Supplementary information

Sepsis-associated skeletal muscle wasting is ameliorated by pharmacological inhibition of the STAT3 signaling pathway in mice

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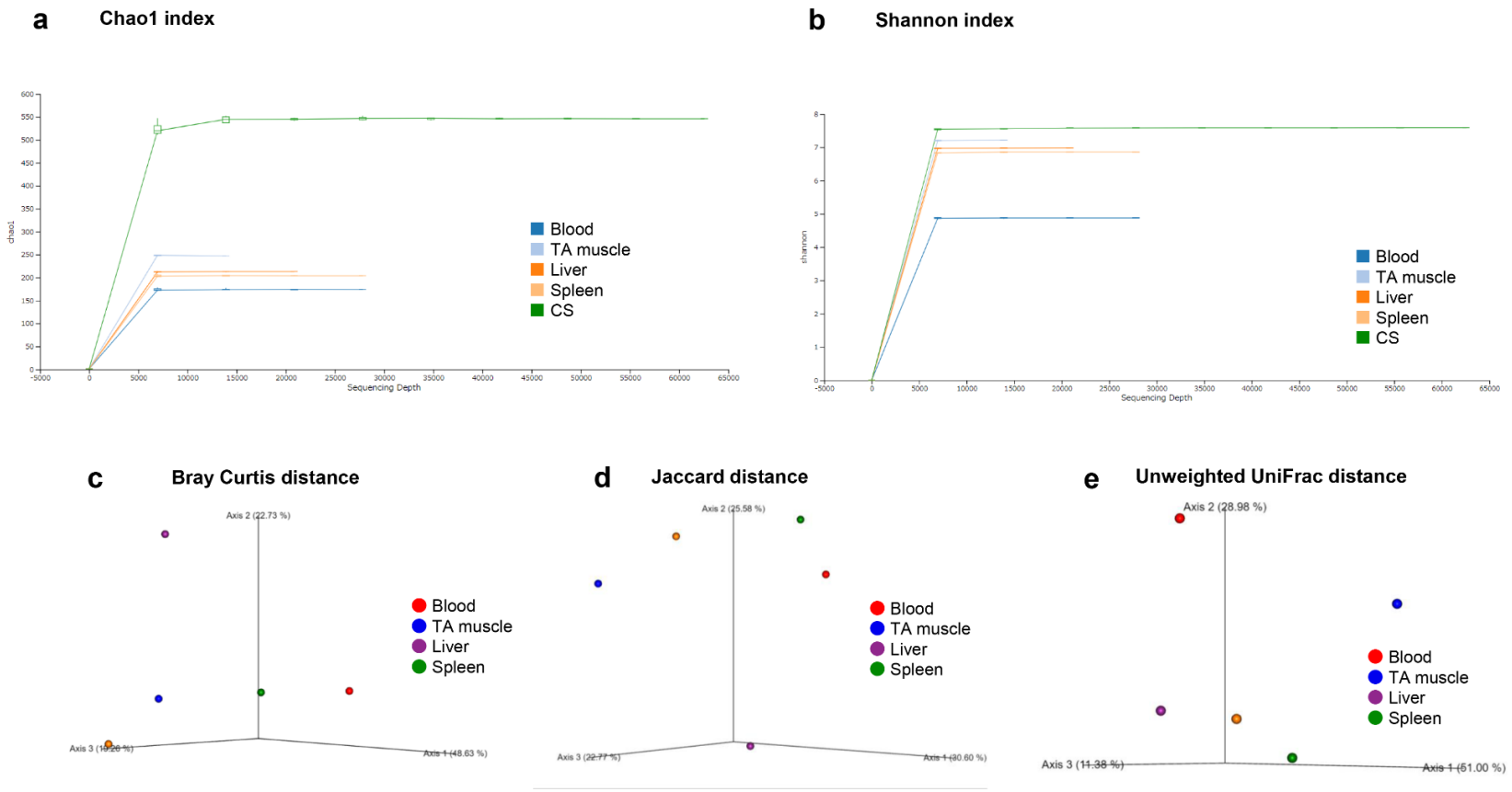
Supplementary information

Contents

Supplementary information

Supplementary information

Supplementary Fig. S1

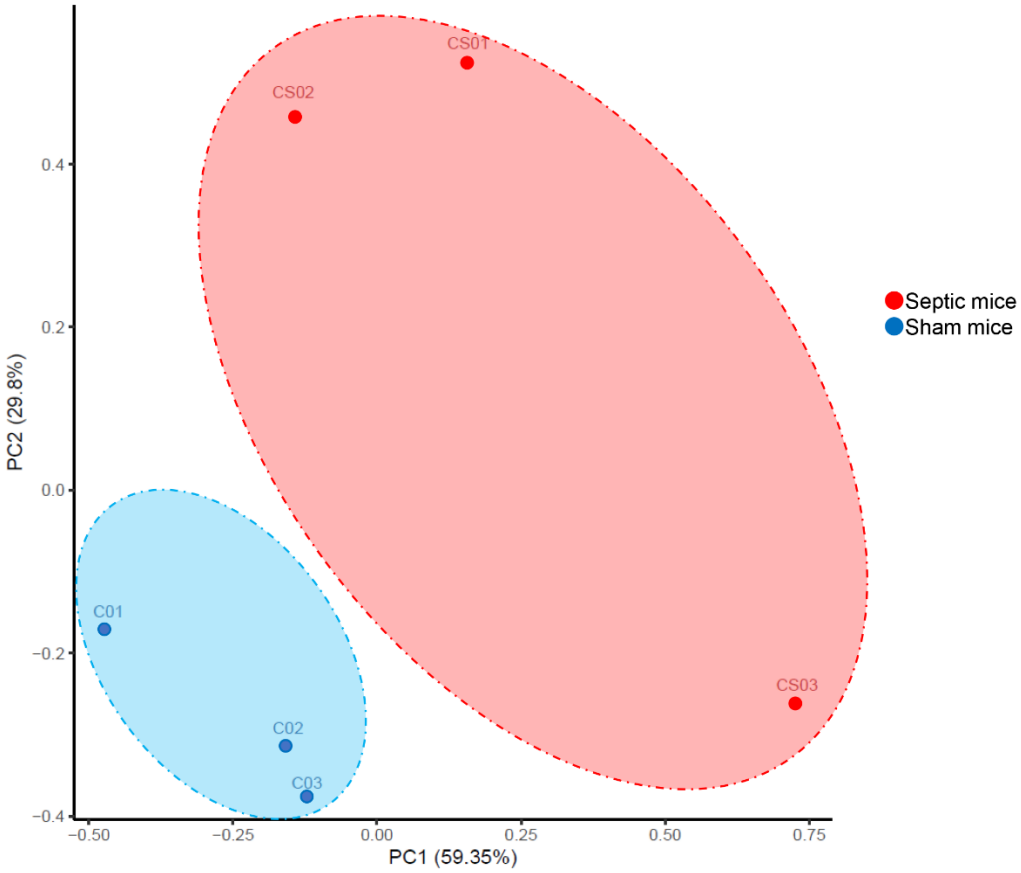


Supplementary Fig. S1. Microbial diversity metrics of the CS preparation and blood, TA muscle, liver, and spleen samples collected from septic mice

Wild-type C57BL/6 mice (8- to 10-week-old males) received an i.p. injection of CS (1.0 mg/g BW) to induce sepsis and were sacrificed 24 h later to harvest blood, TA muscle, liver, and spleen for 16S rRNA gene sequencing analysis of microbial diversity metrics.
(a, b) Microbial diversity within samples (α-diversity) assessed using the Chao1 index (a) and Shannon index (b).
(c–e) Microbial diversity across samples (β-diversity) estimated based on Bray-Curtis distance (c), Jaccard distance (d), and unweighted UniFrac distance (e).

Supplementary information

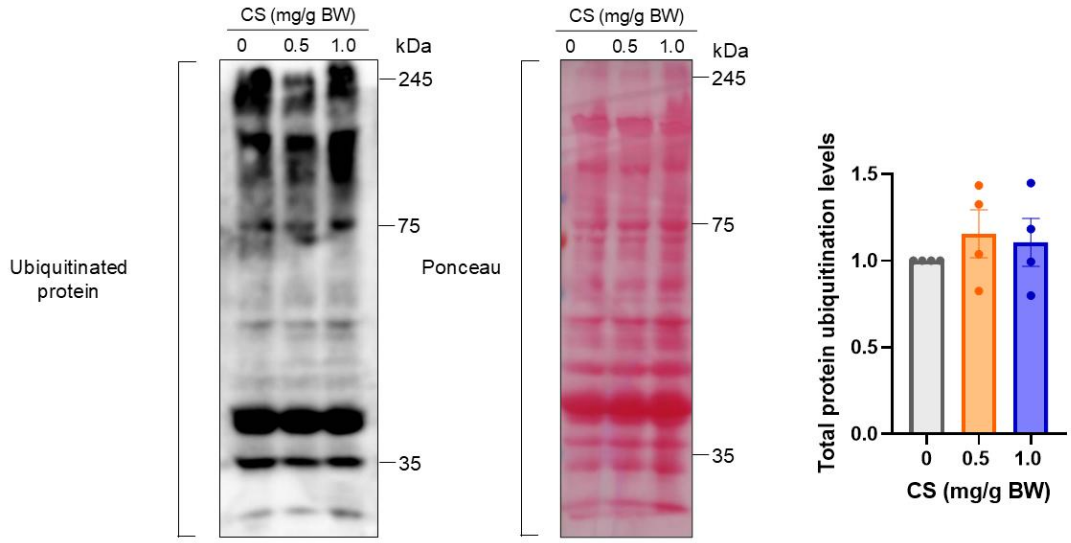
Supplementary Fig. S2



Supplementary Fig. S2. PCA plot of RNA-seq of TA muscles from septic and sham mice, showing a clear separation between the two groups.

Supplementary information

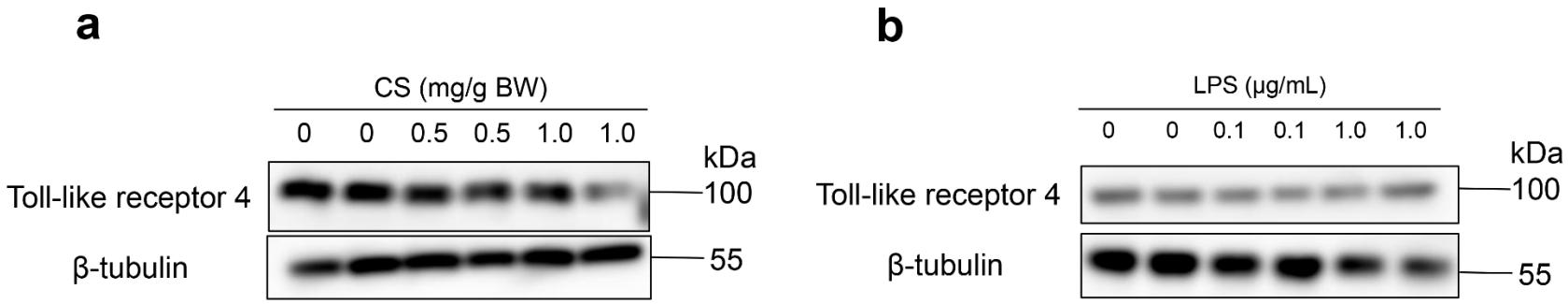
Supplementary Fig. S3



Supplementary Fig. S3. Total protein ubiquitination was not significantly affected in CS-injected mice compared to the sham control mice.
Immunoblotting and corresponding quantification of total ubiquitinated protein in TA muscles of mice at 24 h post injection of CS (0.5 mg/g or 1.0 mg/g BW) or vehicle (N = 4/group). Ponceau S staining was used to normalize the total protein ubiquitination levels, and the ratio in the sham control mice was set to 1. Data are expressed as mean ± SEM. The original, uncropped, and minimally adjusted images of these immunoblots are provided as raw image 13 in the supplementary information.

Supplementary information

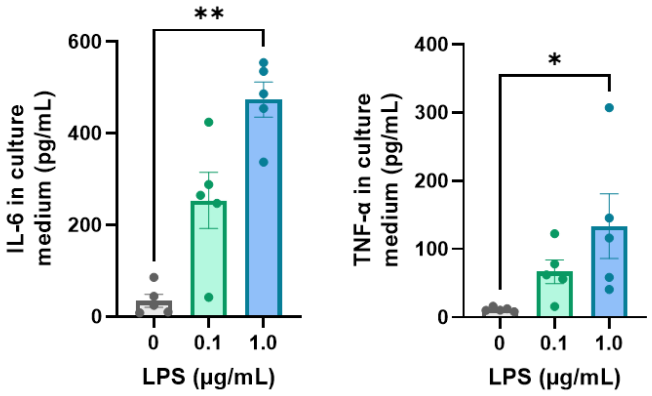
Supplementary Fig. S4



Supplementary Fig. S4. Expression of TLR4 protein in TA muscles and C2C12 myotubes.
(a, b) Immunoblotting detection of TLR4 proteins in the following: TA muscles obtained from mice at 72 h post injection of CS (0.5 mg/g, 1.0 mg/g BW) or vehicle (a); C2C12 myotubes incubated with LPS (0.1 μg/mL, 1.0 μg/mL) or PBS for 48 h (b).
The original, uncropped, and minimally adjusted immunoblot images are presented in Raw image 14 in the supplemental information files.

Supplementary information

Supplementary Fig. S5

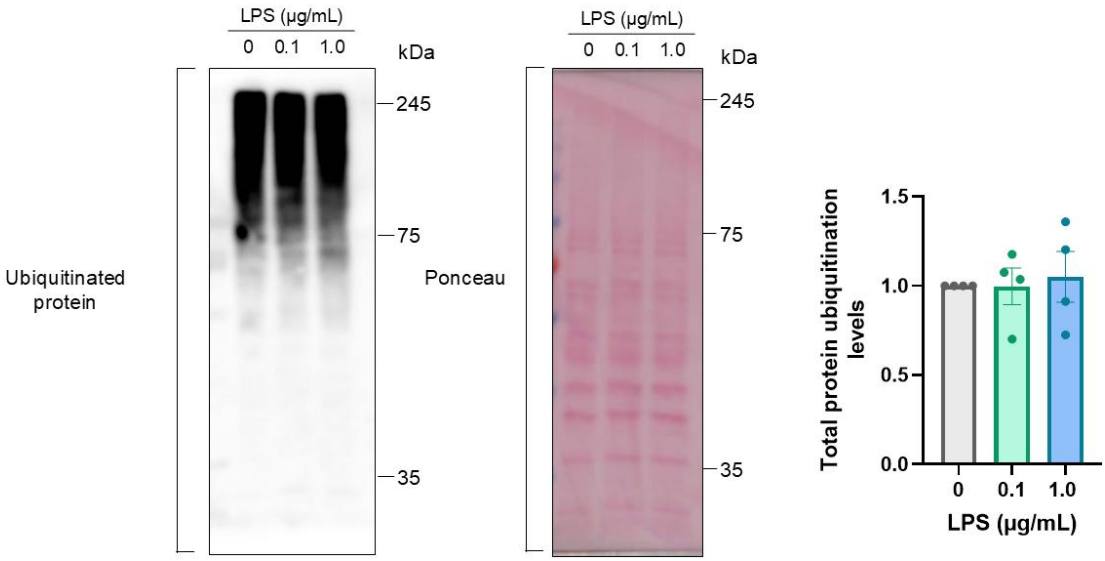


Supplementary Fig. S5. IL-6 and TNF-α concentrations in culture supernatant in LPS-treated C2C12 myotubes

C2C12 myotubes were treated with PBS or LPS (0.1 or 1 μg/mL). After 4 h, cell culture supernatant was collected and IL-6 and TNF-α concentrations were measured by ELISA. N = 5/group. All data are expressed as means ± SEM; **p < 0.01, *p < 0.05.

Supplementary information

Supplementary Fig. S6

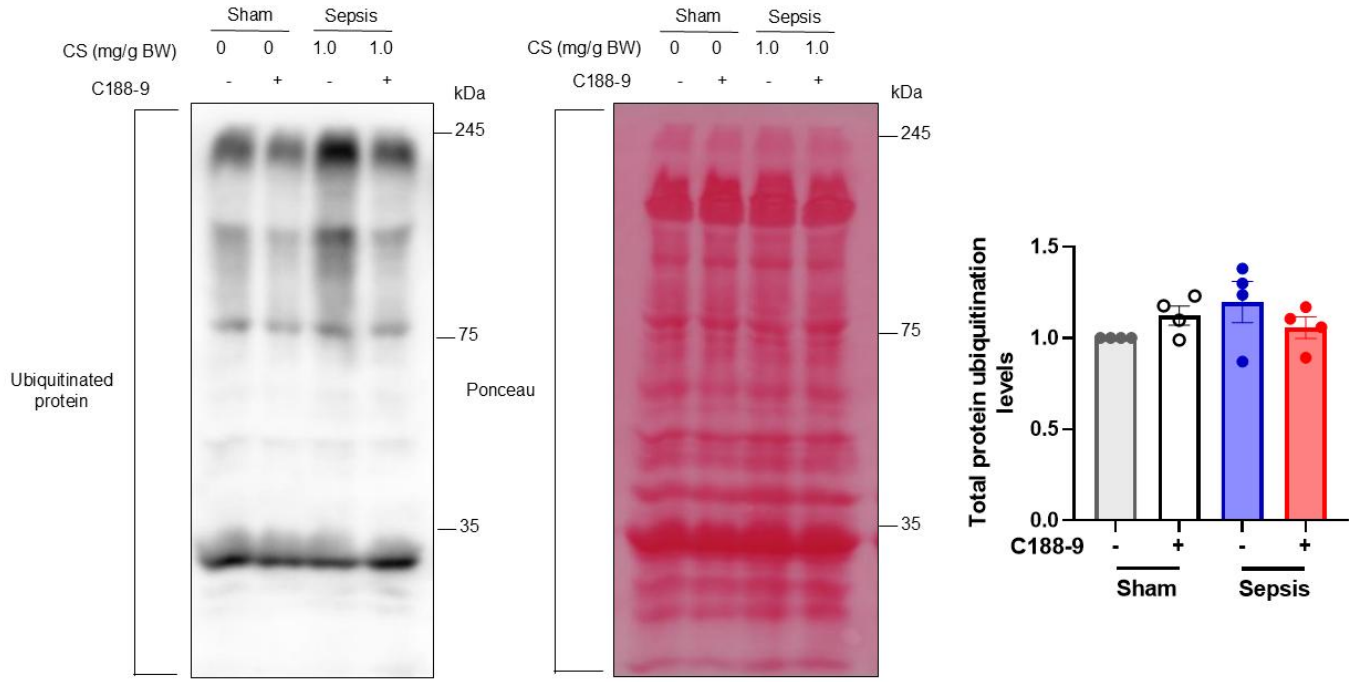


Supplementary Fig. S6. Total protein ubiquitination was not significantly affected in LPS-treated C2C12 myotubes compared with PBS-treated C2C12 myotubes

Immunoblotting and corresponding quantification of total ubiquitinated proteins in C2C12 myotubes cultured in LPS or PBS for 24 h (N = 4/group). Ponceau S staining was used to normalize the total protein ubiquitination levels, and the ratio in the sham control cells was set to 1. Data are expressed as mean ± SEM. The original, uncropped, and minimally adjusted images of these immunoblots are provided as raw image 15 in the supplementary information.

Supplementary information

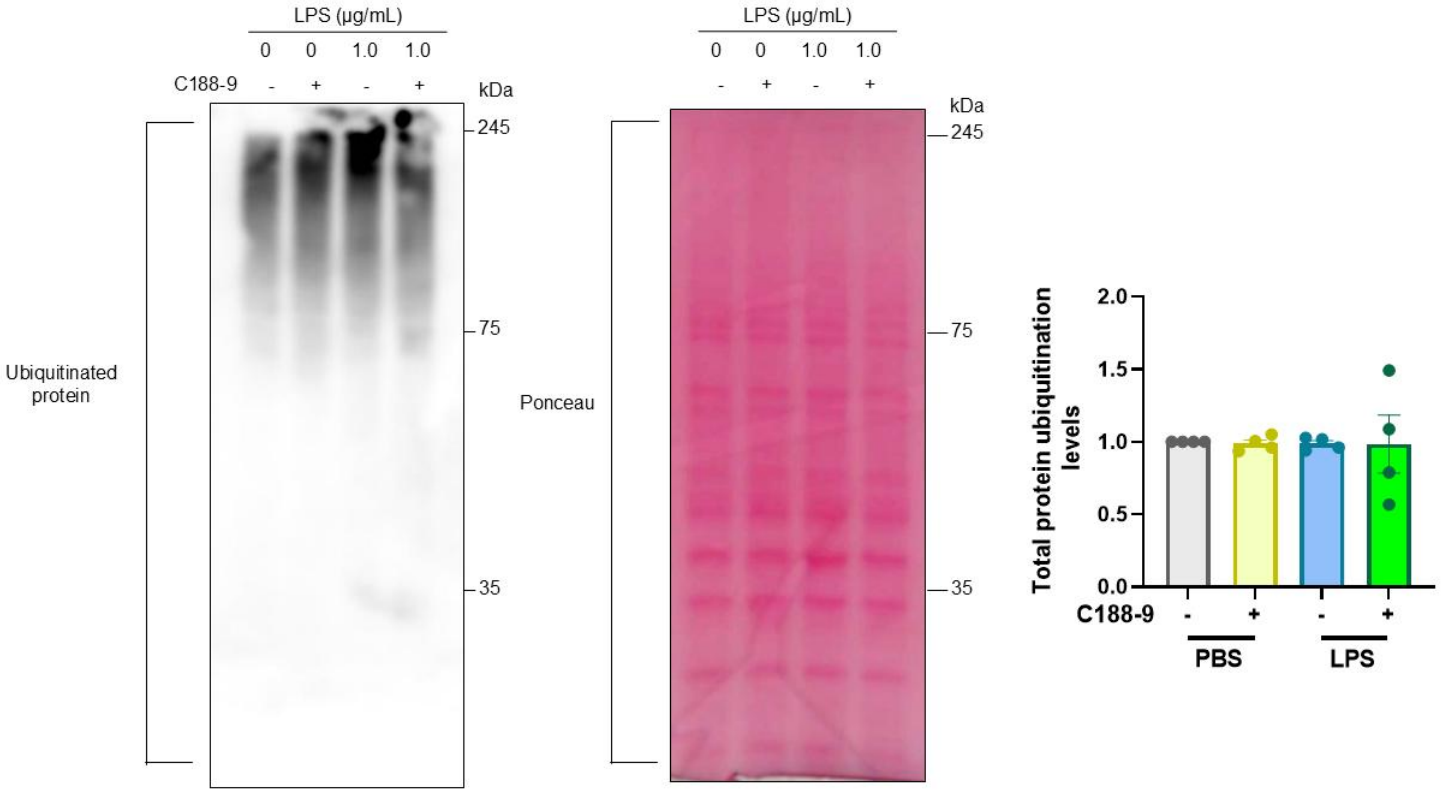
Supplementary Fig. S7



Supplementary Fig. S7 C188-9 treatment did not alter total protein ubiquitination levels in the septic mice
Immunoblotting and corresponding quantification of the total ubiquitinated protein levels in the TA muscles of the septic and sham mice harvested 24 h after the indicated treatments (N = 4/group). Ponceau S staining was used to normalize the total protein ubiquitination levels, and the ratio in the sham vehicle-treated mice was designated as 1 (N = 4/group). Data are expressed as mean ± SEM. The original, uncropped, and minimally adjusted images of these immunoblots are provided as raw image 16 in the supplementary information.

Supplementary information

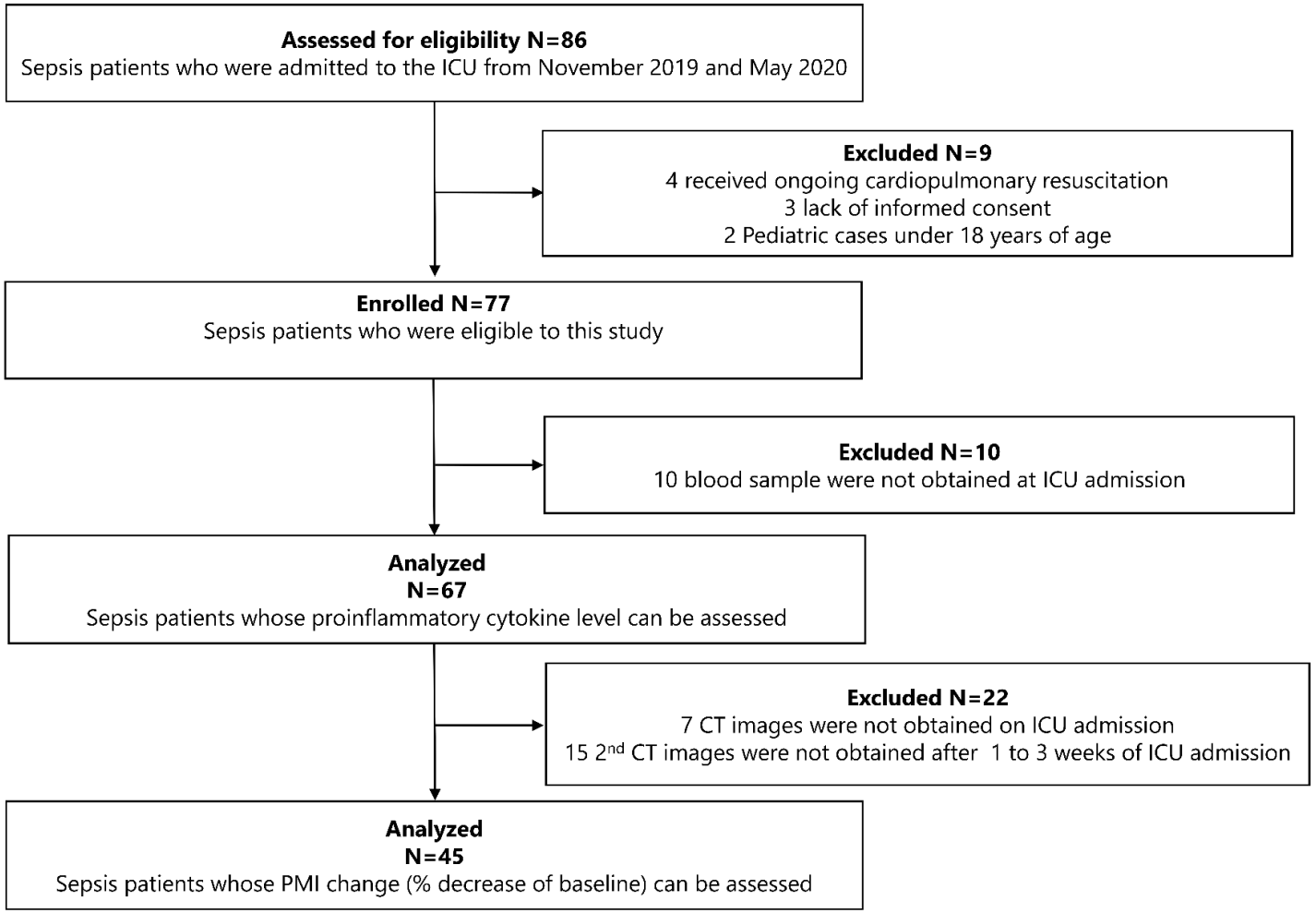
Supplementary Fig. S8



Supplementary Fig. S8 C188-9 treatment did not alter the total protein ubiquitination levels in the LPS-treated C2C12 cells
Immunoblotting and corresponding quantification of total ubiquitinated proteins in C2C12 myotubes cultured with vehicle or C188-9 and exposed to LPS for 24 h (N = 4/group). Ponceau S staining was used to normalize total protein ubiquitination levels, and the ratio in sham control cells was set at 1. was designated as 1 (N = 4/group). Data are expressed as mean ± SEM.
The original, uncropped, and minimally adjusted images of these immunoblots are provided as raw images 17 in the supplementary information.

Supplementary information

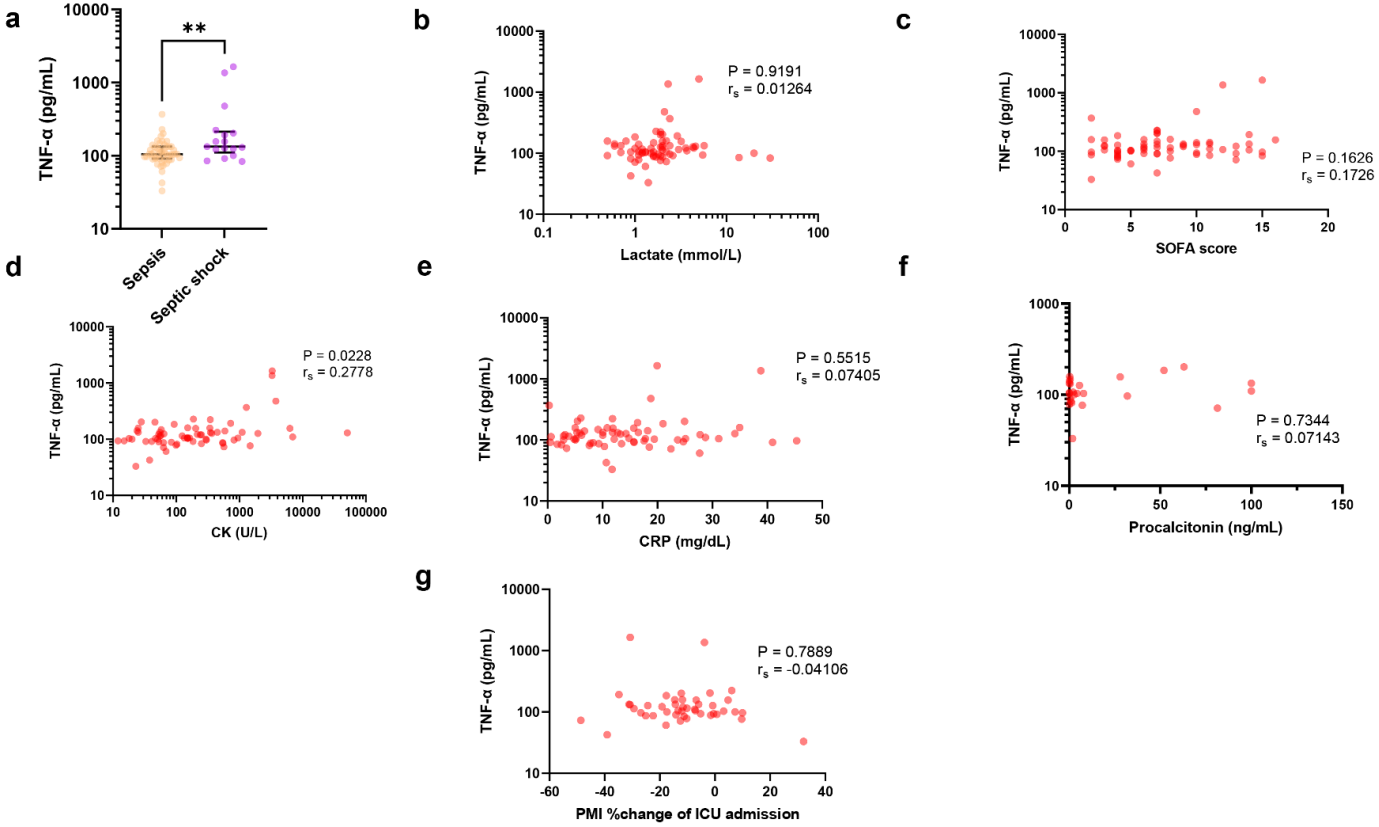
Supplementary Fig. S9



Supplementary Fig. S9. Flow chart of participants in the prospective observational study of ICU sepsis patients

Supplementary information

Supplementary Fig. S10



Supplementary Fig. S10. Correlation between TNF-α levels at ICU admission and disease severity and skeletal muscle atrophy in sepsis patients

(a) Column scatter plot of TNF-α levels in plasma from patients with sepsis (N = 50) and septic shock (N = 17). Circles, horizontal bars, and vertical bar represent the data distribution, median, and interquartile range, respectively. (b–f) Correlation analysis of TNF-α levels at ICU admission with lactate levels (b), SOFA scores (c), and CK (d), CRP (e), and procalcitonin (f) levels in sepsis patients. (b–e) N = 67; (f) N = 25. Plasma procalcitonin concentrations were not measured at ICU admission in 42 patients with sepsis. Correlations evaluated using Spearman's rank-order coefficient (r_s). (g) Correlation analysis of TNF-α levels at ICU admission with changes in PMI between ICU admission and weeks 1–3 post ICU admission in patients with sepsis (N = 45). Correlations evaluated using Spearman's rank-order coefficient (r_s). The minimally anonymized clinical dataset used to generate Supplementary Fig.S10 can be found in Supplementary Data S4.

Supplementary information

Supplementary Table S1. Clinical characteristics of the study participants

Characteristics	Sepsis patients (n=67)
Age	72 (65-79)
Sex	
Male	44 (65.7)
Female	23 (34.3)
Height (cm)	163.0 (150.5-169.0)
Body weight (kg)	52.0 (43.8-61.4)
Body mass index (kg/m^2)	20.5 (18.0-22.7)
Severity	
Septic shock on admission to ICU	17 (25.4)
SOFA score on admission to ICU	7 (4-10)
Laboratory data on admission	
CRP	11.9 (6.0-19.1)
Procalcitonin	2.1 (0.4-28.0)
Lactate	1.9 (1.2-2.4)
CK	152 (54-495)
Comorbidity	
Hypertension	21 (31.3)
Diabetes	18 (26.9)
Cancer	16 (23.9)
Autoimmune disorder	15 (22.4)
Stroke	11 (16.4)
Renal failure	8 (11.9)
Chronic obstructive pulmonary disease	6 (9.0)
Asthma	4 (6.0)
Mental disorder	3 (4.5)
Dementia	2 (3.0)
Liver failure	2 (3.0)
Hematological disease	2 (3.0)
Central **n**ervous **s**ystem degenerative disease	2 (3.0)
Endocrine disorder	2 (3.0)
Medication before admission	
Immunosuppressant	4 (6.0)

Supplementary information

Characteristics	Sepsis patients (n=67)
Steroids	10 (14.9)
Anticancer drugs	5 (7.5)
Primary causes of sepsis	
Pneumonia	27 (40.3)
Intra-abdominal infection	11 (16.4)
Urinary **t**ract **i**nfection	8 (11.9)
Soft tissue infection	6 (9.0)
Catheter related bloodstream infection	2 (3.0)
Others	14 (20.9)
Blood cultures positive	37 (55.2)
Identified microorganisms on blood culture	
Gram-positive bacteria	19 (28.4)
Staphylococcus aureus	7 (10.4)
Enterococcus faecalis	3 (4.5)
Staphylococcus epidermidis	2 (3.0)
Clostridium perfringens	1 (1.5)
Staphylococcus hominis	1 (1.5)
Streptococcus constellatus	1 (1.5)
Streptococcus dysgalactiae	1 (1.5)
Streptococcus mitis group	1 (1.5)
Streptococcus pneumoniae	1 (1.5)
Streptococcus pyogenes	1 (1.5)
Gram-negative bacteria	20 (29.9)
Escherichia coli	10 (14.7)
Klebsiella pneumoniae	5 (7.4)
Haemophilus influenzae	2 (2.9)
Pseudomonas aeruginosa	1 (1.5)
Enterobacter cloacae	1 (1.5)
Raoultella planticola	1 (1.5)
Fungus	6 (9.0)
Candida albicans	4 (6.0)
Candida glabrata	1 (1.5)
Candida tropicali	1 (1.5)
Treatment	

Supplementary information

Characteristics	Sepsis patients (n=67)
Mechanical ventilator (%)	29 (43.3)
Emergency operation (%)	3 (4.5)
Renal replacement therapy (%)	9 (13.4)
Outcomes	
Length of hospital stay (day)	32 (18-43)
Hospital mortality (%)	12 (17.9)
2-year mortality rate	33 (49.2)

Abbreviations: CK, creatine kinase; CRP, c-reactive protein; ICU, intensive care unit; SOFA, sequential organ failure assessment.

Supplementary information

Supplementary Table S2. Sequences of primers used for qRT-PCR

	Forward primer (5'→3')	Reverse primer (5'→3')
Atrogin-1/MAFbx	CACATTCTCTCCTGGAAGGGC	TTGATAAAGTCTTGAGGGGAAAGTG
MuRF1	CACGAAGACGAGAAGATCAACATC	AGCCCCAAACACCTTGCA
TNF-α	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC
IL-6	CCGGAGAGGAGACTTCACAG	GGAAATTGGGGTAGGAAGGA
GAPDH	ACCACAGTCCATGCCATCAC	CACCACCCTGTTGCTGTAGCC

Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin 6; qRT-PCR, real-time reverse-transcription polymerase chain reaction; TNF-α, tumor necrosis factor-α.

Supplementary information

Supplementary Table S3 Details of primary and secondary antibodies

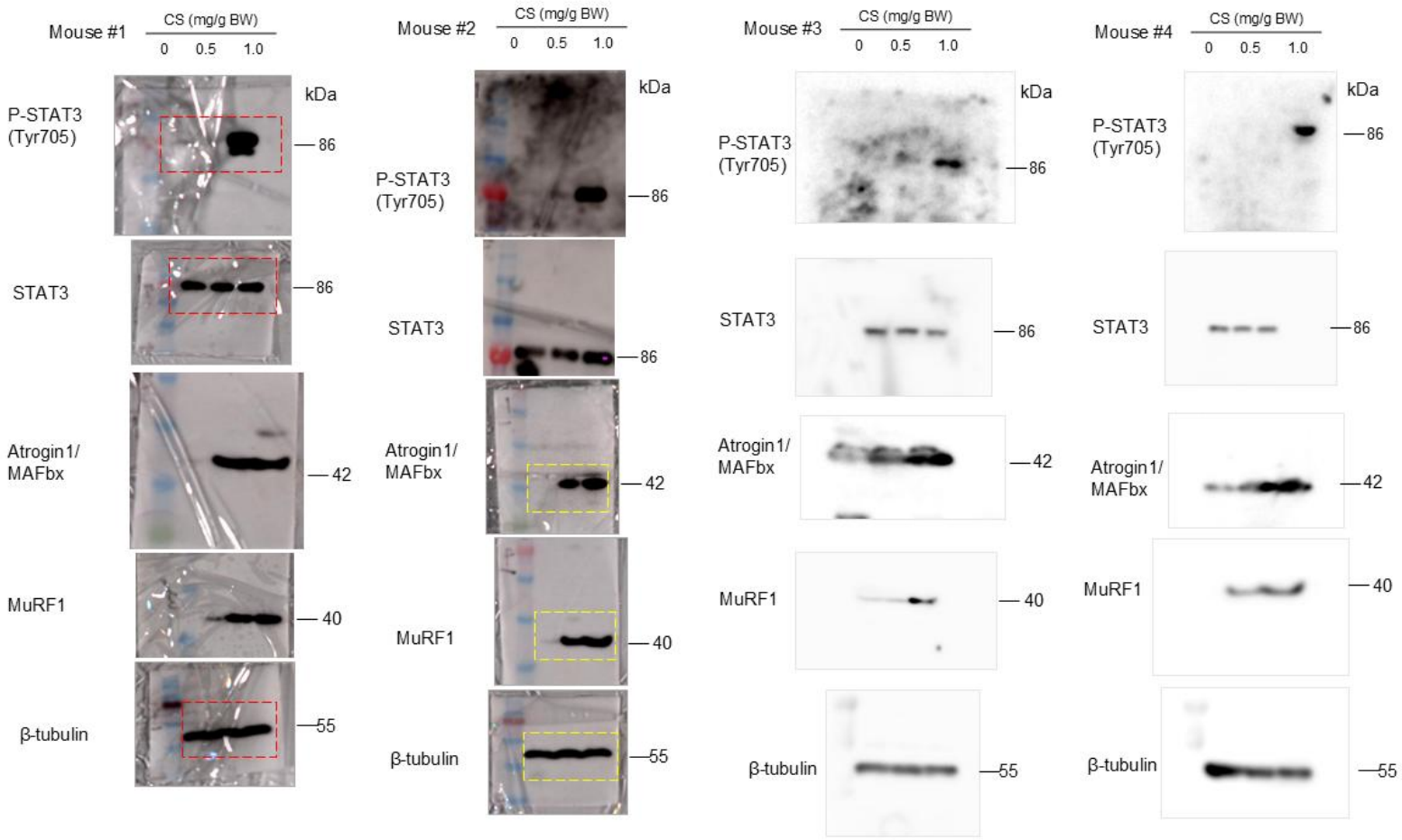
Antibodies	Supplier	Identifier
Anti-Laminin (LAMA1) antibody, rabbit poly clonal, Immunofluorescence staining: 1:50 dilution	MilliporeSigma	Cat# L9393, RRID:AB_477163
Phospho-Stat3 (Tyr705) (D3A7) XP Rabbit mAb, rabbit monoclonal,1:1000 dilution	Cell Signaling Technology	Cat# 9145, RRID:AB_2491009
Stat3 (79D7) Rabbit mAb, rabbit monoclonal, 1:3000 dilution	Cell Signaling Technology	Cat# 4904, RRID:AB_331269
Phospho-p70 S6 Kinase (Thr389) (108D2) Rabbit mAb, rabbit monoclonal, 1:500 dilution	Cell Signaling Technology	Cat# 9234, RRID:AB_2269803
p70 S6 Kinase (49D7) Rabbit mAb, rabbit monoclonal,1:1000 dilution	Cell Signaling Technology	Cat# 2708, RRID:AB_390722
Phospho-Akt (Ser473) Antibody, rabbit monoclonal,1:500 dilution	Cell Signaling Technology	Cat# 9271, RRID:AB_329825
Akt (pan) (11E7) Rabbit mAb, rabbit monoclonal, 1:1000 dilution	Cell Signaling Technology	Cat# 4685, RRID:AB_2225340
Bax Antibody, rabbit polyclonal, 1:1000 dilution	Cell Signaling Technology	Cat# 2772, RRID:AB_10695870
Bcl-2 (D17C4) Rabbit mAb, rabbit monoclonal, 1:1000 dilution	Cell Signaling Technology	Cat# 3498, RRID:AB_1903907
Caspase-3 (D3R6Y) Rabbit mAb, rabbit monoclonal, 1:1000 dilution	Cell Signaling Technology	Cat# 14220, RRID:AB_2798429
TLR4 (25), mouse monoclonal, 1:200 dilution	Santa Cruz Biotechnology	Cat# sc-293072, RRID:AB_10611320
Myosin 4 Monoclonal Antibody (MF20), eBioscience, mouse monoclonal, Western blot: 1:500 dilution, Immunofluorescence staining: 1:100 dilution	Thermo Fisher Scientific	Cat# 14-6503-82, RRID:AB_2572894
Anti-LC3B antibody, rabbit polyclonal, Western blot: 1:3000 dilution, Immunofluorescence staining: 1:2000 dilution	Abcam	Cat# ab51520, RRID:AB_881429
Anti-beta Tubulin antibody - Loading Control, rabbit polyclonal, 1:1000 dilution	Abcam	Cat# ab6046, RRID:AB_2210370
MAFbx Antibody (F-9), mouse monoclonal, 1:50 dilution	Santa Cruz Biotechnology	Cat# sc-166806, RRID:AB_2246982
MuRF1 Antibody (C-11), mouse monoclonal, 1:50 dilution	Santa Cruz Biotechnology	Cat# sc-398608, RRID:AB_2819249
K48-linkage Specific Polyubiquitin (D9D5) Rabbit mAb, rabbit monoclonal, 1:1000 dilution	Cell Signaling Technology	Cat# 8081, RRID: AB_10859893

Supplementary information

Antibodies	Supplier	Identifier
Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP, goat polyclonal secondary, 1:3000 dilution	Thermo Fisher Scientific	Cat# 31460, RRID:AB_228341
Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP, goat polyclonal secondary, 1:3000 dilution	Thermo Fisher Scientific	Cat# 31430, RRID:AB_228307
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, goat polyclonal secondary, 1:400 dilution	Thermo Fisher Scientific	Cat# A-11029, RRID:AB_2534088
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, goat polyclonal secondary, 1:400 dilution	Thermo Fisher Scientific	Cat# A-11008, RRID:AB_143165
Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594, goat polyclonal secondary, 1:400 dilution	Thermo Fisher Scientific	Cat# A-11005, RRID:AB_2534073

Supplementary information

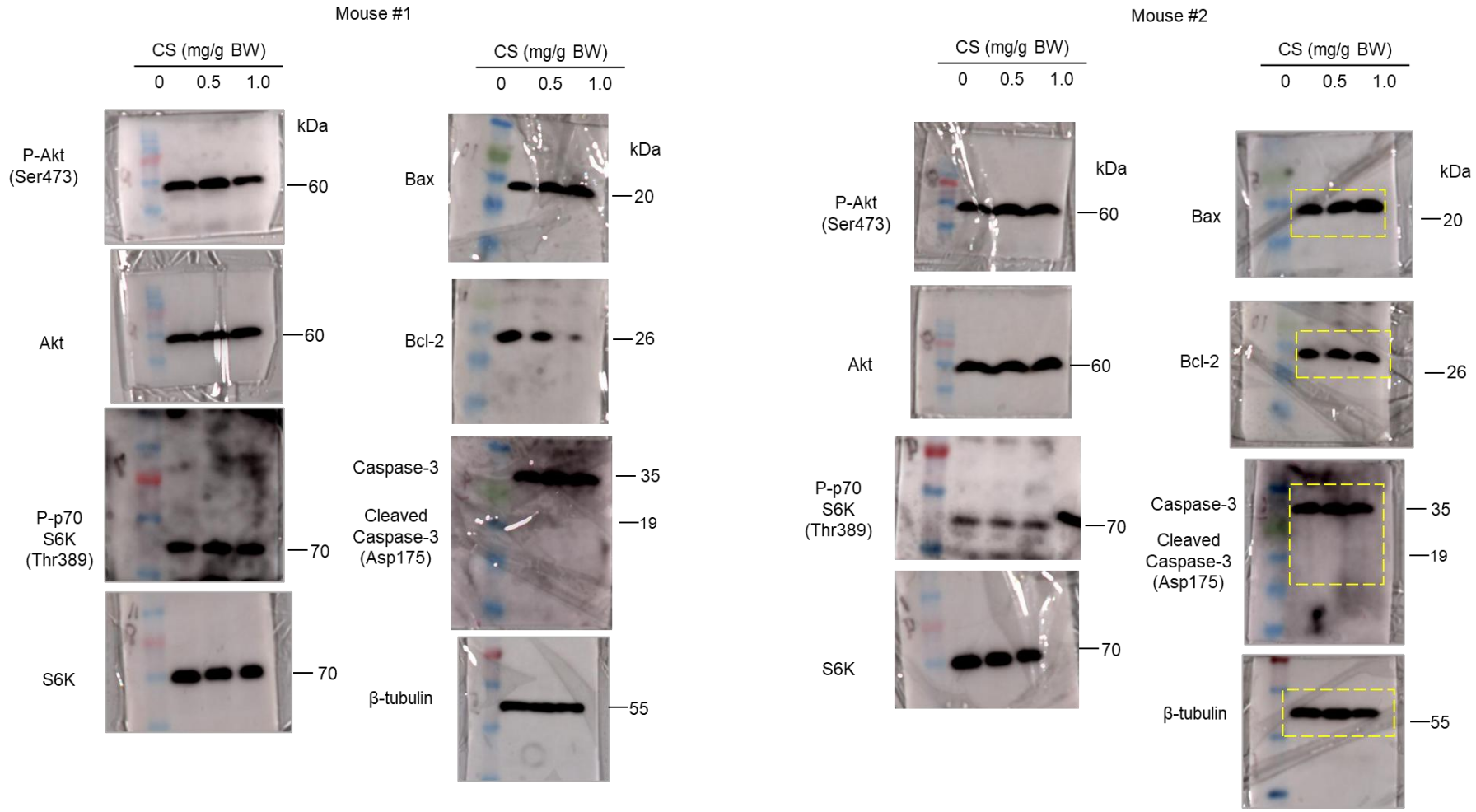
Raw image 1



Raw image 1. Original immunoblots presented in Figs. 3c and 3e.
The dashed line represents the cropped region shown in Fig. 3c (red) and Fig. 3e (yellow).

Supplementary information

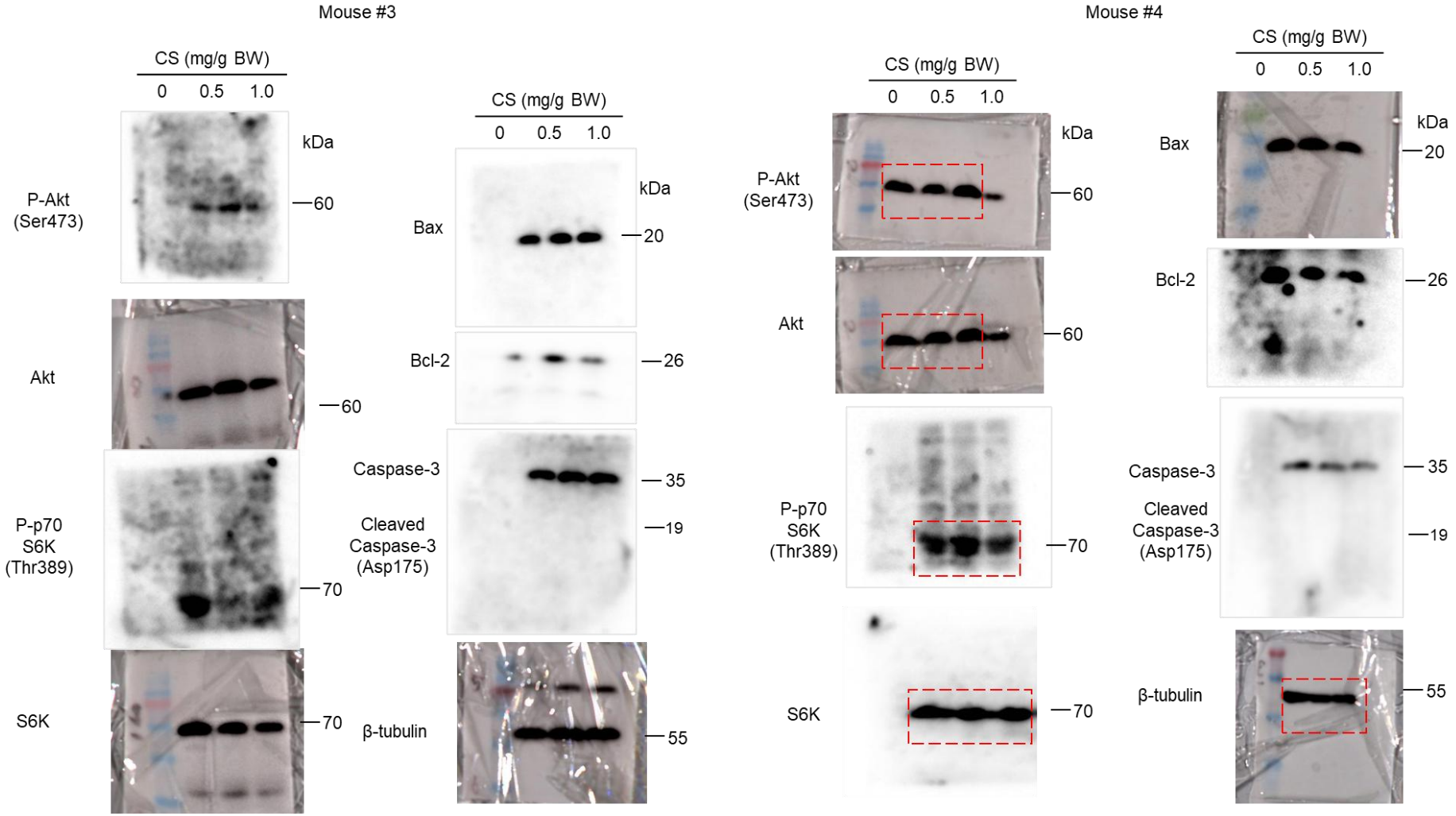
Raw image 2



Raw image 2. Original immunoblots presented in Figs. 3f and 3g.
The dashed line represents the cropped region shown in Fig. 3f (red) and Fig. 3g (yellow).

Supplementary information

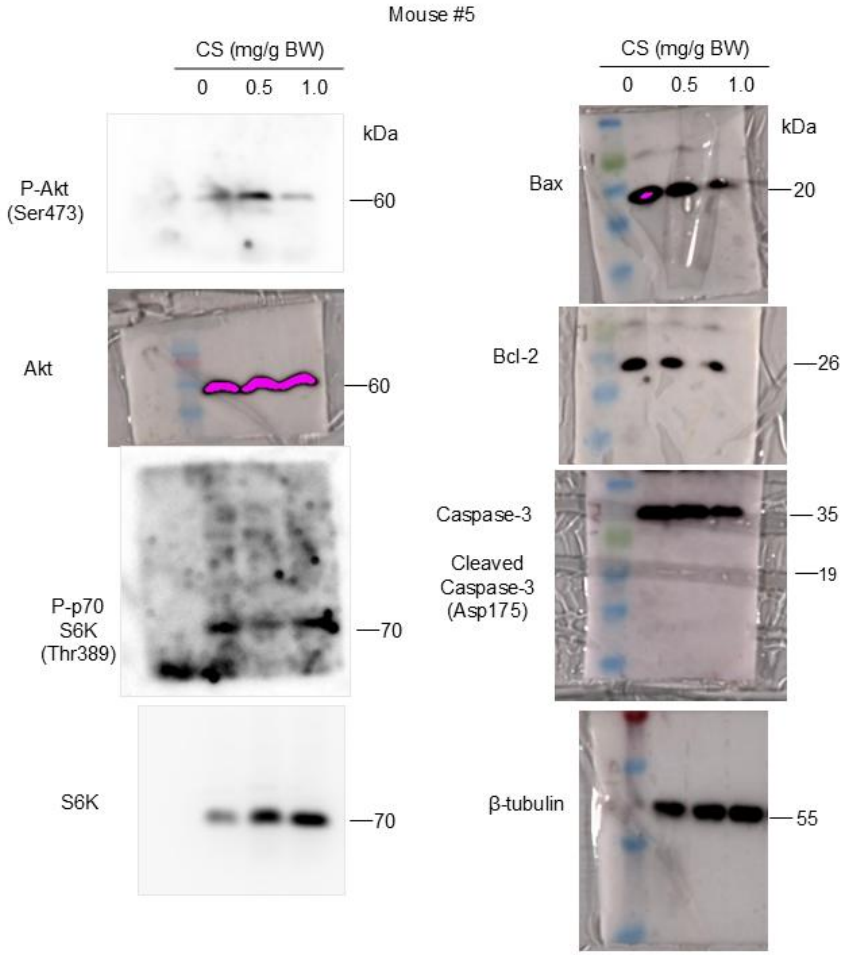
Raw image 2 (continued)



Raw image 2. Original immunoblots presented in Figs. 3f and 3g.
The dashed line represents the cropped region shown in Fig. 3f (red) and Fig. 3g (yellow).

Supplementary information

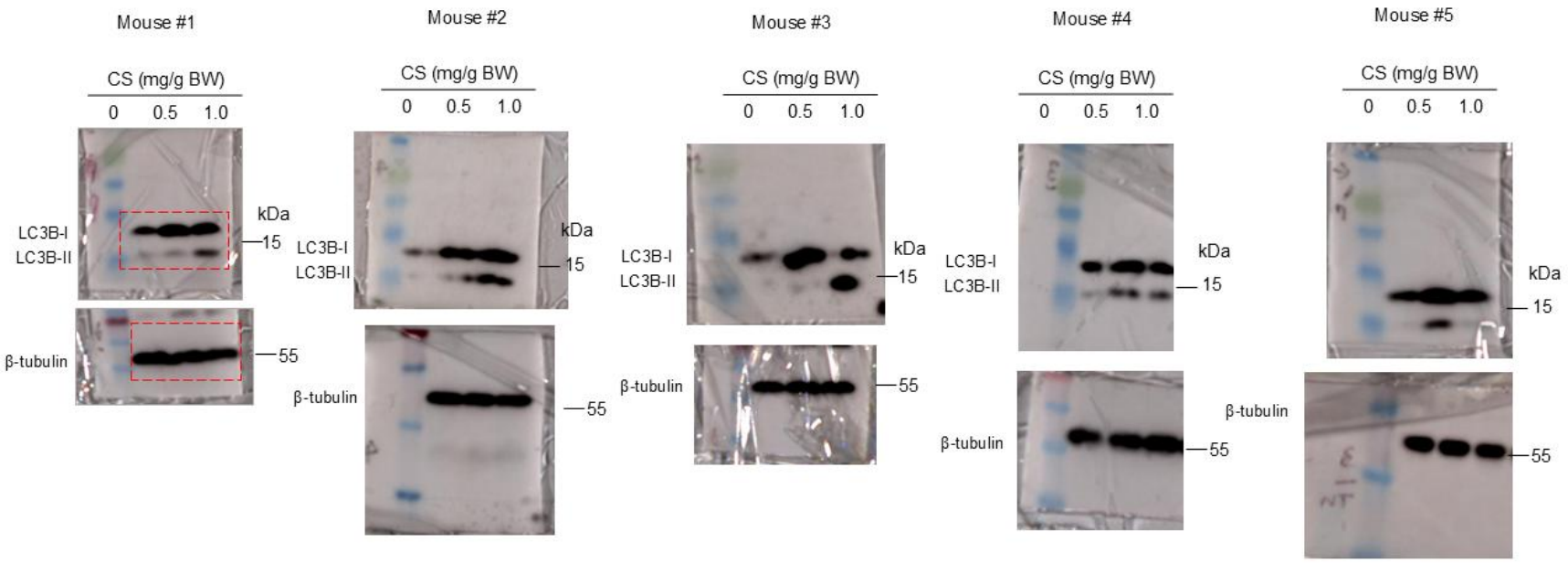
Raw image 2 (continued)



Raw image 2. Original immunoblots presented in Figs. 3f and 3g.
The dashed line represents the cropped region shown in Fig. 3f (red) and Fig. 3g (yellow).

Supplementary information

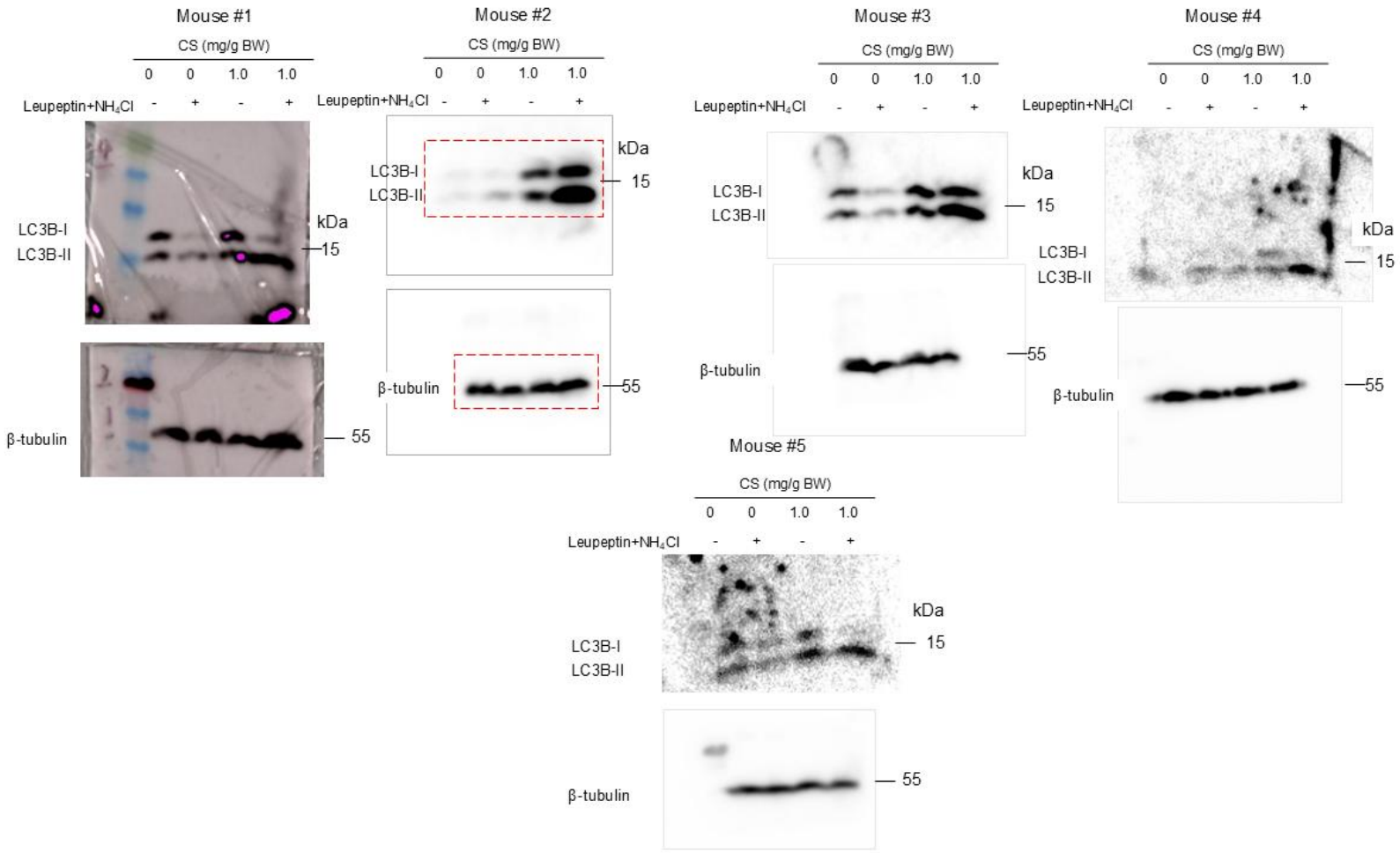
Raw image 3



Raw image 3. Original immunoblots presented in Fig. 3h.
The dashed line represents the cropped region shown in Fig. 3h (red).

Supplementary information

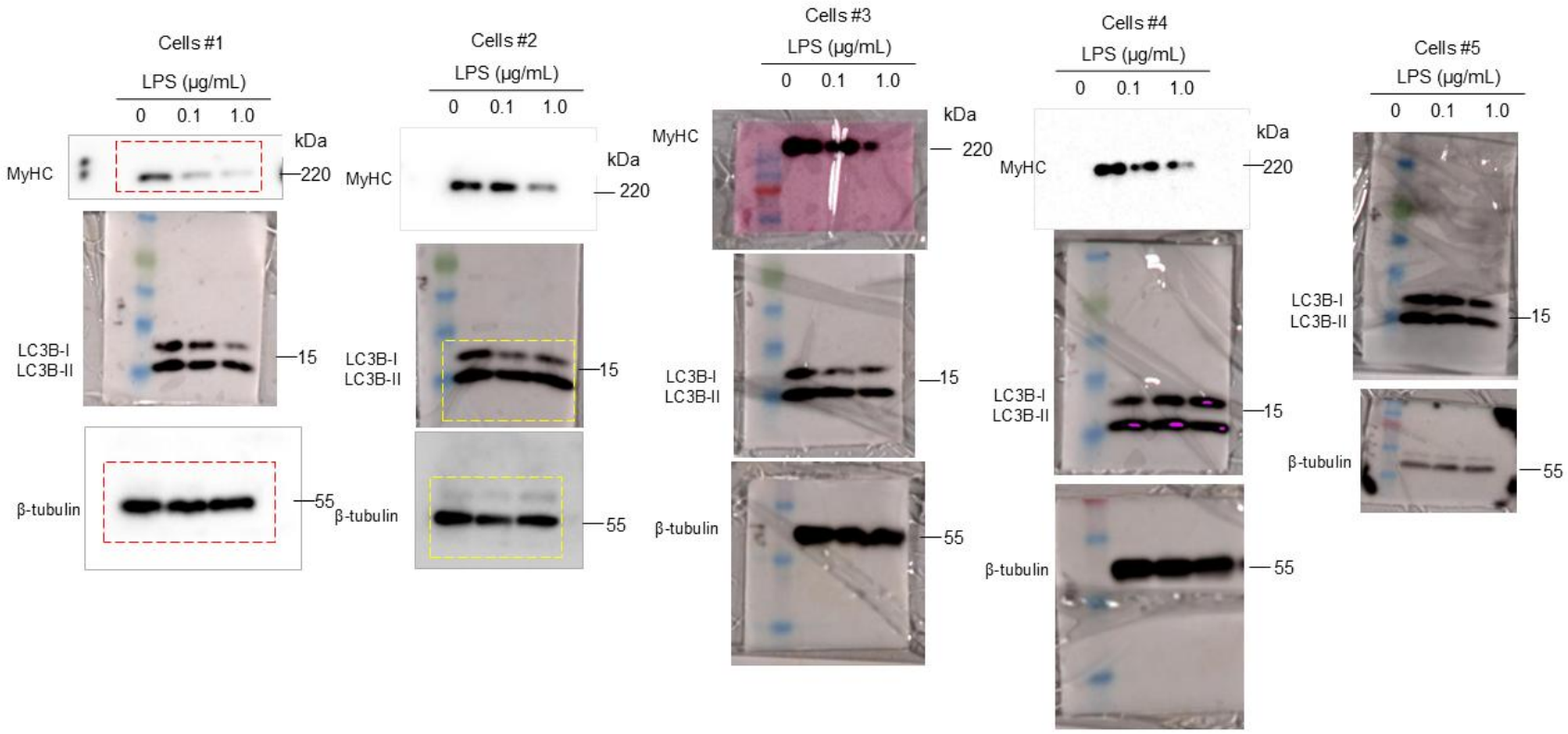
Raw image 4



Raw image 4. Original immunoblots presented in Fig. 3j.
The dashed line represents the cropped region shown in Fig. 3j (red).

Supplementary information

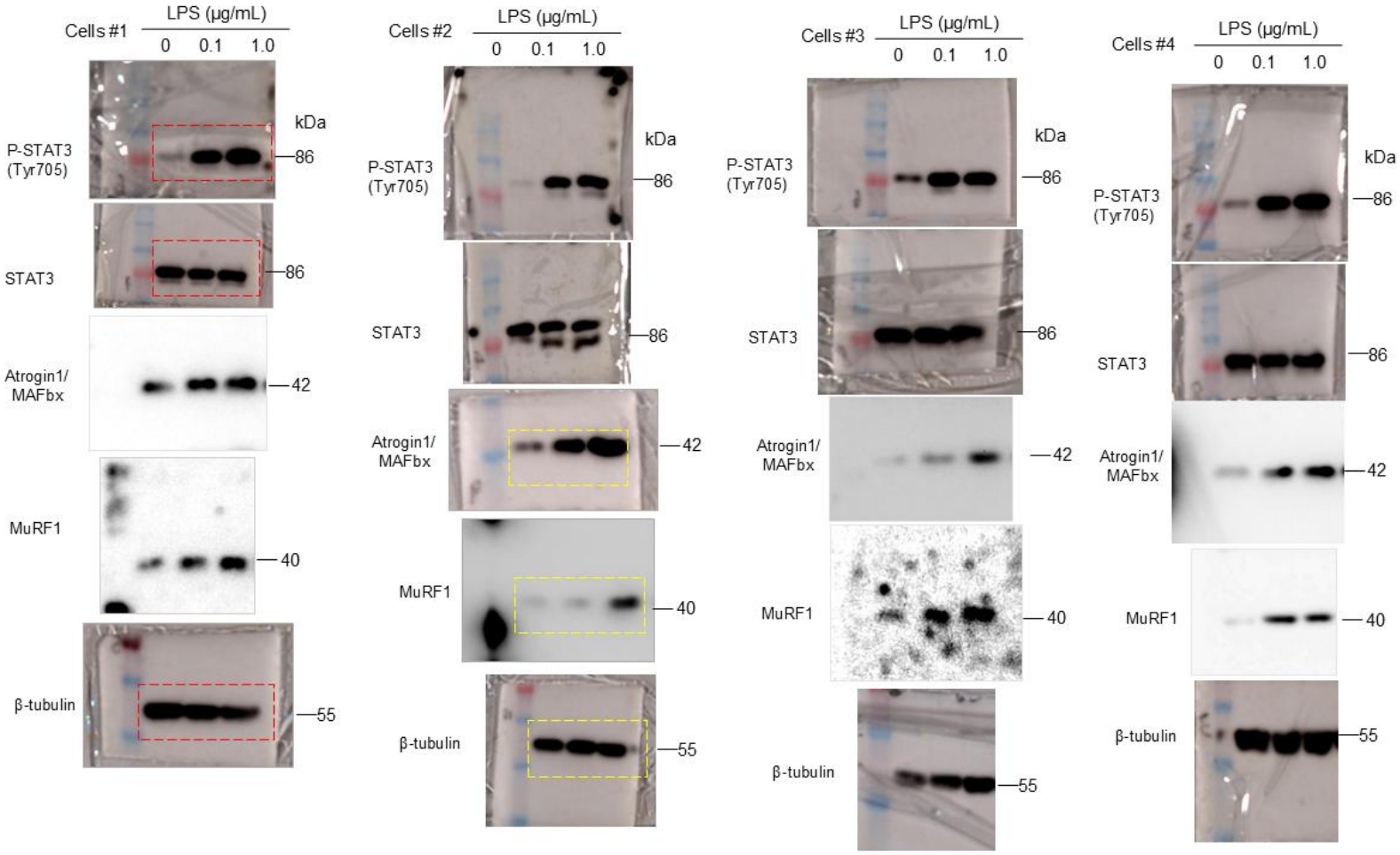
Raw image 5



Raw image 5. Original immunoblots presented in Figs. 4e and 4k.
The dashed line represents the cropped region shown in Fig. 4e (red) and Fig. 4k (yellow).

Supplementary information

Raw image 6



Raw image 6. Original immunoblots presented in Figs. 4g and 4h.
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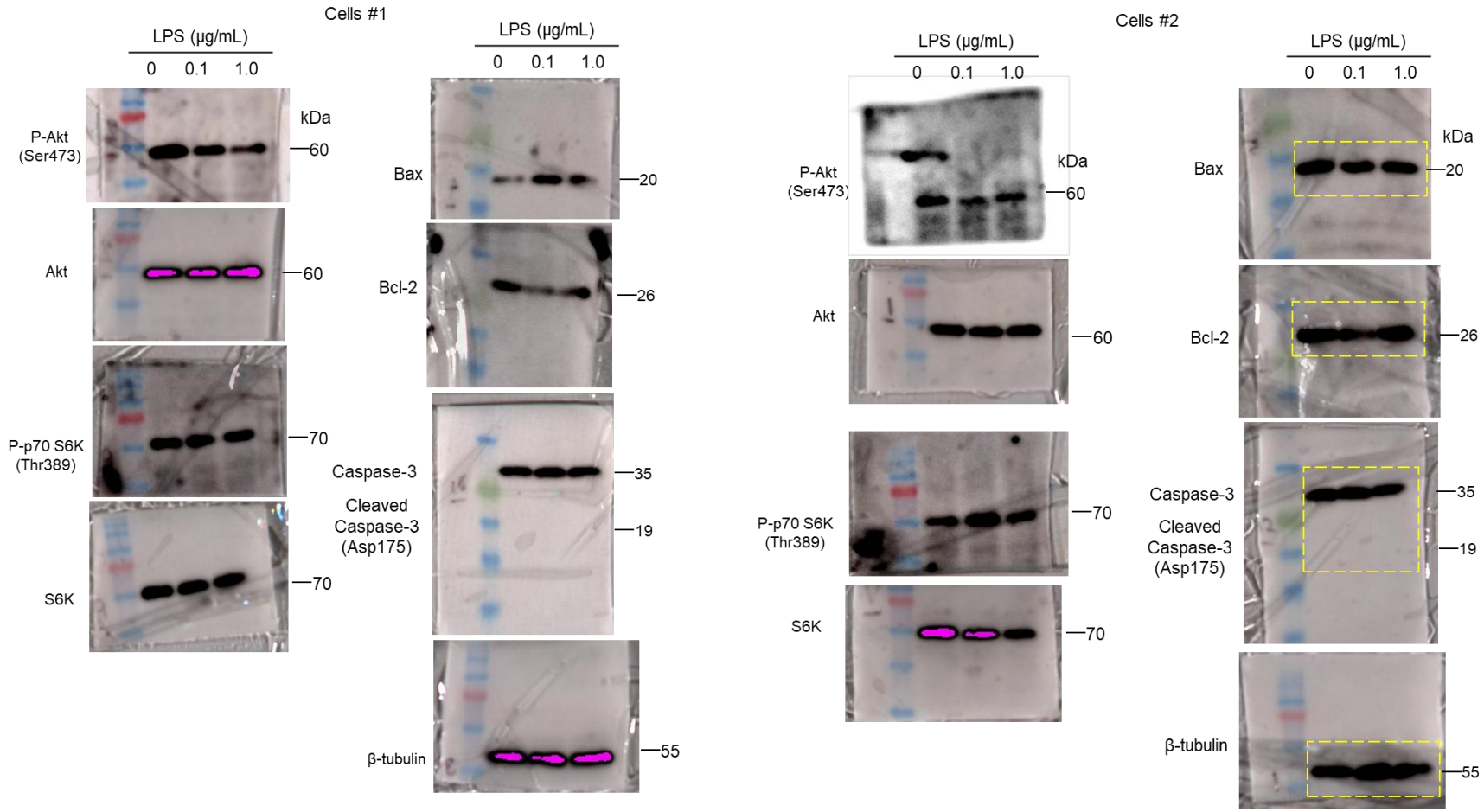
Supplementary information

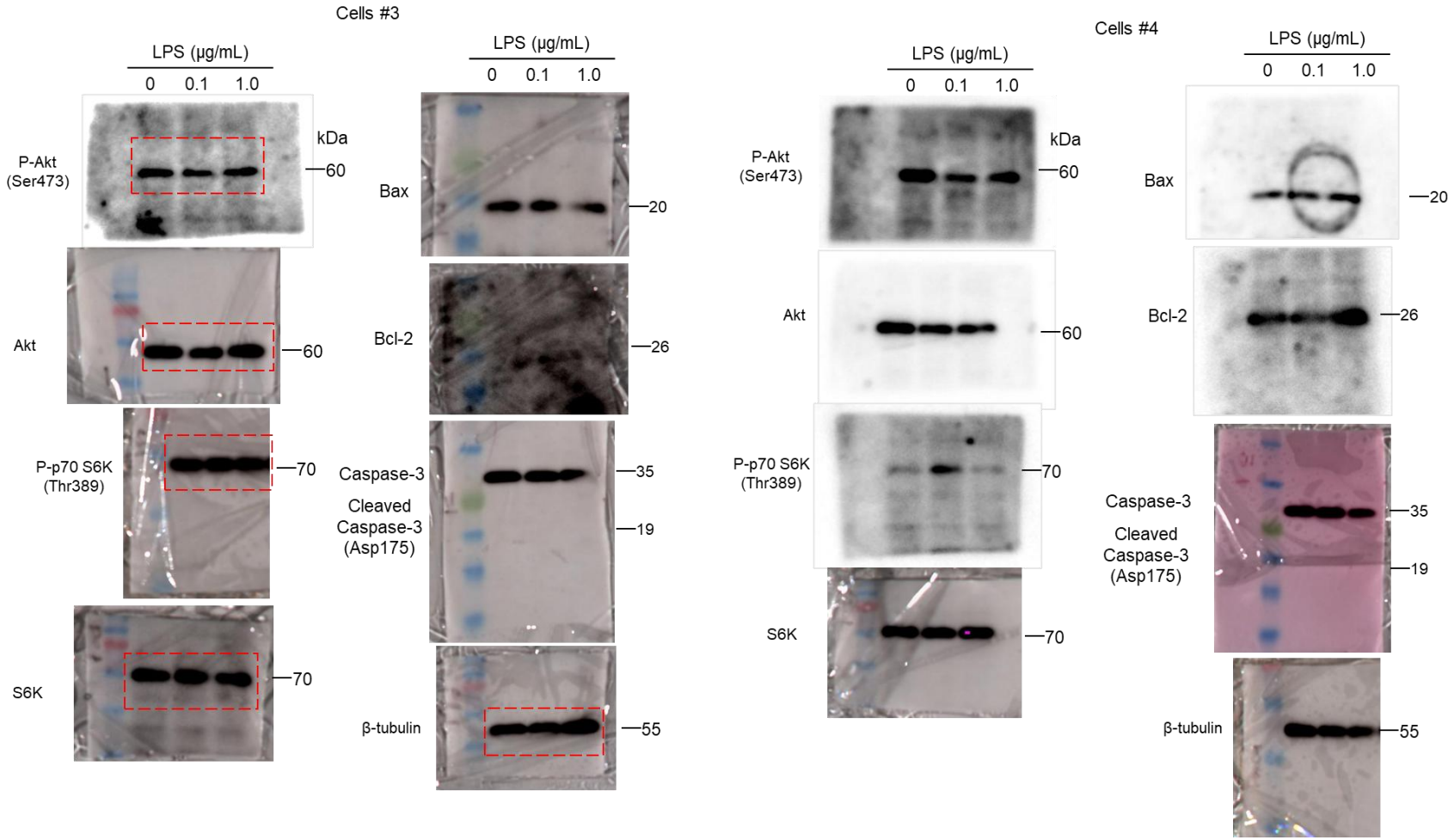
Raw image 7



Raw image 7. Original immunoblots presented in Figs. 4i and 4j.
The dashed line represents the cropped region shown in Fig. 4i (red) and Fig. 4j (yellow).

Supplementary information

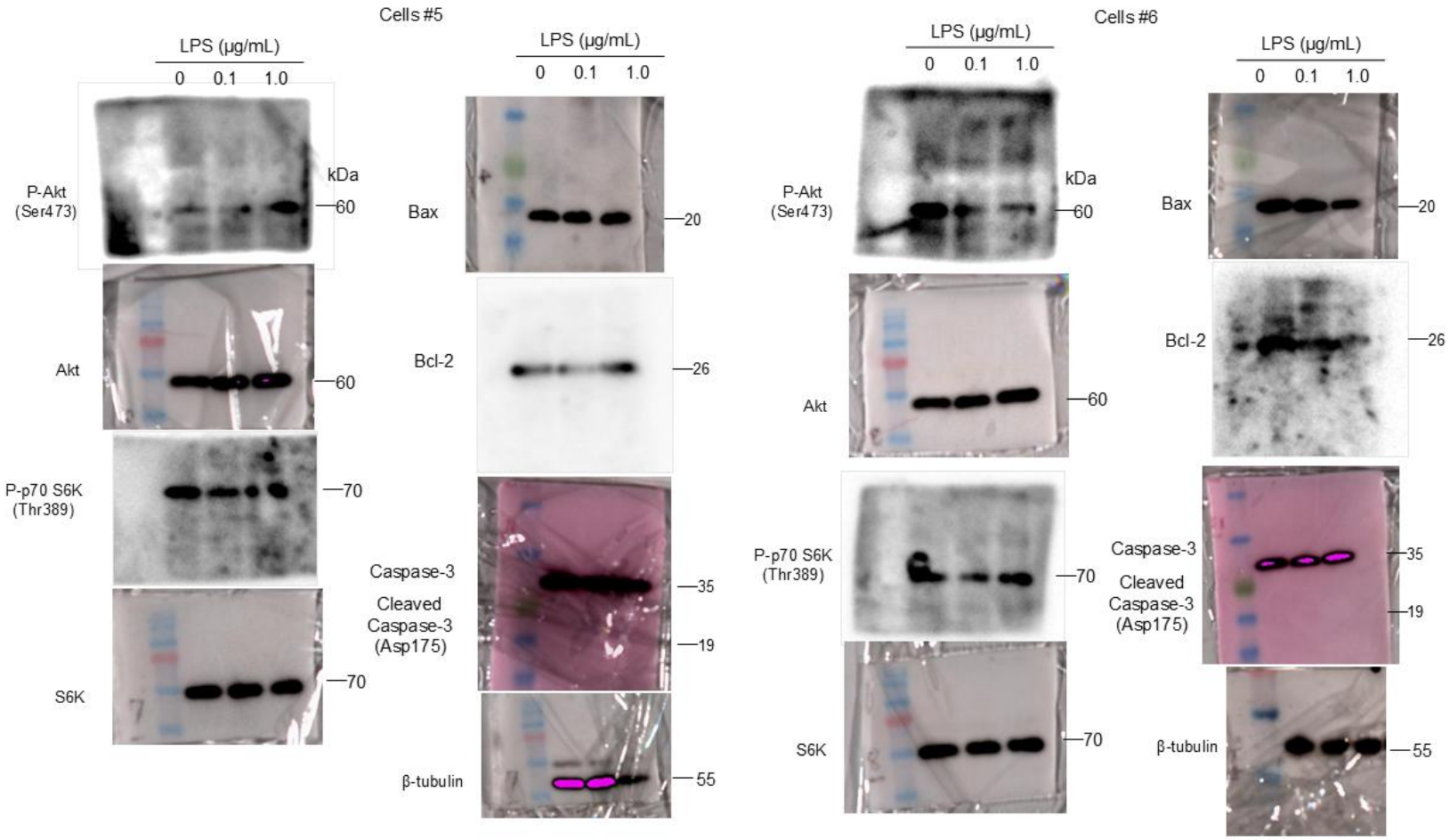
Raw image 7 (continued)



Raw image 7. Original immunoblots presented in Figs. 4i and 4j.
The dashed line represents the cropped region shown in Fig. 4i (red) and Fig. 4j (yellow).

Supplementary information

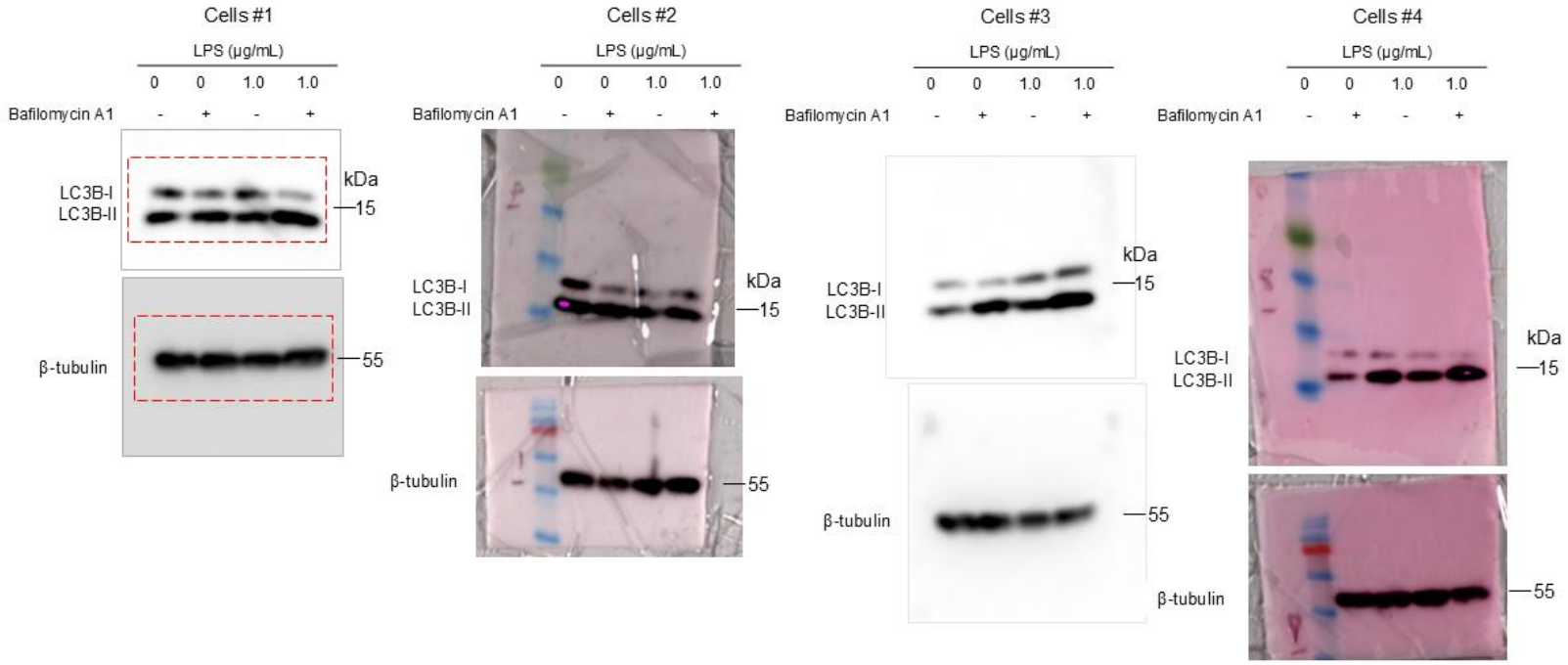
Raw image 7 (continued)



Raw image 7. Original immunoblots presented in Figs. 4i and 4j.
The dashed line represents the cropped region shown in Fig. 4i (red) and Fig. 4j (yellow).

Supplementary information

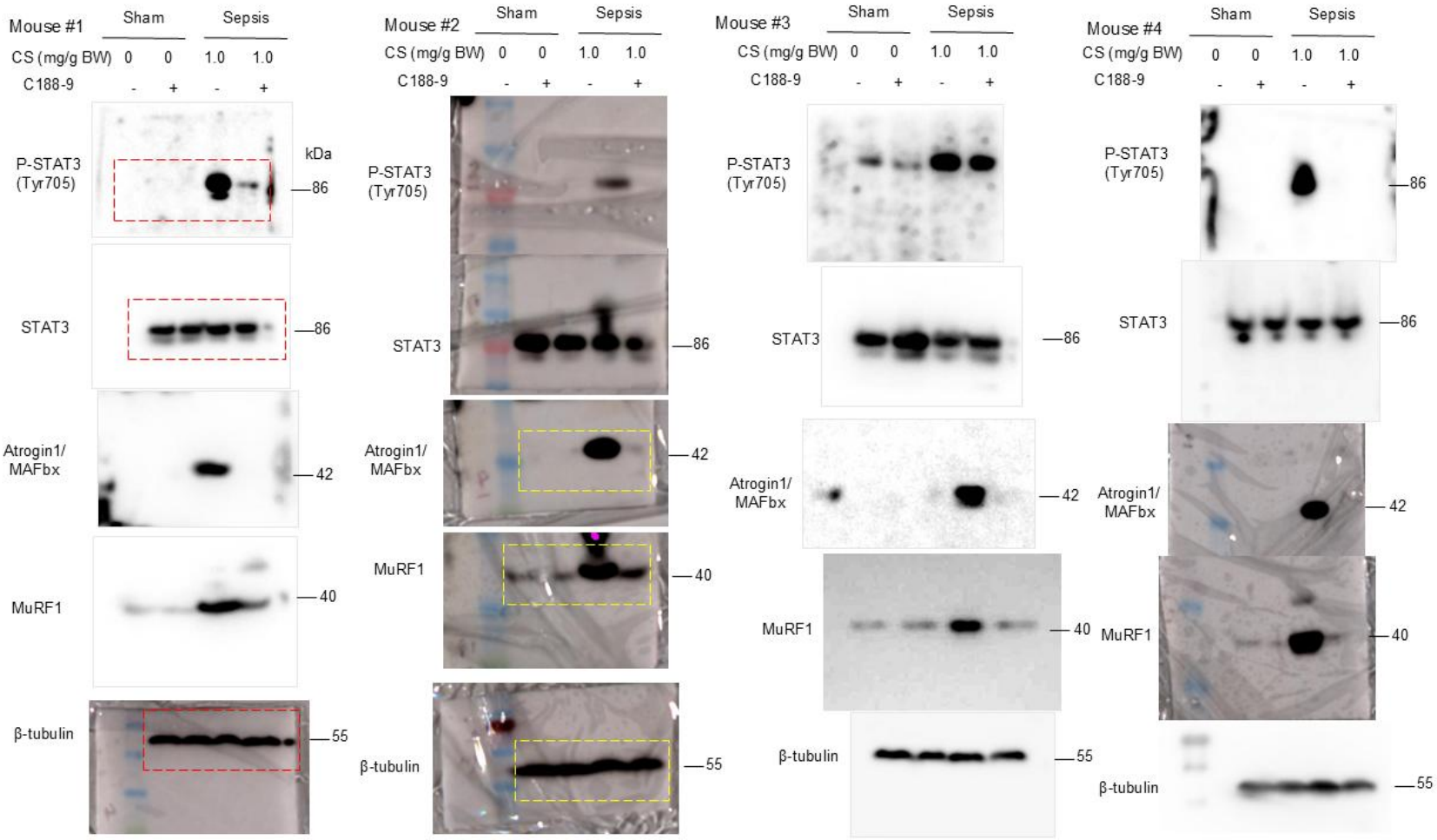
Raw image 8



Raw image 8. Original immunoblots presented in Fig. 4m.
The dashed line represents the cropped region shown in Fig. 4m (red).

Supplementary information

Raw image 9



Raw image 9. Original immunoblots presented in Figs. 6c and 6d.
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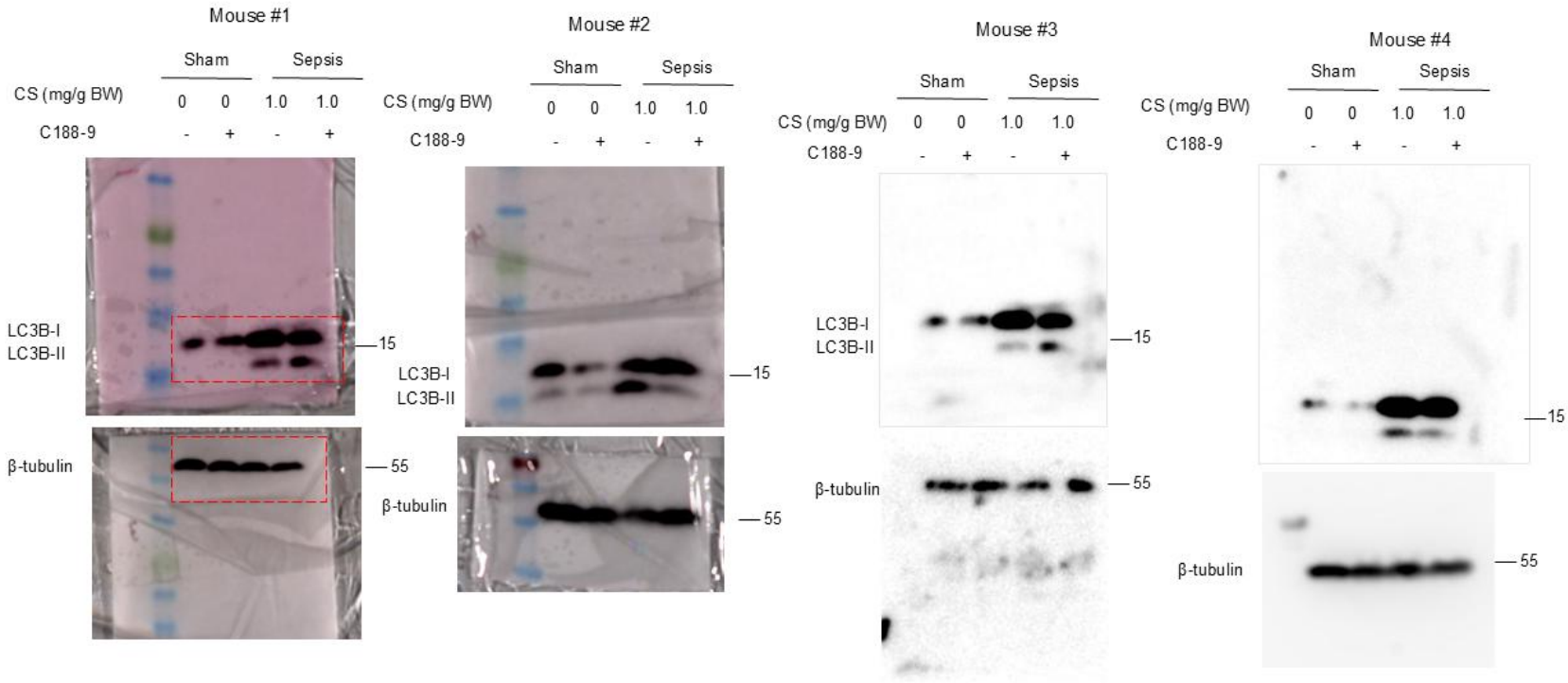
Supplementary information

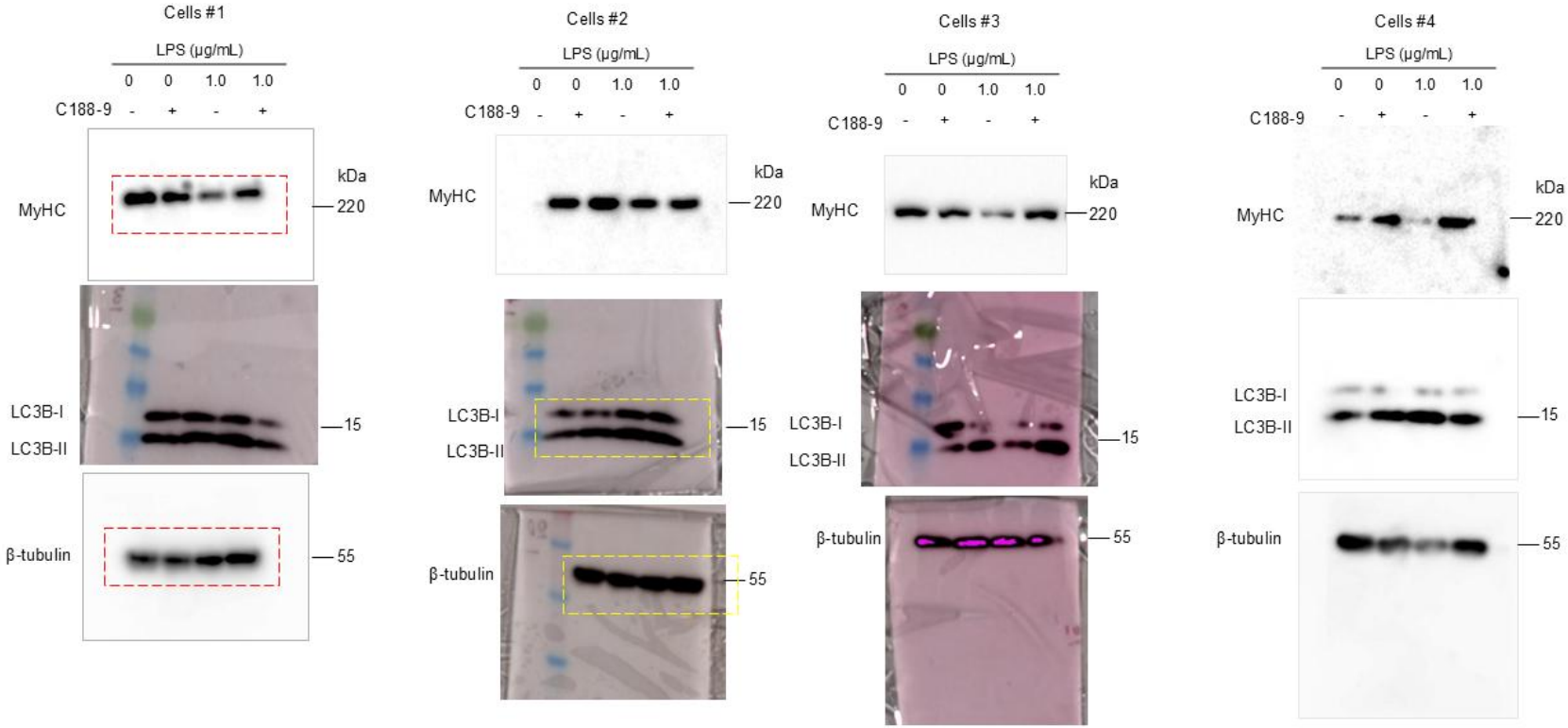
Raw image 10



Raw image 10. Original immunoblots presented in Fig. 6e.
The dashed line represents the cropped region shown in Fig. 6e (red).

Supplementary information

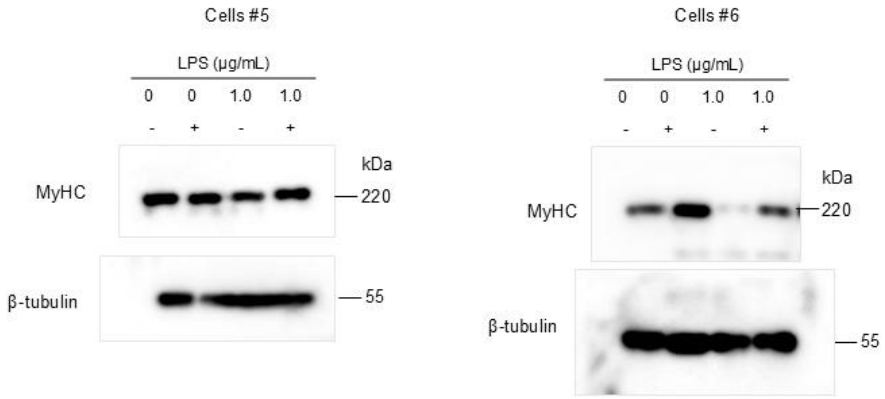
Raw image 11



Raw image 11. Original immunoblots presented in Figs. 7e and 7i.
The dashed line represents the cropped region shown in Fig. 7e (red) and Fig. 7i (yellow).

Supplementary information

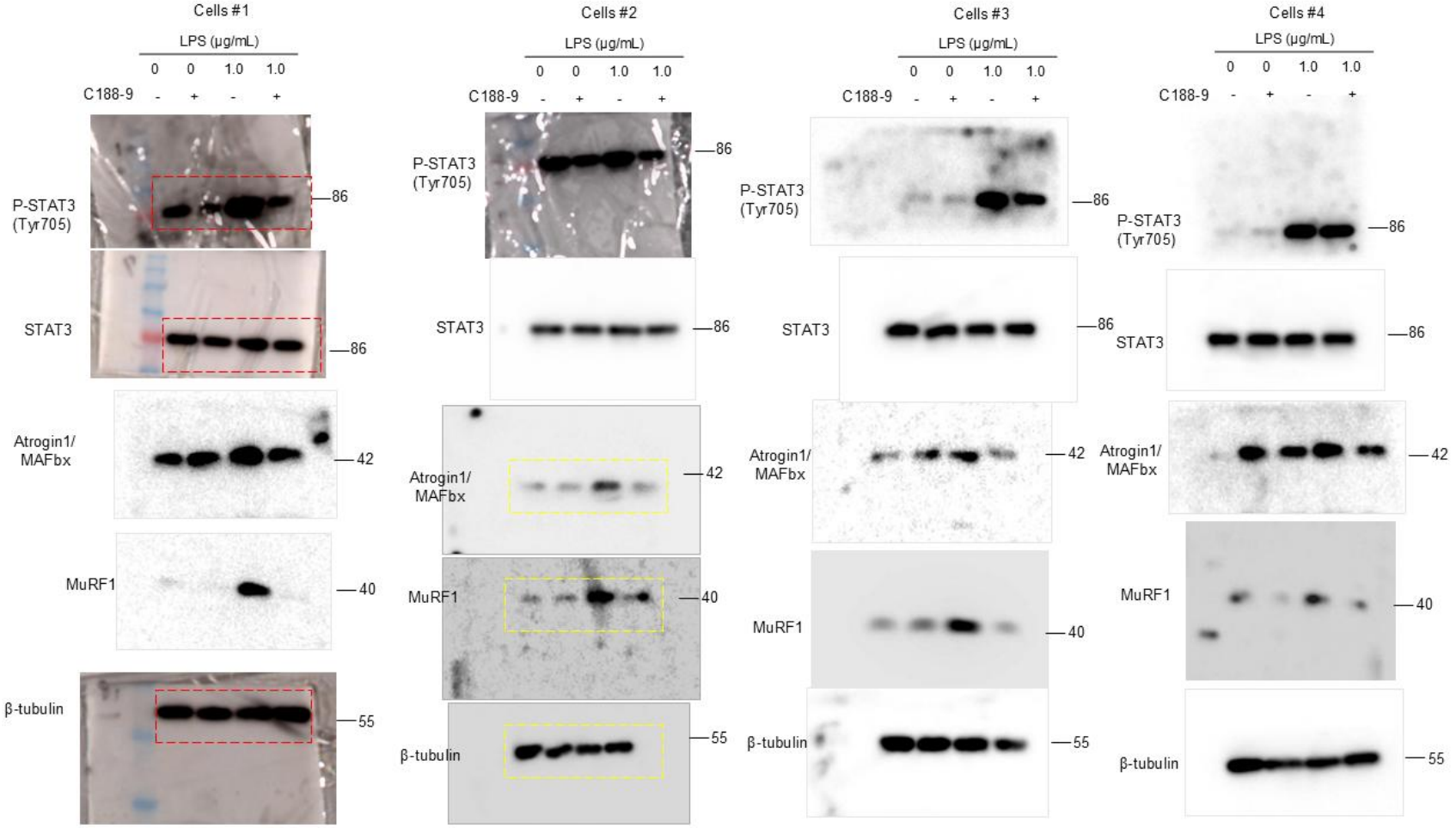
Raw image 11 (continued)



Raw image 11. Original immunoblots presented in Figs. 7e and 7i.
The dashed line represents the cropped region shown in Fig. 7e (red) and Fig. 7i (yellow).

Supplementary information

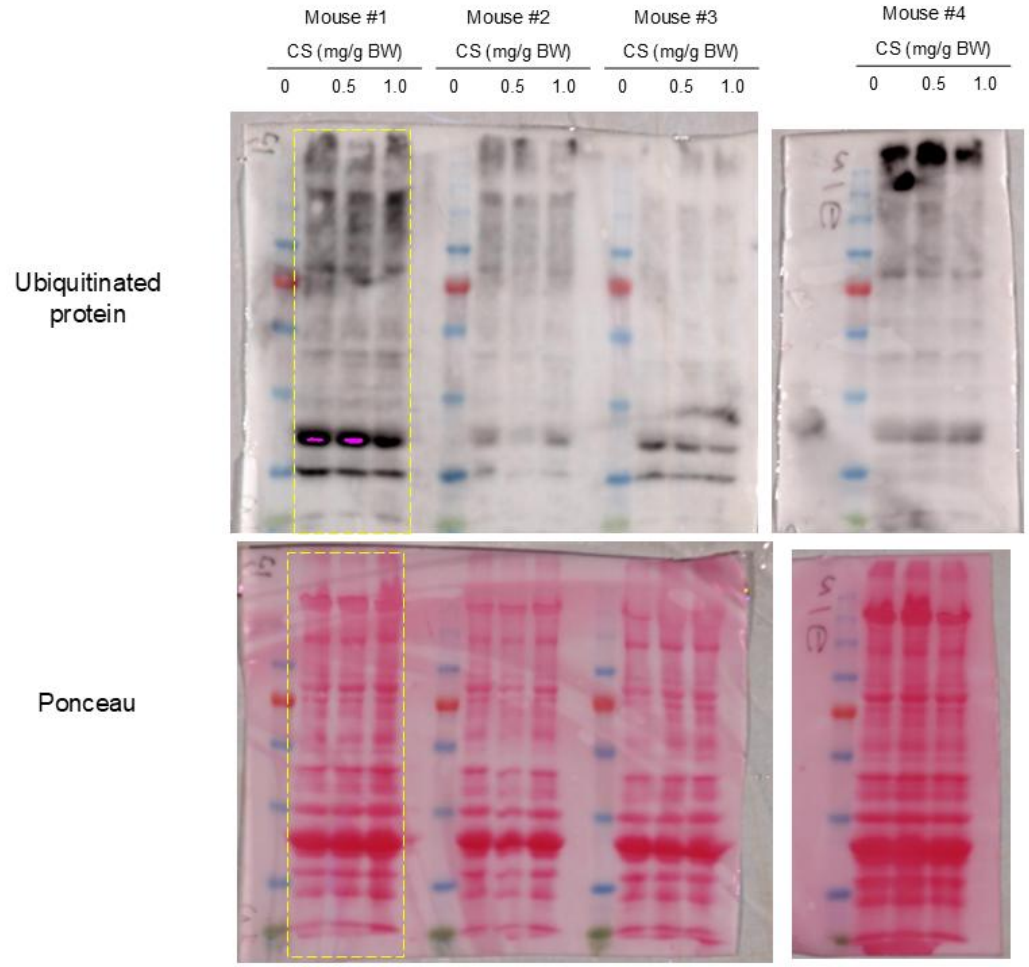
Raw image 12



Raw image 12. Original immunoblots presented in Figs. 7g and 7h.
The dashed line represents the cropped region shown in Fig. 7g (red) and Fig. 7h (yellow).

Supplementary information

Raw image 13

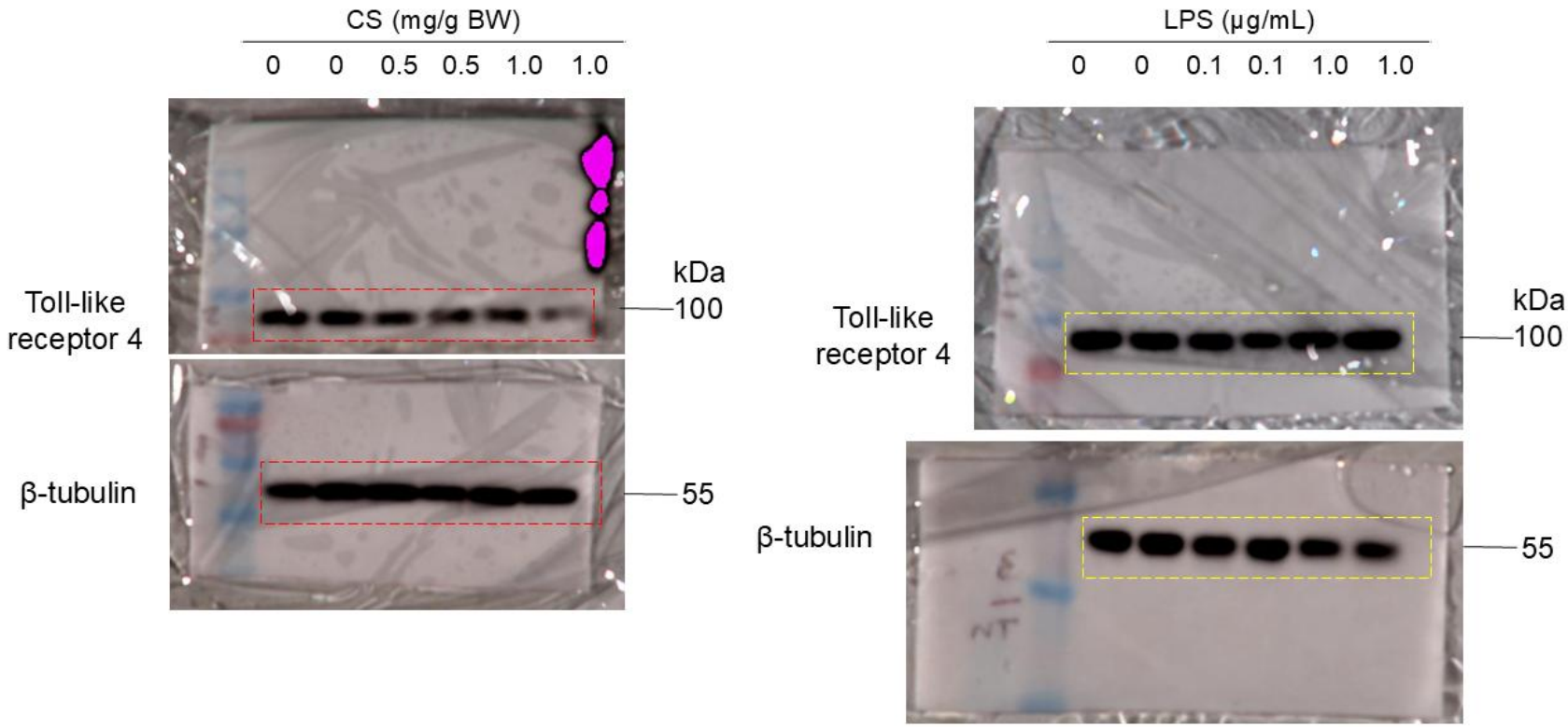


Raw image 13. Original immunoblots presented in Supplementary Fig. S3.

The yellow dashed line represents the cropped region shown in Supplementary Fig. S3.

Supplementary information

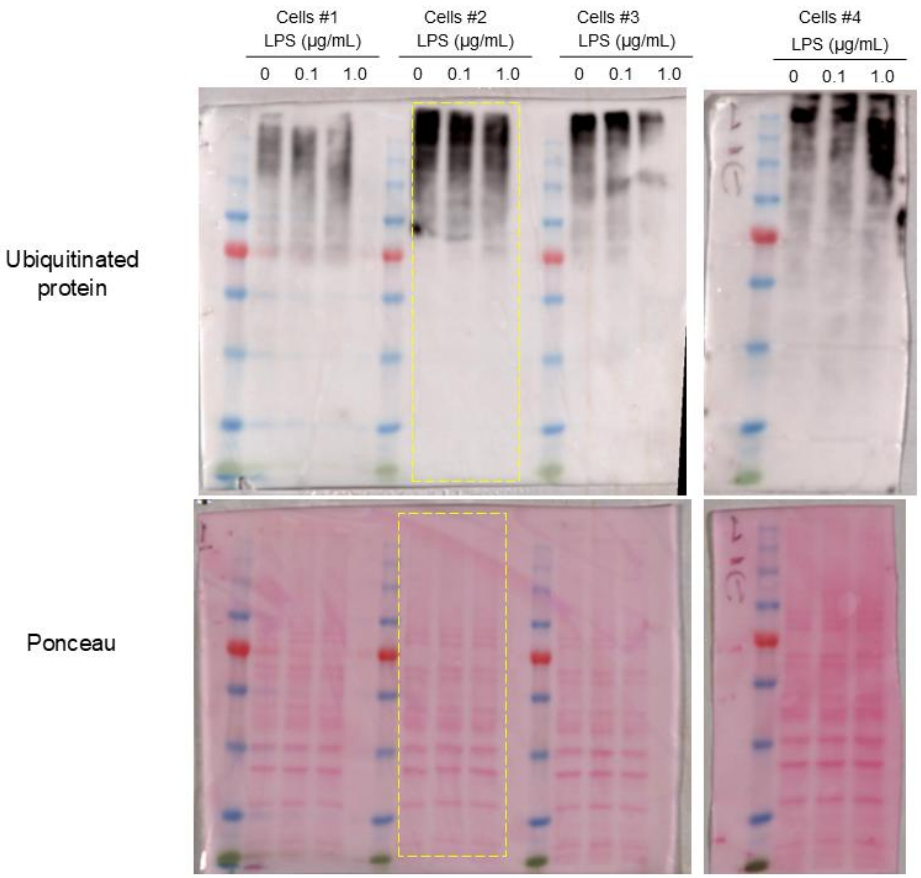
Raw image 14



Raw image 14. Original immunoblots presented in Supplementary Fig. S4.
The dashed lines represent the cropped regions shown in Supplementary Fig. S4a (red) and S4b (yellow).

Supplementary information

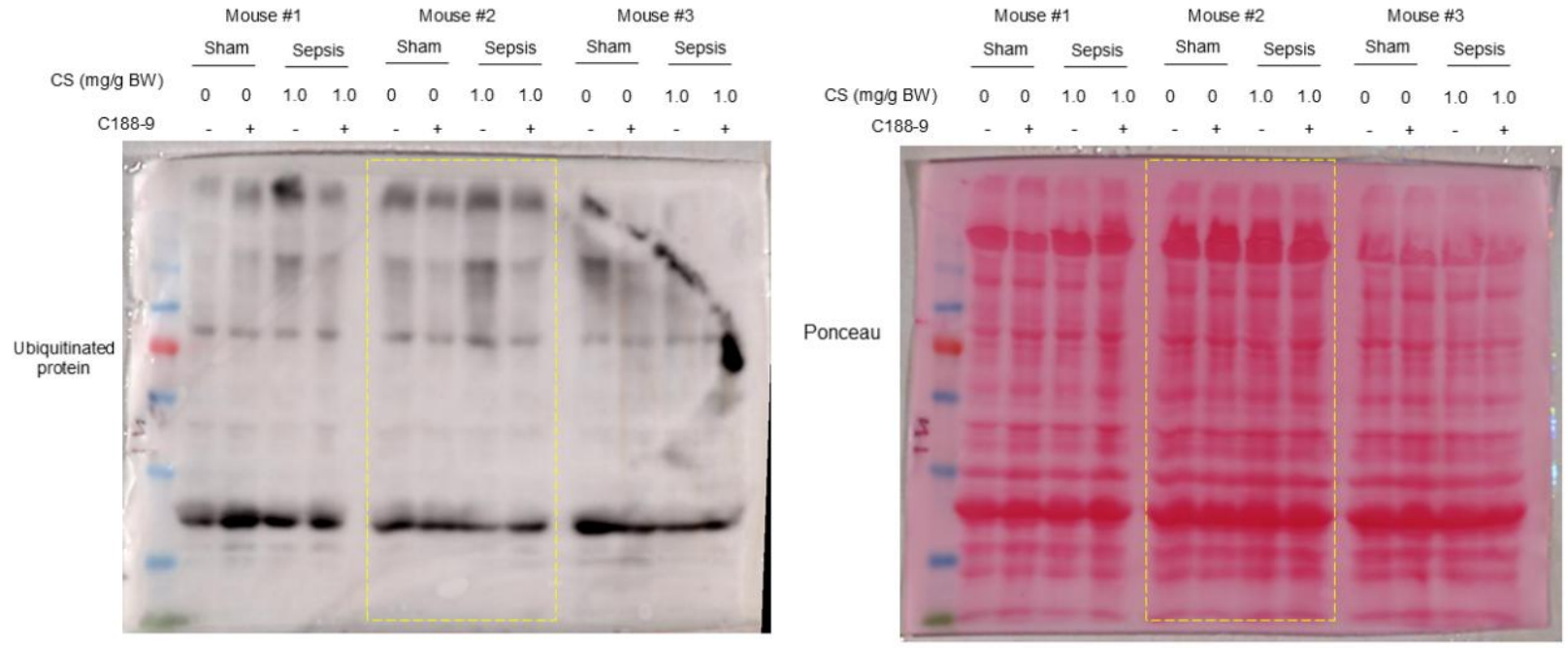
Raw image 15



Raw image 15. Original immunoblots presented in Supplementary Fig. S6.
The yellow dashed line represents the cropped region shown in Supplementary Fig. S6.

Supplementary information

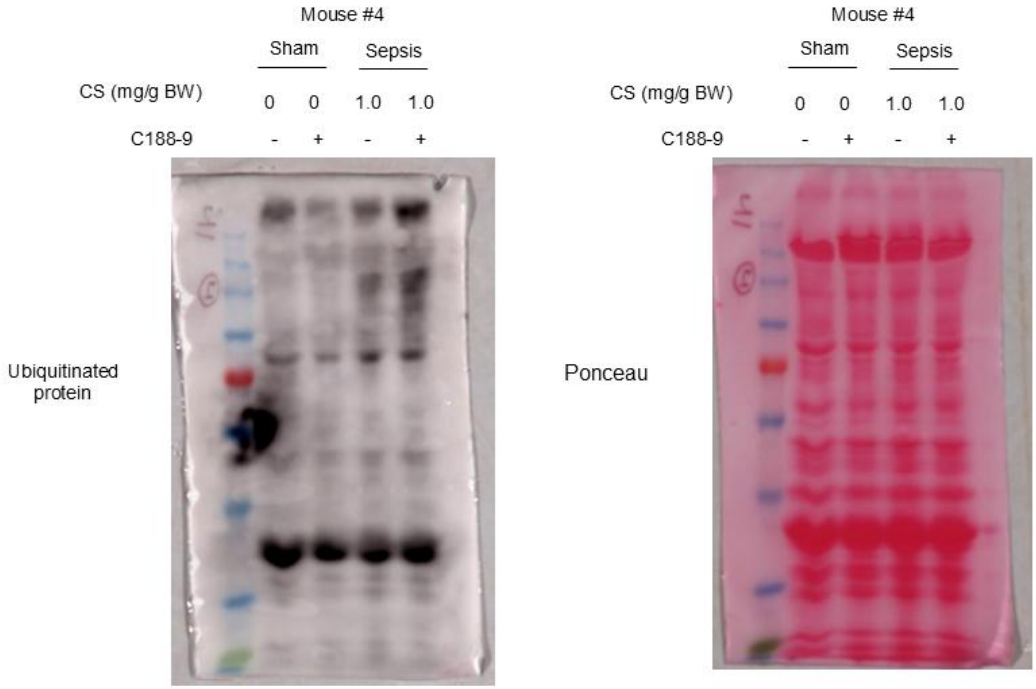
Raw image 16



Raw image 16. Original immunoblots presented in Supplementary Fig. S7.
The yellow dashed line represents the cropped region shown in Supplementary Fig. S7.

Supplementary information

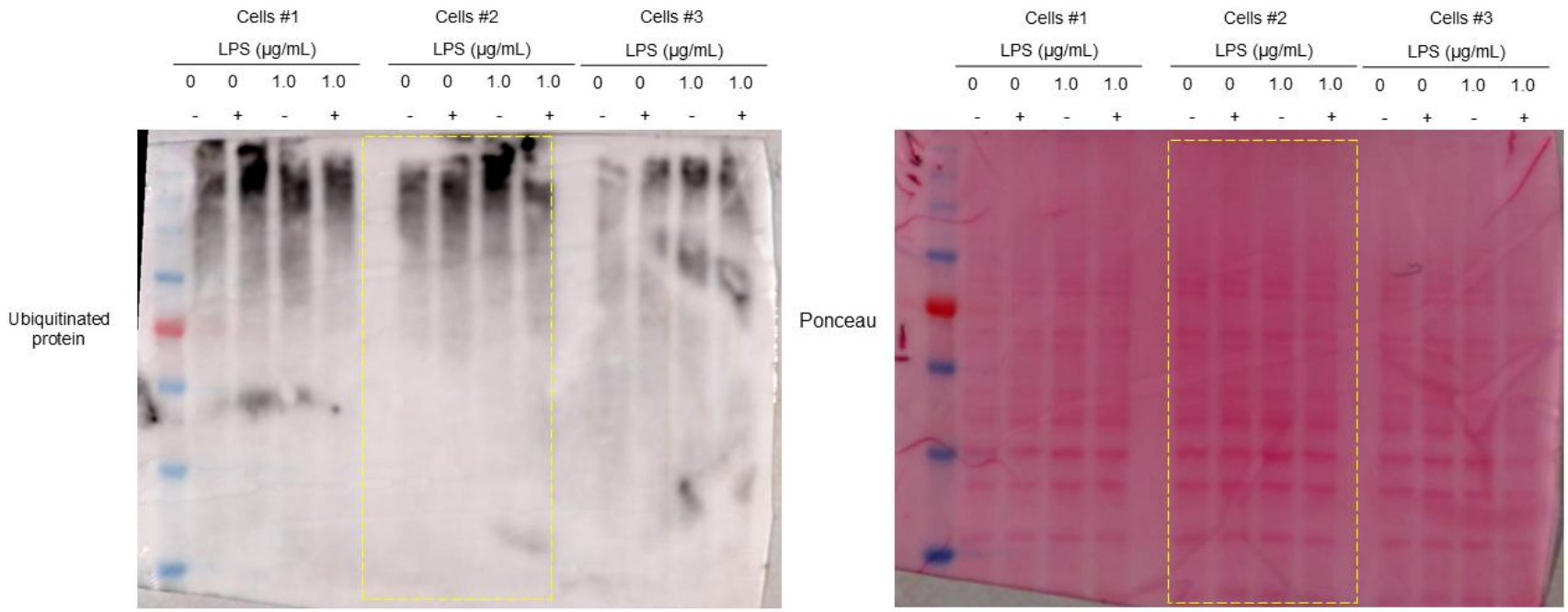
Raw image 16 (continued)



Raw image 16. Original immunoblots presented in Supplementary Fig. S7.
The yellow dashed line represents the cropped region shown in Supplementary Fig. S7.

Supplementary information

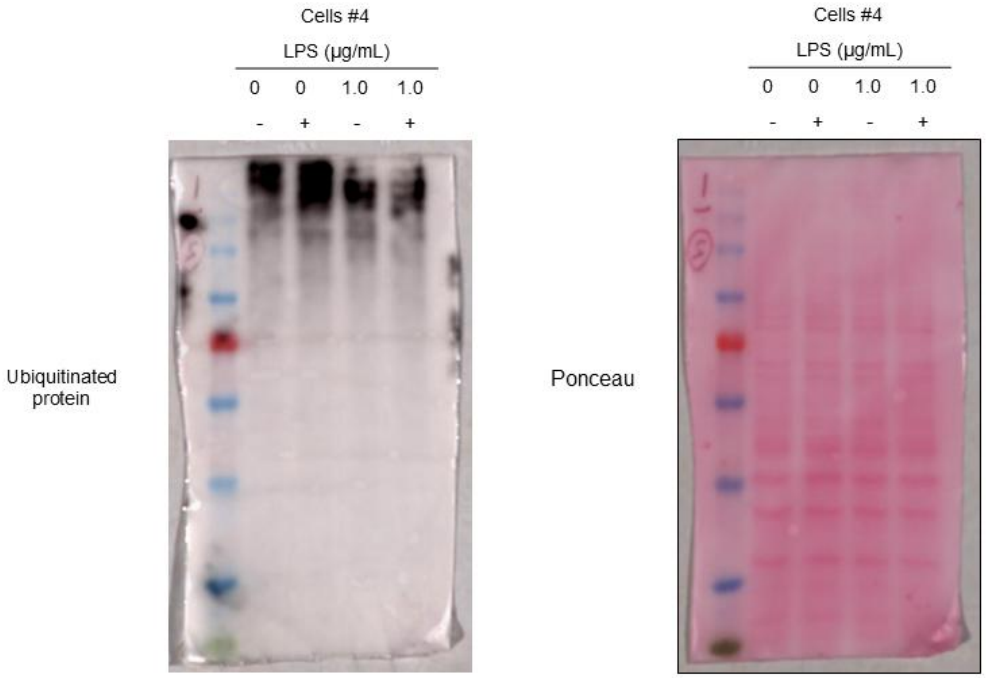
Raw image 17



Raw image 17. Original immunoblots presented in Supplementary Fig. S8.
The yellow dashed line represents the cropped region shown in Supplementary Fig. S8.

Supplementary information

Raw image 17 (continued)



Raw image 17. Original immunoblots presented in Supplementary Fig. S8.
The yellow dashed line represents the cropped region shown in Supplementary Fig. S8.