Antcin K ameliorates cardiotoxin-induced skeletal muscle injury and inflammation via IL-10 regulation

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Supplementary Materials and Methods

Quantitative polymerase chain reaction (qRT-PCR)

Total RNA was extracted using the TRIzol™ reagent, and 1 μg of total RNA was reverse transcribed into cDNA. qRT-PCR was performed in a 20 μL reaction volume containing cDNA template, various primers, PCR Supermix and nuclease-free water. The reaction was carried out in a StepOnePlus™ Real-Time PCR System with the following cycling conditions: 95°C for 10 minutes (polymerase activation), followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute (fluorescence detection). All results were analyzed with StepOne software (Version 2.3). GAPDH or beta-actin was used as the internal control. All results are calculated by StepOne software version 2.3, and obtained from 6 independent experiments at least performed in duplicate.

Western blot analysis

All samples were lysed using RIPA buffer containing protease and phosphatase inhibitors to extract total protein, and equal amounts of protein were prepared for subsequent Western blot analysis. Briefly, the equal amounts of protein were separated by SDS-PAGE electrophoresis and then transferred to Immobilon® poly-vinylidene fluoride membrane (PVDF) membranes. Primary and secondary antibodies were applied as in Supplementary Table S2. The intensity of the blotting signals was captured and quantified using the ImageQuant™ LAS 4000 biomolecular imager (GE Healthcare, Little Chalfont, UK).

Immunofluorescence staining

The C2C12/Control or C2C12/IL-10-/+ mouse myoblasts or the differentiated myocytes were fixed, permeabilized, and labeled with various primary antibodies and secondary antibodies Alexa-Fluor® 488 or 594 conjugate (Thermo Fisher Scientific, UK). 4,6-Diamidino-2-phenylindole (DAPI) was used for staining nuclei. All fluorescent intensities of the indicated proteins were calculated and analyzed by using ImageJ software.

Immunohistochemical staining

For immunohistochemical staining, TA muscles from hind limbs of the mouse model were prepared for paraffin-embedded sections, according to previous reports [2, 3]. After the specimens were rehydrated and stained with indicated antibodies, immunohistochemistry (IHC) was performed using an IHC Kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions.

Cell viability assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to assess cell viability in C2C12 and G7 cell lines following Antcin K treatment in a dose-dependent manner for 24 hours. After treatment, MTT solution (0.5 mg/mL) was added to the cells and incubated to allow formazan formation. The absorbance of formazan crystals, reflecting the number of viable cells, was measured at 550 nm using a microplate reader (Bio-Tek, Winooski, VT, USA).

Supplementary Figures

Figure S1

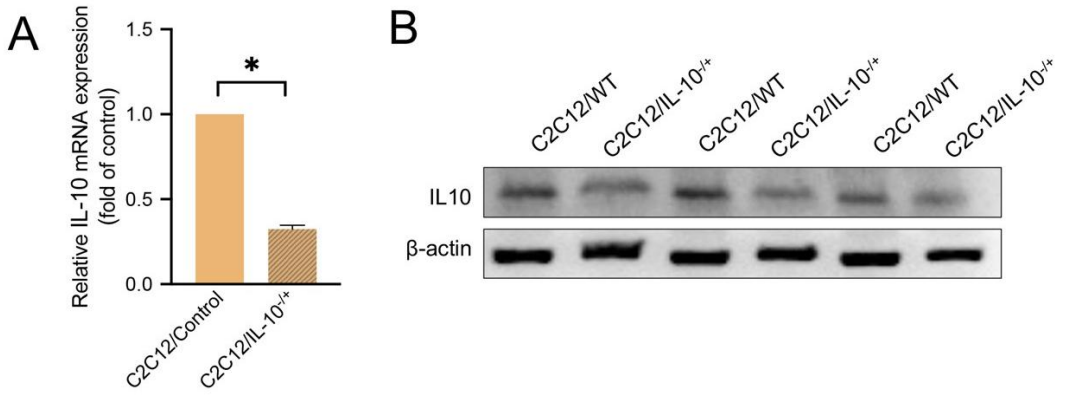

Figure S1. The knockdown efficacy of knockdown IL-10 (IL-10$^{-/+}$) examined by qRT-PCR (A) and western blotting (B).

Figure S2

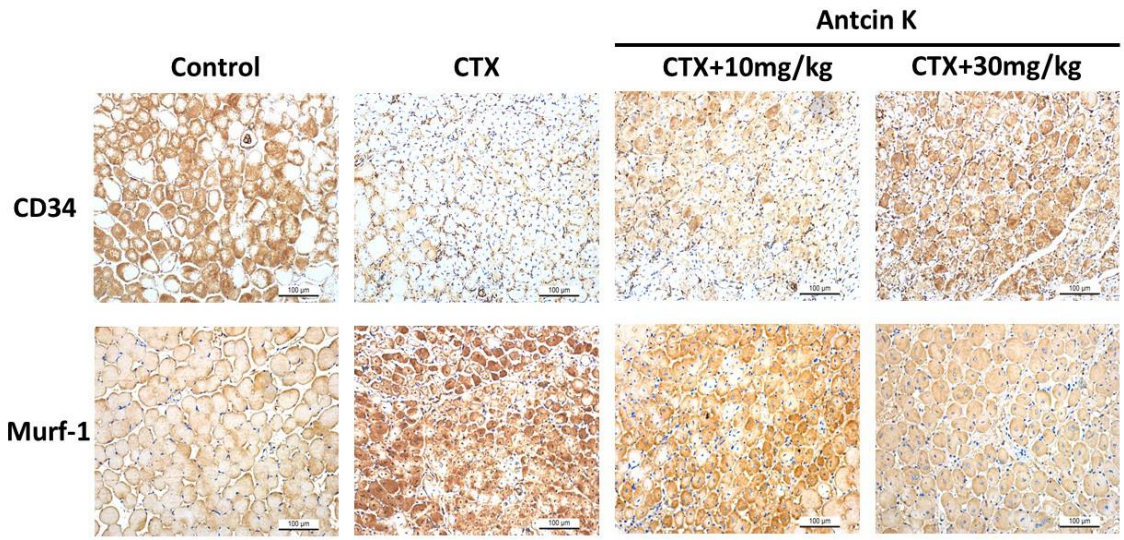

Figure S2. IHC staining for tibialis anterior skeletal muscle *in vivo*. CD34 is a marker of muscle stem cells. MuRF-1 is a marker of muscle atrophy.

Figure S3

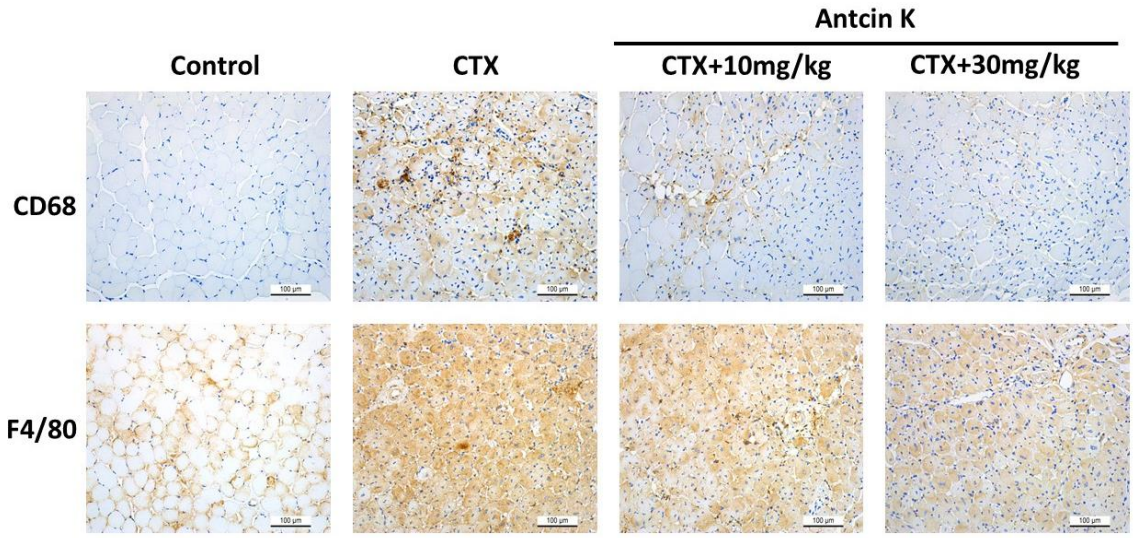

Figure S3. IHC staining for tibialis anterior skeletal muscle *in vivo*. CD68 is a marker of macrophages. F4/80 is a marker of leukocytes.

Figure S4

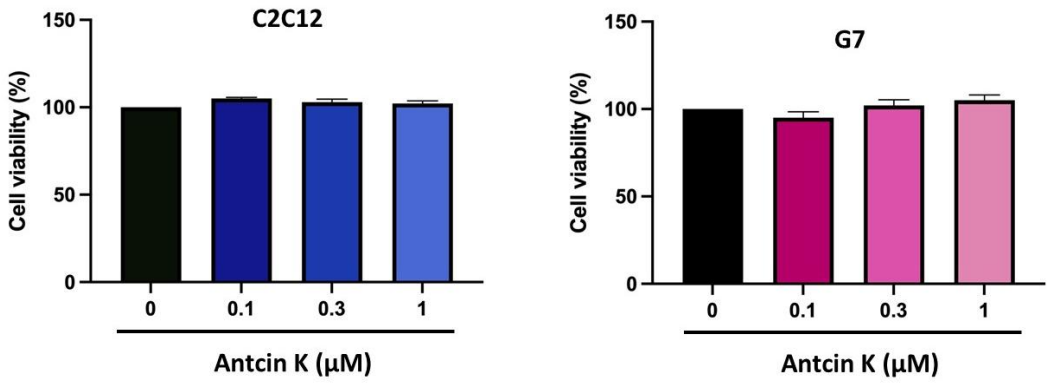

Figure S4. MTT assay was used for the cell viability of Antcin K in C2C12 and G7 cell lines for 24 hours.

Figure S5

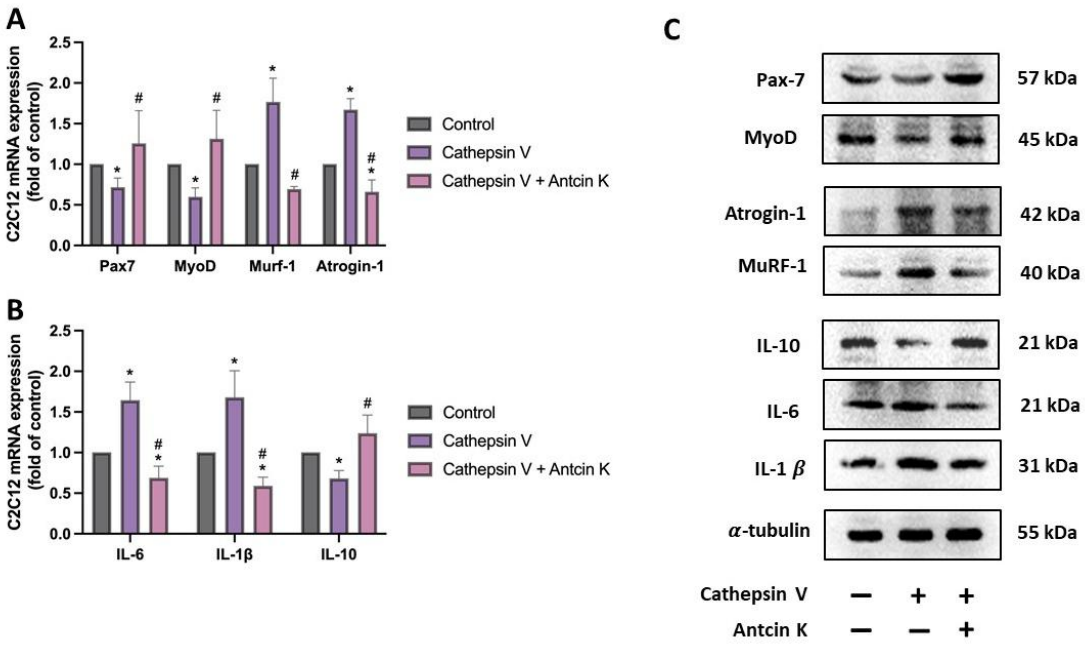

Figure S5. Antcin K alleviated cathepsin V-induced inflammation and enhanced differentiation. (A and B) The C2C12 was treated with the cathepsin V (10 μM) for 24 h, followed by Antcin K (1 μM) for another 24h, and the mRNA levels of proinflammatory cytokines (IL-6, IL-1β), myogenesis (MyoD, Pax-7), and atrophy markers (MuRF-1, Atrogin-1) were measured by qRT-PCR. **(C)** The protein expression of IL-10, proinflammatory markers (IL-6 and IL-1β) myogenesis markers (MyoD, Pax-7) atrophy markers (MuRF-1, Atrogin-1) was analyzed by western blotting in C2C12 cell lines.

Supplementary Tables

Table S1. List of primers used in qRT-PCR

Gene	Sequences (5'-3')
Atrogin-1	Forward: GAGTGGCATCGCCCAAAAGA Reverse: TCTGGAGAAGTTCCCGTATAAGT
MuRF-1	Forward: GTGTGAGGTGCCTACTTGCTC Reverse: GCTCAGTCTTCTGTCCTTGGA
MyoD	Forward: GAGGATCCGATGGAGCTTCTATCG Reverse: CGGATCCTCTCAAAGCACCTGATA
Pax-7	Forward: GGTCC CCAGG ATGAT GAGA Reverse: TTGAT GAAGA CCCCA CCAAG
IL-6	Forward: GATGGTCTTGGTCCTTAGCC Reverse: GGGAAATCGTGGAAATGAGA
IL-1β	Forward: GCAACTGTTCCTGAACTCAACT Reverse: ATCTTTTGGGGTCCGTCAACT
IL-10	Forward: GCTCTTACTGACTGGCATGAG Reverse: CGCAGCTCTAGGAGCATGTG
GAPDH	Forward: TGTGTCCGTCGTGGATCTGA Reverse: TTGCTGTTGAAGTCGCAGGAG

Table S2. List of reagents, resources, and antibodies

ANTIBODIES	HOST/ISOTYPE	SOURCE	IDENTIFIER
MyoD (G-1) monoclonal antibody	Mouse / IgG	Santa Cruz Biotech	Cat#sc-1637
Pax-7 polyclonal cntibody	Rabbit / IgG	Abcam	Cat#ab187339
MAFbx/Atrogin-1 (F-9) monoclonal antibody	Mouse / IgG	Santa Cruz Biotech	Cat#sc-166806
MuRF-1 monoclonal antibody (C-11)	Mouse / IgG	Santa Cruz Biotech	Cat#sc-398608
IL-6 polyclonal antibody	Rabbit / IgG	Genetex	Cat#GTX110527
IL-1β monoclonal antibody	Rabbit / IgG	Genetex	Cat#GTX636887

β-Actin monoclonal antibody	Mouse / IgG	Sigma-Aldrich	Cat#A5441
Neutralizing IL-10 monoclonal antibody	Rat / IgG	R&D system	Cat#MAB417
α-Tubulin monoclonal antibody	Mouse / IgG	Abcam	Cat#ab7291
Myosin skeletal muscle polyclonal antibody	Mouse / IgG	R&D system	Cat#MAB4470
Phospho-PI3 kinase p85(Tyr458)/p55(Tyr199) polyclonal antibody	Rabbit / IgG	Cell Signaling Tech	Cat#4228S
PI3-kinase p85α (B-9) monoclonal antibody	Mouse / IgG	Santa Cruz Biotech	Cat#sc-1637
p-Akt1/2/3 (Thr308) monoclonal antibody	Rabbit / IgG	Santa Cruz Biotech	Cat#sc-16646-R
Akt1 (B-1) monoclonal antibody	Mouse / IgG	Santa Cruz Biotech	Cat#sc-5298
Desmin polyclonal antibody	Rabbit / IgG	Abcam	Cat#ab216616
Dystrophin polyclonal antibody	Rabbit / IgG	Abcam	Cat#ab15277
Rabbit anti-goat IgG-HRP conjugated secondary antibody	Rabbit / IgG	Sigma-Aldrich	Cat#A8919
Mouse anti-rabbit IgG-HRP conjugated secondary antibody	Mouse / IgG	Santa Cruz Biotech	Cat#sc-2357
Goat anti-rabbit IgG (H+L), Alexa Fluor 488 conjugated secondary antibody	Goat / IgG	Thermo Fisher Scientific	Cat#A32731
Goat anti-rabbit IgG (H+L), Alexa Flour 594 conjugated secondary antibody	Goat / IgG	Thermo Fisher Scientific	Cat#A32740
CD34	Rabbit / IgG	Abcam	Cat#ab81289
CD68	Mouse / IgG1 kappa	Novus Biologicals	Cat#NB100-683
F4/80	Mouse / IgG_1	Santa Cruz Biotech	Cat#SC-377009

CHEMICALS, PLASMID	SOURCE	IDENTIFIER
wortmannin (PI3K inhibitor)	Santa Cruz Biotech	Cat#SC-3505A
Akt inhibitor	Sigma-Aldrich	Cat#A6730
Mouse shRNA IL-10 plasmid	National RNAi Core Facility (Sinica, Taiwan)	Cat#TRCN0000365913
CMV promoter plasmid	Addgene	Cat#169739
MD plasmid	Addgene	Cat#20864
DAPI	Sigma-Aldrich	Cat#D9564
Cardiotoxin	LATOXAN	Cat#L8102-1MG
Immobilon Western Chemiluminescent HRP substrate	Merck millipore	Cat#WBKLS0500
IHC Kit	Sigma-Aldrich	Cat#00-4955-58
Cathepsin V overexpression	National RNAi Core Facility (Sinica, Taiwan)	Cat#NJ0090248-1
CELL LINES	**SOURCE**	**IDENTIFIER**
C2C12 mouse myoblast	ATCC	Cat#CRL-1772
G7 mouse myoblast	ATCC	Cat#CRL-1447
293 T	Thermo Fisher Scientific	Cat#R700007
CULTURE MEDIUM	**SOURCE**	**IDENTIFIER**
Dulbecco's Modified Eagle's Medium (DMEM)	Gibco	Cat#41966-029
Fetal bovine serum (FBS)	Gibco	Cat#21875-034
Horse serum (HS)	Gibco	Cat#16050-122
Trypsin	Gibco	Cat#15090-046