Cathelicidin-related antimicrobial peptide mediates skeletal muscle degeneration caused by injury and Duchenne muscular dystrophy in mice

Moon-Chang Choi¹, Jiwon Jo¹, Myeongjin Lee¹, Jonggwan Park², Tso-Pang Yao³ & Yoonkyung Park¹*

¹Department of Biomedical Science, Chosun University, Gwangju, South Korea; ²Department of Bioinformatics, Kongju National University, Kongju, South Korea; ³Department of Pharmacology and Cancer Biology, Duke University, Durham, NC, USA

Abstract

Background  Cathelicidin, an antimicrobial peptide, plays a key role in regulating bacterial killing and innate immunity; however, its role in skeletal muscle function is unknown. We investigated the potential role of cathelicidin in skeletal muscle pathology resulting from acute injury and Duchenne muscular dystrophy (DMD) in mice.

Methods  Expression changes and muscular localization of mouse cathelicidin-related antimicrobial peptide (Cramp) were examined in the skeletal muscle of normal mice treated with chemicals (cardiotoxin and BaCl₂) or in dystrophic muscle of DMD mouse models (mdx, mdx/Utrn⁻/⁻ and mdx/Utrn⁻/⁻/C₀⁻/⁻). Cramp penetration into myofibres and effects on muscle damage were studied by treating synthetic peptides to mouse skeletal muscles or C2C12 myotubes. Cramp knockout (KO) mice and mdx/Utrn/Cramp KO lines were used to determine whether Cramp mediates muscle degeneration. Muscle pathophysiology was assessed by histological methods, serum analysis, grip strength and lifespan. Molecular factors targeted by Cramp were identified by the pull-down assay and proteomic analysis.

Results  In response to acute muscle injury, Cramp was activated in muscle infiltrating neutrophils and internalized into myofibres. Cramp treatments of mouse skeletal muscles or C2C12 myotubes resulted in muscle degeneration and myotube damage, respectively. Genetic ablation of Cramp reduced neutrophil infiltration and ameliorated muscle pathology, such as fibre size (P < 0.001; n = 6) and fibrofatty infiltration (P < 0.05). Genetic reduction of Cramp in mdx/Utrn⁻/⁻ mice not only attenuated muscle damage (35%, P < 0.05; n = 9–10), myonecrosis (53%, P < 0.05), inflammation (37–65%, P < 0.01) and fibrosis (14%, P < 0.05) but also restored muscle fibre size (14%, P < 0.05) and muscle force (18%, P < 0.05). Reducing Cramp levels led to a 63% (male, P < 0.05; n = 10–14) and a 124% (female, P < 0.001; n = 20) increase in the lifespan of mdx/Utrn⁻/⁻ mice. Proteomic and mechanistic studies revealed that Cramp cross-talks with Ca²⁺ signalling in skeletal muscle through sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase1 (SERCA1). Cramp binds and inactivates SERCA1, leading to the activation of Ca²⁺-dependent calpain proteases that exacerbate DMD progression.

Conclusions  These findings identify Cramp as an immune cell-derived regulator of skeletal muscle degeneration and provide a potential therapeutic target for DMD.

Keywords  Cathelicidin; Cramp; Duchenne muscular dystrophy; Muscle degeneration; Serca1

Received: 6 January 2022; Revised: 6 July 2022; Accepted: 20 July 2022

*Correspondence to: Yoonkyung Park, Ph.D., Department of Biomedical Science, Chosun University, 309 Pilmun-daero, Dong-gu, Gwangju 61452, South Korea. Tel: +82-62-230-6854; Fax: +82-62-225-6758. Email: y_k_park@chosun.ac.kr

© 2022 The Authors. Journal of Cachexia, Sarcopenia and Muscle published by John Wiley & Sons Ltd on behalf of Society on Sarcopenia, Cachexia and Wasting Disorders. This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
Introduction

Skeletal muscles respond to damage by modulating cross-talk between intrinsic muscle and environmental factors. When acutely injured, muscle undergoes degeneration and repair via sequential exertion of calcium (Ca\(^{2+}\))-dependent myofibre damage, removal of damaged fibres by inflammatory cells, satellite cell (SC)-dependent new fibre formation and maturation of regenerated muscle.\(^1\) In contrast, chronic dystrophic muscles represent progressive necrosis and inflammation that overtakes the restorative capacity of muscle. Duchenne muscular dystrophy (DMD) is the most common and severe form of muscular dystrophy and is caused by mutations in the dystrophin gene on the X chromosome.\(^2\)

Ca\(^{2+}\)-dependent proteolytic cascades trigger injury-induced muscle degeneration and are abnormally activated in dystrophic muscles of DMD. Upon injury, Ca\(^{2+}\) influx stimulates Ca\(^{2+}\)-dependent calpain proteases and the mitochondrion uptake of Ca\(^{2+}\), activating cellular necrosis and pro-apoptotic signalling.\(^3\) Cytosolic Ca\(^{2+}\) levels in muscle are largely modulated by Ca\(^{2+}\)-handling proteins located in sarcoplasmic reticulum (SR), including sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA), calsequestrin (CSQ) and ryanodine receptor (RyR).\(^3\) Of these, SERCA1 Ca\(^{2+}\) pump plays a critical role in reducing cytosolic Ca\(^{2+}\) levels by taking Ca\(^{2+}\) up into the SR in fast-twitch skeletal muscles.\(^4\) Previous investigations established that preserving SERCA activity or its forced expression significantly attenuates DMD severity.\(^5,6\) However, the molecular factors linking Ca\(^{2+}\) signalling and myonecrosis are not fully understood.

Antimicrobial peptides (AMPs) are small protein molecules that kill a wide range of microorganisms.\(^8\) Cathelicidins and defensins are two major families of endogenous AMPs in mammals. Mouse cathelicin-related antimicrobial peptide (Cramp) and its human homologue LL-37 are generated through cleavage of their precursor proteins, pro-Cramp and hCAP18, respectively. Unlike cathelicidins, defensins comprise a large family of cationic peptides including many α-defensin and β-defensin isoforms.\(^9\) These AMPs function as potent immune modulators by killing bacteria, neutralizing bacterial lipopolysaccharide, functioning as chemotactants for immune cells and/or activating innate immune responses.\(^10,11\) The pleiotropic promotion or protective roles of cathelicidins have recently been elucidated in the context of tissue degeneration associated with sterile inflammation.\(^12–15\) The role of cathelicidins in skeletal muscle pathology and their molecular targets have yet to be explored.

Here, we report that antimicrobial peptide Cramp actively participates in the injury-induced degeneration of skeletal muscle. We show that immune cell-derived Cramp translocate to muscle cells and disturb Ca\(^{2+}\) signalling by inactivating SERCA1. Therefore, our study uncovers the roles of Cramp in muscle pathology and may provide a therapeutic target for muscular dystrophy.

Methods

Mice and treatments

Cramp knockout (KO) (B6.129X1-Camp\(^{tm2Nig}\)/J) and mdx/Utrn-/- (B10ScSn.Cg-Utrn\(^{tm2Ked}\)/Dmd\(^{m085j/J}\)) mice were purchased from Jackson Laboratory. To generate mdx/Utrn/Cramp KO lines, male Cramp\(^{-/-}\) mice were crossed with female mdx\(^{-/-}\)/Utrn\(^{-/-}\) mice. The F1 male mdx\(^{+/-}\)/Utrn\(^{+/-}\)/Cramp\(^{-/-}\) mice were backcrossed with female mdx\(^{-/-}\)/Utrn\(^{-/-}\) mice to produce male mdx\(^{+/-}\)/Utrn\(^{+/-}\)/Cramp\(^{-/-}\) and female mdx\(^{-/-}\)/Utrn\(^{-/-}\)/Cramp\(^{+/-}\) mice, and the resultant F2 offspring were intercrossed until experiments were conducted. Age-matched, sex-matched and background-matched mice were used in all experiments. Roughly similar numbers of male mice were used in most cases. Sample size was determined according to our prior research experiences. Mouse survival was recorded as time to natural death. Each figure legend indicates the mouse gender, age and treatments. All experiments were approved by the Chosun University Institutional Animal Care and Use Committee (IACUC approval no. CIACUC2018-A0029).

To induce acute muscle injury, mice were anaesthetised through isoflurane inhalation and TA muscles were treated with 50 μL of 10 μM cardiotoxin (CTX) or 1.2% barium chloride (BaCl\(_2\)) via intramuscular (IM) injection in one site of TA muscle.\(^16\) Reagents and antibodies used are listed in Tables S1 and S2, respectively. For neutrophil depletion, mice were intraperitoneally treated with 400 μg of control or Ly6G antibody. The biological analysis service for IVIS Spectrum was provided by the staff of the Korea Basic Science Institute (KBSI). Neutrophil influx was visualized under IVIS Spectrum in mice 10 min after IP injection of luminol.

Cell culture and treatments

C2C12 myoblasts maintained in 10% FBS/DMEM were differentiated into myotubes in 2% horse serum/DMEM for 4–5 days. To stain myotubes and proliferating cells, cells were treated with 10 μM EdU for 1 h, fixed with 4% paraformaldehyde (PFA), permeabilized with 0.2% TX-100 and stained with MF20 Ab and the EdU imaging kit.

Histology and immunostaining

Muscles were frozen using optimal cutting temperature (OCT) solution in isopentane pre-cooled in liquid nitrogen and sectioned into 10 μm thickness. Histological analysis using haematoxylin and eosin (H&E) or oil red O staining was performed as described previously.\(^16\) Fibrosis was detected using picrosirius red staining kit. Alizarin red staining was performed using a standard method.
For immunostaining, frozen sections were sequentially incubated for 10 min each with 4% PFA, 0.2% TX-100 and 0.3–3% H2O2 at room temperature and then blocked with 10% normal goat serum for 1 h. Sections were incubated with each primary antibody overnight at 4°C and processed using an IHC staining kit or with fluorescent-dye conjugated secondary antibodies for IF. The eMyHC and Pax7 staining were performed using a tyramide signal amplification (TSA) kit as described previously. The TSA kit was also used for Ly6G, CD68 and myosin Ila. To detect myosin heavy chains, sections were fixed in cold acetone but not in PFA. Images were captured using an EVOS FL Auto2 microscope (Invtrogen). All histological images were blinded before they were obtained, except for cell culture experiments. Fibre cross-sectional area measurements were performed in blinded studies using laminin staining as described previously. Histological data were quantified using ImageJ program.

**RNA analysis**

Muscles were homogenized in Tri reagent and total RNA was isolated using a standard method. cDNA was synthesized using oligo dT primer and reverse transcriptase. Quantitative polymerase chain reaction was carried out using SYBR green premix on a 7500 Real-Time PCR System (Applied Biosystems). Values were normalized to Gapdh. Primers are summarized in Table S3.

**Western blot analysis**

Whole muscle lysates were prepared as described previously. Equal amounts of extracts were resolved on tris-glycine or tris-tricine (for Cramp) SDS-PAGE gels, transferred onto nitrocellulose membranes and probed with antibodies. Quantifications were performed using ImageJ program.

**Pull-down experiments and in vitro binding assays**

Muscles were homogenized in suspension buffer containing 50 mM NaCl, 20 mM Tris (pH 7.4), 0.3 mM DTT, 1 mM PMSF and protease/phosphatase inhibitor cocktail. 10X detergents were added to final concentrations of 1% NP-40 and 1% TX-100. Lysates were pre-cleared using streptavidin-coated beads for 2 h. Supernatants were incubated with biotinylated peptides overnight. Streptavidin beads were added and further incubated for 2 h. Beads were washed four times with lysis buffer, once with 150 mM NaCl, and protein complexes were eluted with 2X sample buffer. Samples were resolved on SDS-PAGE gels and stained with imperial stain kits. We used biological analysis services for proteomic analysis, which were conducted by staff at the KBSI. For this, the gel slices were sent to the KBSI for in-gel digestion and LC-MS analysis.

To analyse in vitro interactions, HEK293 cells transfected with expression constructs were lysed in extraction buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and cocktails of protease and phosphatase inhibitors). HA-tagged proteins were captured using anti-HA beads and incubated with 10 μg Cramp in extraction buffer for 1 h at 4°C. Additionally, 4 μg of Serca1 recombinant protein tagged with C-terminal Myc/DDK pre-bound to anti-Flag beads was incubated with 4 μg Cramp in extraction buffer. Beads were washed four times with extraction buffer, once with 150 mM NaCl, and then eluted with 2X sample buffer.

**Serca1 ATPase activity assays**

Serca1 activity was measured in SR membrane fractions or whole-muscle homogenates using an ATPase activity assay kit (Sigma-Aldrich). Enriched SR fraction was isolated from GA muscles in accordance with previous reports. The procedures were identical except for initial homogenization using glass-to-glass homogenizer (10 strokes) and final solubilization in assay buffer (40 mM Tris, 80 mM NaCl, 8 mM MgAc2, 1 mM EDTA, pH 7.5). To extract whole-muscle homogenates, TA muscles were homogenized in assay buffer using glass-to-glass homogenizer (15 strokes). Homogenates were cleared by centrifugation (3000 rpm for 10 min, then 13 000 rpm for 20 min) and quantified using BCA assay. SR membrane fractions (100 ng) or muscle homogenates (500 ng) were incubated with increasing concentrations of CaCl2 and 750 nM A23187 in assay buffer for 10 min. ATP (1 mM final concentration) was added to initiate the reaction and incubated for 30 min, and then the reagent was added and the mixture incubated for 30 min to generate the colorimetric product. Relative Serca1 activity was plotted with added Ca2+ concentration or maximal Serca1 activity was determined.

**Grip strength test and serum creatine kinase activity assay**

Mouse muscle force was measured based on forelimb grip strength using a grip strength meter (Bioseb, BIO-GS3). Mice were allowed to grip the metal bar and then pulled horizontally by the tail. The test was performed three times, and the average force was recorded for each mouse. Measurements of grip strength were performed in blinded studies. Values were normalized to the body weight.

Whole-body muscle breakdown was measured in serum using the creatine kinase (CK) activity assay kit (BioVision). Blood was harvested at the end of experiments, and serum samples were obtained by allowing the blood to clot at room
temperature for 40 min and centrifuging at 4000 rpm for 15 min. CK enzyme activity was determined using a colorimetric method.

**Calpain activity assay**

Calpain activity was measured using a manufacturer-recommended protocol (BioVision). Muscles were homogenized in extraction buffer with 15 strokes in glass-to-glass homogenizer and centrifuged for 5 min at 4°C. Protein amounts were measured using the Bradford assay. Calpain activity was determined using a fluorometric method.

**Statistical analysis**

Data are expressed as individual values. The mean and SEM values were computed using GraphPad Prism V8.0.2. The number of biological replicates and P values are indicated in the figures. n indicates the number of mice or independent experiments (in vitro experiments). Student’s unpaired two-tailed t-test was used for comparisons between two conditions. A one-way analysis of variance with Dunnett’s post hoc test was used for multiple comparison. Survival curves were analysed using the Gehan–Breslow–Wilcoxon test. Kruskal–Wallis test with Dunn’s correction was used to obtain the median lifespan of mdx/Utrn<sup>−/−</sup> mice. Statistical significance is defined as P < 0.05.

**Results**

**Acute muscle injury increases Cramp levels in neutrophils and damaged myofibres**

To explore the potential roles of AMPs in muscle pathology, we treated mouse tibialis anterior (TA) muscles with BaCl<sub>2</sub> for 3 or 8 days to induce degeneration and regeneration, respectively, and examined the mRNA levels of defensins and Cramp (Figure S1A). Compared with untreated muscles, BaCl<sub>2</sub>-injured muscles showed a robust and transient up-regulation of Cramp on Day 3, whereas defensins, including Defa2-Defb4, were unchanged or moderately increased. Western blot analysis confirmed strong induction of both precursor and peptide forms of Cramp 3 days after injury (Figure 1A). Immunostaining revealed a prominent accumulation of Cramp in degenerating myofibres (Figure 1B). Cramp levels returned to basal levels 8 days after injury.

Analysis of CTX-injured muscles at earlier time points showed rapid induction on Day 1, followed by a gradual reduction in both Cramp mRNA and protein levels (Figure S1B and S1C). Immunohistochemical studies showed high levels of Cramp in whole muscle regions (Figure 1C) and in structures like neutrophil granules (Figure 1D) 1 day after injury. Two to three days after injury, Cramp displayed additional punctate localization patterns. Interestingly, Cramp was also concentrated within the degenerating myofibres (Figure 1C).

To verify the cellular source of elevated Cramp in damaged muscle, we co-stained Cramp with several neutrophils, macrophages, SCs or T cell-specific markers. Notably, the majority of Cramp was detected in neutrophils in 1-day injured muscles (Figure 1E). Although a small population of macrophages was positive for Cramp (Figure S1D), it was not detected in SCs or T cells (Figure S1E). We used bioluminescence imaging to quantitatively monitor tissue-infiltrating neutrophils<sup>19</sup> and found that the injections of neutrophil-neutralizing antibody reduced both neutrophil accumulation and Cramp induction in damaged muscles (Figures 1F and S1F). These results suggest that Cramp is produced by locally recruited neutrophils and then localized to degenerating myofibres.

**Cramp is internalized by skeletal muscle and causes muscle damage**

To examine whether neutrophil-derived Cramp translocated to myofibres, we traced the internalization of biotinylated Cramp (Biotin-Cramp). Two unrelated AMPs, HP (2-20) or magainin2, which have potent antimicrobial activities,<sup>20</sup>21 were used as control peptides. Notably, Biotin-Cramp was internalized into MF20-positive long myotubes rather than MF20-negative single cells (Figure 2A). Further, Cramp injected into TA muscles was distributed across individual long myofibres rather than being randomly diffused (Figure 2B), confirming that Cramp infiltrates myofibres.

We next investigated the effects of internalized Cramp on muscle damage and architecture. One day after administering Cramp, Cramp-transduced myofibres appeared to be damaged, as determined by IgG uptake (Figure 2B), a marker of myonecrosis. On Day 10, Cramp induced massive necrosis with severe inflammation (Figure 2C and 2D). Interestingly, alizarin red staining showed abnormal calcification in Cramp-treated muscle. In contrast, no obvious changes in muscle integrity were found in muscles treated with control peptides, except for local activation of muscle regeneration following injection.

To test if Cramp is also toxic to cultured myotubes, we treated C2C12 myotubes with Cramp for 24 h and analysed the number of MF20-positive differentiated cells and EdU-incorporated proliferating cells. Treatment with 10–20 μM of Cramp significantly damaged myotubes without affecting myoblast proliferation (Figure 2E and 2F), whereas a higher concentration of Cramp (40 μM) decreased both myotube integrity and cell proliferation, indicating specific induction of myotube damage with 10–20 μM of Cramp.
LL-37 also decreased myotube integrity (Figure S2). Thus, neutrophil-derived Cramp infiltrates myofibres and promotes muscle damage.

Cramp deficiency reduces muscle damage and neutrophil infiltration in the early phase of muscle injury

To define the function of Cramp in muscle degeneration, we used mice lacking Cramp in whole-body. Analysis of myosin IIa, IIx and IIb in TA muscles showed no appreciable difference between Cramp WT and homozygous (HOMO) KO mice (Figure 3A–C), indicating that loss of Cramp does not affect normal muscle development.

We analysed the early cellular and molecular events occurring in damaged muscles of Cramp WT and KO mice. WT TA muscles showed necrotic fibres with inflammatory cells 2 days after injury, whereas Cramp-null muscles showed slightly elevated levels of inflammatory cells (Figure 3A). Given that Cramp administration promotes muscle damage (Figure 2B), we asked if loss of Cramp diminishes muscle damage caused by injury. Analysis of IgG intensity showed reduced membrane permeability in Cramp KO muscles (Figure 3A), indicating that muscles lacking Cramp are more resistant to damage.

Neutrophils contribute to muscle damage by secreting toxic effectors while promoting muscle regeneration by removing damaged debris and can express IL-1β in skeletal muscles. Because Cramp and LL-37 have chemotactic activity towards neutrophils, we examined if reduced muscle damage by Cramp KO is associated with a decrease in neutrophil recruitment. Indeed, Cramp KO significantly decreased the number of neutrophil infiltrates at the site of muscle trauma 1-day post-injury, and this was sustained on Day 2 (Figure 3B). This finding was reproduced via bioimaging analysis of living mice (Figure 3C). Further, the absence of Cramp decreased IL-1β immunoreactivity and mRNA levels in CTX-injured muscle (Figure 3D). These results suggest that neutrophil-expressing Cramp further recruits peripheral neutrophils, contributing to muscle damage in response to acute injury.

Figure 1 Neutrophils deposit Cramp at sites of muscle injury. (A,B) Transient up-regulation of Cramp in degenerating muscle. TA muscles from 8-week-old male mice treated with BaCl2 for 3 or 8 days were analysed for protein expression (A) and intramuscular localization (B) of Cramp. Arrowhead and arrow indicate the precursor and peptide forms of Cramp, respectively. Difference in migration between the detected bands and the synthetic Cramp is likely due to salt content and protein quantities in the samples. Macrophage marker CD68 antibody and DAPI were used to detect inflamed regions and DNA, respectively. (C) Patterns of Cramp expression and distribution in early degenerating muscle. TA muscles of 9-week-old male mice treated with cardiotoxin (CTX) for the indicated time periods were analysed. (D) Cramp localization in neutrophil granule-like structures. (E) Co-localization of Cramp and neutrophil marker Ly6G. (F) Effect of neutrophil depletion on Cramp levels. Eight-week-old female mice were treated with control IgG or Ly6G Ab (clone 1A8) to deplete circulating neutrophils and with CTX to induce muscle damage. Reduced neutrophil invasion of damaged muscle (left) results in decreased Cramp levels (right). UD, undamaged. Scale bars, 200 μm.
Figure 2. Cramp penetrates myofibres and causes damage. (A) Internalization of exogenous Cramp into C2C12 myotubes. Myotubes were treated with 10 μM of biotinylated Cramp or control HP (2-20) peptide for 10 min, washed, fixed 3 h later and co-stained with FITC-Streptavidin and muscle differentiation marker MF20 Ab. (B) Internalization of exogenous Cramp into mouse muscle. Longitudinal sections of TA muscles derived from Cramp HOMO KO mice treated with 4 μg of Cramp for 24 h were immuno-stained with Cramp Ab for IHC (left) or with IgG for IF (right) to indicate increased membrane permeability. (C,D) Effects of Cramp administration on muscle damage. TA muscles of 8-week-old male mice were treated with 50 μL of vehicle or 0.1 mM peptides (approximately 20 μg Cramp) for 10 days. Muscle inflammation and calcification were determined via CD68 and alizarin red staining, respectively. (E,F) Effects of Cramp treatment on C2C12 myotube damage. Myotube integrity (MF20) and proliferation (EdU) were determined in myotubes treated with vehicle or indicated peptides for 24 h. Approximately 1600 nuclei were counted per well (F). Scale bars, 200 μm.
Five days after injury, small new fibres with centrally positioned myonuclei appeared and temporally expressed embryonic myosin (eMyHC), a marker of regenerating fibres (Figure 3E). We found that the degree of muscle regeneration was decreased in Cramp-null muscles, as indicated by lower eMyHC intensity and the presence of residual macrophages (Figures 3E and 3F and S4). Measurements of the cross-sectional area and the numbers of new fibres revealed smaller, but similar number of myofibres in Cramp KO compared with WT (Figure 3F), indicating delayed muscle regeneration. These results suggest that dysregulation of neutrophil influx due to Cramp deficiency may slow down muscle damage and inflammatory responses, thus retarding the clearance of damaged fibres and subsequent myogenesis in acutely injured muscle.

**Cramp deficiency improves muscle integrity after long-term injury**

To evaluate the function of Cramp in late stage muscle regeneration, we analysed 12-day treated TA samples from Cramp WT and HOMO KO mice. Whereas damaged muscles from both genotypes were regenerated by this time point, muscles with Cramp KO showed significant reduction in interstitial space between new myofibres (Figures 4A and S5A). A similar low-grade inflammation and slightly smaller fibres were found in Cramp KO muscle (Figures 4A and S5B).

Four weeks after muscle injury, Cramp loss resulted in a slight increase in muscle weight and fibre size compared with WT (Figure 4B). Histological analysis showed that Cramp KO partially reduced the development of fibrotic and adipose tissues within skeletal muscle (Figure S6A and S6B). To further investigate the role of Cramp in chronic muscle injury, we administered three injections of BaCl₂ (BaCl₂-3X) and analysed the muscles 4 weeks after the last injection (Figure 4C). Notably, muscle weight and fibre size were significantly increased in Cramp KO-injured muscles. Further analysis of fibre size distribution showed a typical rightward shift towards larger fibres in Cramp-null injured muscles (Figure 4D), indicating that regeneration-associated muscle hypertrophy was accelerated by Cramp inactivation. Furthermore, fibrofatty tissues were significantly diminished in Cramp-deficient muscles (Figure 4E and 4F). Together, the larger myofibres and less...
fi brofatty tissues in muscles lacking Cramp indicate the improved pathology of skeletal muscle in response to long-term multiple injury. We also assessed whether Cramp haploinsufficiency played a similar immunomodulatory role after injury. The findings showed that heterozygous (HET) KO of Cramp did not significantly affect neutrophil invasion on Day 2 following injury, nor macrophage accumulation on Day 5 (Figure S7A and S7B). Thus, complete inactivation of Cramp is required for the regulation of muscle inflammation induced by acute injury.

**Cramp is activated in dystrophic muscles during DMD development**

DMD is associated with muscle wasting, cardiac failure and premature death. Several mouse models have been used...
to identify mechanisms underlying DMD as well as potential treatments. Mdx mice, the most widely used model, show relatively mild phenotypes compared with fatal human DMD. Mice with additional KO of utrophin (mdx/Utrn^{-/-}) display severe disease phenotypes with shortened lifespans, whereas utrophin haploinsufficiency (mdx/Utrn^{+/+}) mice exhibit intermediate phenotypes between mdx and mdx/Utrn^{-/-}. Analysis of TA muscles from 4-week-old mdx mice revealed Cramp up-regulation in dystrophic muscles and strong accumulation in dystrophic areas (Figure 5A and 5B). At 8 weeks, elevated Cramp levels were detected in all three dystrophic types, with positive correlation between Cramp levels and disease severity (Figure 5C). Cramp was also induced in both skeletal and cardiac muscles of mdx/Utrn^{+/+} mice at 24 weeks of age (Figure 5D). Thus, Cramp is activated in dystrophic muscle developed in the mouse model of DMD.

Inactivation of Cramp mitigates DMD phenotypes

To investigate the functional significance of Cramp in DMD, we generated diseased mice lacking Cramp and produced diverse genotypes by crossing mdx^{+/Y}/Utrn^{+/+}/Cramp^{+/+} male and mdx^{+/Y}/Utrn^{+/+}/Cramp^{-/-} female mice. We examined muscle physical activity and whole-body muscle breakdown by measuring forelimb grip strength and serum CK activity, respectively. Eight-week-old mdx/Cramp^{-/-} mice showed enhanced grip strength compared with WT counterparts, whereas the improvement was less prominent in mdx/Cramp^{+/+} mice (Figure 6A). Analysis of older mice with more severe phenotypes (24-week-old mdx/Utrn^{+/+}) revealed that grip strength was markedly increased by Cramp haploinsufficiency and moderately increased by Cramp deficiency (Figure 6B). Serum CK activity was also significantly decreased by Cramp HET KO. Cramp loss in either mdx/Utrn^{+/+} or mdx mice diminished serum CK activity, although this reduction was not statistically significant (Figures 6B and S8). Moreover, myofibre size reduction occurred in mdx/Utrn^{+/+} mice was alleviated by both Cramp HET and HOMO KO. These findings indicate that reducing Cramp levels improves DMD pathophysiology.

As a further assessment of muscle abnormalities, we analysed histological features. Strikingly, either reduction or lack of Cramp substantially reduced membrane breakdown, infusions of neutrophils and macrophages and fibrosis of muscle interstitium (Figures 6C and S9). Fat accumulation was not severe, even in mdx/Utrn^{+/+}/Cramp^{+/+} muscles, and no differences were detected between groups.

Figure 5 Cramp is persistently elevated in skeletal muscle of DMD mouse models. (A) Up-regulation of Cramp in TA muscle of 4-week-old mdx male mice. Arrowhead and arrow indicate the precursor and peptide forms of Cramp, respectively. (B) Accumulation of Cramp around CD68-positive inflamed regions in TA muscle of 4-week-old mdx male mice. (C) TA muscles from 8-week-old mdx, mdx/Utrn^{+/+} and mdx/Utrn^{-/-} male mice were analysed for Cramp protein expression. (D) Skeletal TA muscles and cardiac muscles from 24-week-old mdx/Utrn^{+/+} male mice were analysed for Cramp expression. WT indicates normal C57BL/6 mice. Non-specific signal (NS) was used as a protein loading control. Scale bars, 200 μm.
Mdx/Utrn−/− mice phenocopy the severe features of patients with DMD, including premature death. To verify whether targeting Cramp could be a potential treatment for DMD, we monitored the lifespans of Mdx/Utrn−/−/Cramp KO lines. We found that Cramp haploinsufficiency markedly extended the lifespans of both male and female mice (Figure 6D). Loss of Cramp significantly delayed the death of female mice, but only slightly delayed the death of male mice. Spe-
cifically, the median lifespans of mdx/Utrn−/−/Cramp+/+, mdx/Utrn−/−/Cramp+/− and mdx/Utrn−/−/Cramp−/− mice were 10.2, 16.6 and 15.3 weeks, respectively, for males and 11.9, 26.6 and 20.2 weeks, respectively, for females (Figure 6E). Collectively, these results suggest that Cramp inhibition attenuates manifestations of dystrophic disease in DMD mouse models.

**Cramp binds to muscle proteins associated with Ca<sup>2+</sup> signalling**

Our results show that Cramp HET KO does not inhibit neutrophil invasion following acute injury but profoundly suppresses DMD progression. Accordingly, neutrophil inhibition by Cramp can only contribute to, but cannot fully explain, the molecular mechanisms targeted by Cramp. Moreover, treatment of muscle cell culture with Cramp resulted in muscle damage, implying the existence of intrinsic muscle factors mediating Cramp action. To screen Cramp-interacting muscle proteins, we performed pull-down experiments using biotinylated peptides. Biotin-Cramp was incubated with muscle lysates, and the protein complexes captured by streptavidin beads were subjected to SDS-PAGE and liquid chromatography–mass spectrometry (LC-MS) analysis (Figure 7A). Interestingly, the protein complex contained Serca1 and its isoforms, Dpf1, Cpt1b, Slc25a12, Jph2, Aifm1, Atp5f1a, Titin, Fmrd4b, Pttds2, Cttnap1, Slc25a4 and the previously recognized Gapdh in monocytes. Many of these have previously been implicated in Ca<sup>2+</sup> signalling, mitochondrial functioning or both. Western blot analysis confirmed the binding of several proteins (Figure 7B), including SR Ca<sup>2+</sup> pump Serca1, Cpt1b associated with mitochondrial fatty acid oxidation, Ca<sup>2+</sup>-binding mitochondrial carrier protein Slc25a12 and Slc25a4 associated with mitochondrial permeability transition.

**Figure 7** Cramp binds to skeletal muscle proteins associated with SR Ca<sup>2+</sup> signalling and mitochondrial functions. (A,B) Identification of Cramp-binding skeletal muscle proteins via in vitro screening. Schematic illustration of the experimental strategy and proteins identified using LC–MS are shown. Presence of Cramp and green-coloured proteins: Serca1, Cpt1b, Slc25a12 and Slc25a4 in the precipitants were confirmed using Western blot analysis (B). (C) In vivo interaction between Cramp and SR Ca<sup>2+</sup>-handling proteins (left) and mitochondrial proteins (right). TA muscles in 8-week-old Cramp HOMO KO male mice were injected with Biotin-HP (2-20) or Biotin-Cramp (approximately 10 μg) for 24 h. Protein complexes in lysates were precipitated using streptavidin-coated beads and subjected to Western blot analysis using the indicated antibodies. (D) In vitro binding of Cramp to Serca1-HA and Csq1-HA purified from HEK293 cells transfected with expression constructs.
To verify the interactions in vivo, we administered Biotin-Cramp into TA muscles for 24 h, and proteins bound to Biotin-Cramp were collected using streptavidin beads (Figure 7C). Again, Serca1 was pulled-down with Biotin-Cramp. We further examined the interaction between Cramp and other SR Ca\(^{2+}\) regulatory proteins, such as Ca\(^{2+}\)-buffering protein Csq, and Ca\(^{2+}\) release channel RyR, although they were not detected during the initial screening. As shown in Figure 7C, Csq, but not RyR, was co-precipitated with Biotin-Cramp. Mitochondrial Cpt1b, S1c25a12 and Slc25a4 were also precipitated.

To examine peptide–protein interaction in vitro, we incubated Cramp with hemagglutinin (HA)-tagged Serca1 or Csq1 purified from HEK293 cells transfected with expression constructs and found physical interactions between Cramp and both Serca1 and Csq1 (Figure 7D). Stronger binding to Csq1 than to Serca1 is likely a reflection of higher quantities of Csq1 input. Moreover, Cramp bound to recombinant Serca1 protein in vitro (Figure S10). Together, these results indicate that Cramp binds to proteins associated with SR Ca\(^{2+}\) signalling and mitochondrial functions.

**Cramp inhibits Serca1 activity and promotes calpain activity**

It has been known that Serca1 protects skeletal muscles from DMD damage.\(^5\) To determine whether Cramp regulates Serca1 activity, we analysed Ca\(^{2+}\)-ATPase activity in SR membrane fractions or muscle homogenates incubated with Cramp in vitro. Under both conditions, Cramp markedly decreased Serca1 activity, independent of the quantities of Ca\(^{2+}\) (Figures 8A and S11A) or ATP added (Figure S11B).

We also examined whether Cramp deficiency affects the protein levels of SR Ca\(^{2+}\)-handling factors in TA muscles of mdx/Utrn\(^{+/+}\) mice. We found that reduction or ablation of Cramp significantly or partially elevated Serca1 levels, respectively (Figures 8B and S12). In contrast, the levels of neighbouring SR proteins, Csq and RyR, were affected less by Cramp KO, indicating the specific up-regulation of Serca1.

To further elucidate Serca1 inhibition by Cramp during DMD development, we compared Serca1 activity in muscle homogenates and found that reducing or depleting Cramp levels in mdx/Utrn\(^{+/+}\) muscle restored the overall activity of Serca1 (Figure 8C). Reinforcing this notion, both Cramp HET and KO mice showed reduced calpain activity (Figure 8D). Calpain activity was determined in pooled homogenates (left) or individual homogenate (right) of TA muscles from 24-week-old male mice with the indicated genotypes. WT, 24-week-old C57BL/6J. n = 9 for Cramp WT, n = 10 for HET KO and n = 10 for HOMO KO. (D) Reduced calpain activity in Cramp KO DMD muscles. TA muscles of 24-week-old male mice were used. N = 4 for each group. (E) Decreased Ca\(^{2+}\)-dependent ATPase activity (left) and increased calpain activity (right) in Cramp-transduced TA muscles. Muscle homogenates from 8-week-old C57BL/6 male mice injected with control HP (2-20) or Cramp for the indicated periods were analysed.

![Figure 8](image-url)
and HOMO KO reduced the enzymatic activity of calpains (Figure 8D). The calpain activity due to Cramp loss was also reduced in the 12-week-old mdx mice (Figure S13).

To gain further insights into the roles of Cramp in Ca\(^{2+}\)-dependent myonecrosis, we determined whether Cramp administration into skeletal muscle affects the activities of Serca1 and calpain proteases. We found that Cramp decreased Ca\(^{2+}\)-ATPase activity on both Day 1 and Day 5 after treatment, whereas calpain activity was significantly increased on Day 5 (Figure 8E). Collectively, these findings suggest that functional interaction between Cramp and Serca1 contributes to DMD pathogenesis by exacerbating the Ca\(^{2+}\) -dependent proteolytic pathway.

**Discussion**

Increasing evidence suggesting the role of endogenous AMPs in the pathogenesis of diverse non-infectious diseases prompted us to investigate their function in muscle pathology. Here, we found that Cramp plays a role in multiple facets of skeletal muscle damage in mice, including inflammation, immune cell-muscle cell interaction and myonecrosis. Cramp appeared to promote muscle damage by exacerbating both Ca\(^{2+}\)-dependent myonecrosis and neutrophil-associated inflammation via different mechanisms in different places.

Our results demonstrate that Cramp contributes to acute muscle damage, at least in part, by regulating neutrophil infiltration. Cramp also plays a role in neutrophil recruitment and IL-1\(\beta\) expression in other tissues.\(^{15,34}\) \(\beta_2\) integrin CD18 and formylpeptide receptors bind to Cramp and play a role in neutrophil regulation and thus might be associated with Cramp regulation of neutrophil invasion in skeletal muscle.\(^{15,34–37}\) In this scenario, neutrophil-expressing Cramp further recruits peripheral neutrophils, contributing to muscle damage in response to acute injury.

Typically, neutrophils stimulate the recruitment of macrophages, the immune cells that exert pro-inflammatory function (M1 macrophages) and resolve inflammation with SC stimulation for muscle recovery (M2 macrophages).\(^{38}\) However, despite a decrease in neutrophils, Cramp KO-injured muscle showed increased macrophages. Similarly, a decrease in neutrophil invasion following CD18 blockade after muscle injury resulted in an accelerated influx of macrophages.\(^{26}\) This study also demonstrated that injured muscles deficient CD18 display delayed regeneration during the early period but larger myofibres during the late stage of muscle regeneration, similar to Cramp-null damaged muscles. Hence, enhanced inflammation of macrophages in Cramp-null muscle might be attributed to compensatory activation against abnormal neutrophil suppression, and this later stimulates muscle repair.

Ca\(^{2+}\) overload resulted from unstable sarcolemma integrity of dystrophin-deficient muscle programs DMD progression. Our initial observation that Cramp introduction causes muscle calcification suggested a potential role for Cramp in Ca\(^{2+}\) signalling. Subsequent proteomic and mechanistic studies identified Ca\(^{2+}\)-SERCA1 axis regulated by Cramp. SERCA activity can be impaired in dystrophic muscles by its downregulation, post-translational modification and/or direct inhibition by SERCA inhibitory peptides,\(^3\) including sarcolipin (Sln) and phospholamban (Pln). However, it is unlikely that the Cramp is associated with Sln or Pln in mouse skeletal muscle, because they are barely detectable in the fast-twitch skeletal muscles of rodents.\(^{39,40}\) We note that Cramp also forms complexes with other Ca\(^{2+}\)-associated factors such as Csqa1 and Slc25a12, implying that Ca\(^{2+}\) regulation by Cramp is not limited to the SERCA pump. Therefore, in contrast to conventional SERCA inhibiting micropeptides located in muscle SR membrane, Cramp is an inflammatory cell-derived peptide that disrupts Ca\(^{2+}\) homoeostasis when muscle is injured. Interestingly, Cramp was also found to affect Serca1 levels, at least in TA muscles of the mdx/Utrn\(^{17/–}\) model. Although the mechanistic basis behind this observation remains to be elucidated, this two-way arrangement of Serca1 by Cramp might facilitate efficient suppression of Serca activity in response to chronic damage.

Most notably, this study showed that reducing Cramp levels significantly extends lifespan by protecting dystrophic muscles from deterioration. Unexpectedly, however, the total loss of Cramp appeared not to be more beneficial on alleviating overall DMD than Cramp haploinsufficiency. We assume that a small portion of elevated Cramp may be indispensable for normal Ca\(^{2+}\) homoeostasis or for immune cell regulation. This feature may be relatively common in Serca inhibitory peptides, as Sln HET KO in mdx/Utrn\(^{17/–}\) mice has been shown to alleviate overall DMD more than Sln deficiency.\(^6\) We also noticed that the extended lifespan of mdx/Utrn\(^{17/–}\) mice following Cramp KO was more obvious in female mice than in male mice. Given that female hormones are protective against muscle damage in mdx mice,\(^41\) it is interesting to speculate that Cramp may also be involved in the regulation of sex hormones.

In this study, we provide genetic evidence for the role of Cramp in promoting the progression of DMD. Cramp-deficient mice do not show overt abnormalities at baseline, and the loss of one Cramp allele in mdx/Utrn\(^{17/–}\) mice can prolong survival. In this regard, Cramp inhibition would be an attractive therapeutic approach against DMD with minimal side effects.

**Limitations of the study**

Here, we investigated whether and how Cramp regulates skeletal muscle degeneration in mice. Whether human LL-37 is also up-regulated in the muscles of DMD patients needs to be investigated. In addition, although we showed...
that Cramp inhibits Serca1 activity, we did not investigate the structural inhibition of Serca1 activity. In-depth analyses are necessary to fully elucidate the mechanism underlying regulation of Serca1 by Cramp. Further, the study should be extended to cardiac muscles. It is essential to explore the translational significance of pharmacological inhibition of Cramp and its benefits in DMD and associated cardiomyopathies.

Acknowledgements

We thank Dr Seongsoo Lee for conducting IVIS imaging studies. We also thank Dr Jong Bok Seo for conducting in-gel digestion and LC-MS analysis. This work was supported by the National Research Foundation of Korea (NRF) grants (2018R1D1A1A02042725 and 2021R1A2C1003561 to M.-C. C.; 2019R1A2B5B03070330 and 2017M3A9E4077206 to Y.P.) and Institute for Information & Communications Technology Promotion (IITP) grant (MSIT; No. 2017-0-01714, Development of Antimicrobial Peptide using Deep Learning) funded by the Korean Government. The authors of this manuscript certify that they comply with the ethical guidelines for authorship and publishing in the Journal of Cachexia, Sarcopenia and Muscle.

Conflict of interest

None declared.

Online supplementary material

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

References

1. Laumonier T, Menetrey J. Muscle injuries and strategies for improving their repair. J Exp Orthop 2016;3:15.
2. Duan D, Goemans N, Takeda S, Mercuri E, Aartsma-Rus A. Duchenne muscular dystrophy. Nat Rev Dis Primers 2021;7:13.
3. Mareedu S, Million ED, Duan D, Babu GJ. Abnormal calcium handling in Duchenne muscular dystrophy: Mechanisms and potential therapies. Front Physiol 2021;12:647010.
4. Periasamy M, Kalyanasundaram A. SERCA pump isoforms: Their role in calcium transport and disease. Muscle Nerve 2007;35:430–442.
5. Goonasekera SA, Lam CK, Millay DP, Sargent MA, Hajar RJ, Kranias EG, Molkentin JD. Mitigation of muscular dystrophy in mice by SERCA overexpression in skeletal muscle. J Clin Investig 2011;121:1044–1052.
6. Voit A, Patel V, Pachon R, Shah V, Bakhutma M, Kohlbrenner E, McArdle JJ, Dell’Italia LJ, Mendell JR, Xie LH, Hajar RJ, Duan D, Fraidenraich D, Babu GJ. Reducing sarcolin expression mitigates Duchenne muscular dystrophy and associated cardiomyopathy in mice. Nat Commun 2017;8:1068.
7. Gehrig SM, van der Poel C, Sayer TA, Schertzer JD, Henstridge DC, Church JE, Lamon S, Russell AP, Davies KE, Febbraio MA, Lynch GS. Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. Nature 2012;484:394–398.
8. Prasad SV, Fiedoruk D, Daniluk T, Piall T, Bucki R. Expression and function of host defense peptides at inflammation sites. Int J Mol Sci 2019;21:104.
9. Yamaguchi Y, Ouchi Y. Antimicrobial peptide defense: Identification of novel isoforms and the characterization of their physiological roles and their significance in the pathogenesis of diseases. Proc Jpn Acad Ser B Phys Biol Sci 2012;88:152–166.
10. Alford MA, Baquir B, Santana FL, Haney EF, Hancock REW. Cathelicidin host defense peptides and inflammatory signaling: Striking a balance. Front Microbiol 2020;11:1902.
11. Ganz T. Defensins: Antimicrobial peptides of innate immunity. Nat Rev Immunol 2003;3:710–720.
12. Pircher J, Czermak T, Ehrlich A, Eberle C, Zasloff M. Magainins, a class of antimicrobial peptides and in inflammatory signaling: Some. J Mol Cell Cardiol 2020;139:1523.
13. Pan LL, Liang W, Ren Z, Li C, Chen Y, Niu W, Liu Y, Zhong M, Diana J, Agerberth B, Sun J. Cathelicidin-related antimicrobial peptide protects against ischaemia-reperfusion-induced acute kidney injury in mice. Br J Pharmacol 2020;177:2726–2742.
14. Choi MC, Jo J, Lee M, Park J, Park Y. Intra-articular administration of Cramp into mouse knee joint exacerbates experimental osteoarthritis progression. Int J Mol Sci 2021;22:3429.
15. Wu Y, Zhang Y, Zhang J, Zhai T, Hu J, Luo H, Zhou H, Zhang Q, Zhou Z, Liu F. Cathelicidin aggravates myocardial ischemia/reperfusion injury via activating TLR4 signaling and P2X7R/NLRP3 inflammasome. J Mol Cell Cardiol 2020;139:75–86.
16. Choi MC, Ryu S, Hao R, Wang B, Kapur M, Fan CM, Yao TP. HDAC4 promotes Pax7-dependent satellite cell activation and muscle regeneration. EMBO Rep 2014;15:1175–1183.
17. Choi MC, Cohen TJ, Barrientos T, Wang B, Li M, Simmons BJ, Yang JS, Cox GA, Zhai T, Yao TP. A direct HDAC4-MAP kinase crosstalk activates muscle atrophy program. Mol Cell 2012;47:122–132.
18. Hardy D, Besnard A, Latil M, Jouvin G, Bakhutma M, Kohlbrenner E, McArdle JJ, Sepulchre P, Christien F. Comparative study of injury models for studying muscle regeneration in mice. PLoS ONE 2016;11:e0147198.
19. Tseng JC, Kung AL. In vivo imaging of inflammatory phagocytes. Chem Biol 2012;19:1199–1209.
20. Bylund J, Christophe T, Boulay F, Nystrom T, Karlsson A, Dahlgren C. Proinflammatory activity of a cecropin-like antibacterial peptide from Helicobacter pylori. Antimicrob Agents Chemother 2001;45:1700–1704.
21. Zaslavoff M, Magainins, a class of antimicrobial peptides from Xenopus skin: Isolation, characterization, and expression of two active forms, and partial cDNA sequence of a precursor. Proc Natl Acad Sci U S A 1987;84:5449–5453.
22. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamasp V, Piraino J, Huttner K, Gallo RL. Innate antimicrobial peptide protects the skin from invasive bacterial infection. Nature 2001;414:454–457.
23. Yang W, Hu P. Skeletal muscle regeneration is modulated by inflammation. J Orthop Translat 2018;13:25–32.
24. Tsuchiya M, Sekia S, Hatakeyama H, Koide K, Chawewannakorn C, Yaito F, Tan-No K, Sasaki K, Watanabe M, Sugawara S, Endo Y, Itoe I, Hagiwara Y, Kanzaki M. Neutrophils provide a favorable IL-1-mediated immunomodulatory niche that primes GLUT4...
translocation and performance in skeletal muscles. *Cell Rep* 2018;23:2354–2364.
25. Kawanishi N, Mizokami T, Niihara H, Yada K, Suzuki K. Neutrophil depletion attenuates muscle injury after exhaustive exercise. *Med Sci Sports Exerc* 2016;48:1917–1924.
26. Pizza FX, Peterson JM, Baas JH, Koh TJ. Neutrophils contribute to muscle injury and impair its resolution after lengthening contractions in mice. *J Physiol* 2005;562:899–913.
27. Hodgetts S, Radley H, Davies M, Grounds MD. Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with etanercept in mdx mice. *Neuromuscul Disord* 2006;16:591–602.
28. Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, Yang D. Mouse cathelin-related antimicrobial peptide chemo-attracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J Immunol* 2005;174:6257–6265.
29. Yucel N, Chang AC, Day JW, Rosenthal N, Blau HM. Humanizing the mdx mouse model of DMD: the long and the short of it. *NPJ Regen Med* 2018;3:4.
30. Mookherjee N, Lippert DN, Hamill P, Falsafi R, Nijnik A, Kindrachuk J, Pistolic J, Gardy J, Miri P, Naseer M, Foster LJ, Hancock REW. Intracellular receptor for human host defense peptide LL-37 in monocytes. *J Immunol* 2009;183:2688–2696.
31. Bonnefont JP, Djouadi F, Prip-Buus C, Munnich A, Bastin J. Carnitine palmitoyltransferases 1 and 2: Biochemical, molecular and medical aspects. *Mol Aspects Med* 2004;25:495–520.
32. Brustovetsky N. The role of adenine nucleotide translocase in the mitochondrial permeability transition. *Cell* 2020;9:2686.
33. Döring Y, Drechsler M, Wantha S, Kemmerich K, Lievens D, Vijayan S, Gallo RL, Weber C, Seehnlein O. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res* 2012;110:1052–1056.
34. Sekheri M, Othman A, Filep JG. beta2 Integrin regulation of neutrophil functional plasticity and fate in the resolution of inflammation. *Front Immunol* 2021;12:660760.
35. Liu M, Chen K, Yoshimura T, Liu Y, Gong W, Le Y, Gao JL, Zhao J, Wang JM, Wang A. Formylpeptide receptors mediate rapid neutrophil mobilization to accelerate wound healing. *PLoS ONE* 2014;9:e90613.
36. Babu GJ, Bhupathy P, Carnes CA, Billman GE, Periasamy M. Differential expression of sarcolipin protein during muscle development and cardiac pathophysiology. *J Mol Cell Cardiol* 2007;43:215–222.
37. Anderson DM, Makarewich CA, Anderson KM, Shelton JM, Bezprozvannaya S, Bassel-Duby R, Olson EN. Widespread control of calcium signaling by a family of SERCA-inhibiting micropeptides. *Sci Signal* 2016;9:ra119.
38. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: Update 2021. *J Cachexia Sarcopenia Muscle* 2021;12:2259–2261.