P<0.05
Cd68: leucine effect P<0.01
Socs3 Socs1 Il-6 Ccl2 Cd68 Tnfa Socs3 Socs1 Il-6 Ccl2 Cd68 Tnfa
Basal
Leu
E
pSTAT3
STAT3
Total protein
75 kDa
75 kDa
SOCS3 + - + -
Leu - - + +
LPS + + + +
F
pSTAT3 / STAT3 (norm to WT-CON basal)
Leu x genotype
P<0.001
Basal Leu
WT-CON MKO-CON WT-LPS MKO-LPS

Figure 1: Muscle-specific deletion of SOCS3 exacerbates STAT3 Tyr705 phosphorylation following LPS and leucine administration.

mRNA expression of inflammatory molecules in WT and SOCS3-MKO mice 4 hours after an injection with either saline (A) or LPS (D; 1 mg/kg) and 1 hour after an injection of either saline (Basal) or leucine (Leu). Representative immunoblots and quantification of STAT3 Tyr705 phosphorylation (B, C, E, F). Data are normalized to 'WT-CON basal' for each measurement, and presented as mean ± SEM of 8 animals per group. Significant differences between pSTAT3 in KO-LPS Basal and Leu mice, $P<0.001$. * Significantly different between WT-LPS and KO-LPS treated mice ($P<0.05$). Main effects are in the top left corner of each graph where appropriate. WT-CON, wild-type saline treated control; MKO-CON, SOCS3-MKO saline treated control; WT-LPS, wild-type LPS treated; MKO-LPS, SOCS3-MKO LPS treated; Leu, leucine stimulated.


A
Puromycin
Total protein
Puromycin+ (%) 0 25 50 75 100
Puromycin- (%) 100 75 50 25 0
B
R2=0.989
Staining density
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Total protein
SOCS3
Leu
LPS
D
Leucine effect P<0.05
Muscle protein synthesis (norm to WT-CON basal)
Basal
Leu
E
PHOSPHO
TOTAL
AKT
mTOR
S6
4EBP1
Total protein
50 kDa
250 kDa
37 kDa
25 kDa
F
phospho / total (norm to WT-CON basal)
Akt
mTOR
S6
4EBP1
Basal
mTOR: leucine effect P<0.001
Leu
G
H
4EBP1: leucine effect P<0.05
WT-CON
MKO-CON
WT-LPS
MKO-LPS

Figure 2: LPS attenuates leucine-stimulated protein synthesis but does not impair anabolic signaling in WT or SOCS3-MKO mice.

Representative immunoblots for serial dilutions of puromycin-positive with puromycin-negative samples from quadriceps muscle (A). A standard curve (B) was generated to quantify muscle protein synthesis. Quantification of puromycin-labelled proteins (C) was corrected using representative immunoblots and the puromycin standard curve (D). Anabolic signaling was measured (E, G) and quantified (F, H). Data are normalized to 'WT-CON basal' for each measurement, and presented mean ± SEM of 8 animals per group. Significant differences are shown where appropriate ($P<0.05$). Main effects are in the top left corner of each graph where appropriate. WT-CON, wild-type saline treated control; MKO-CON, SOCS3-MKO saline treated control; WT-LPS, wild-type LPS treated; MKO-LPS, SOCS3-MKO LPS treated; Leu, leucine stimulated.