The NLRP3 inflammasome contributes to inflammation-induced morphological and metabolic alterations in skeletal muscle

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Abstract

Background Systemic inflammation is associated with skeletal muscle atrophy and metabolic dysfunction. Although the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome contributes to cytokine production in immune cells, its role in skeletal muscle is poorly understood. Here, we studied the link between inflammation, NLRP3, muscle morphology, and metabolism in in vitro cultured C2C12 myotubes, independent of immune cell involvement.

Methods Differentiated C2C12 myotubes were treated with lipopolysaccharide (LPS; 0, 10, and 100–200 ng/mL) to induce activation of the NLRP3 inflammasome with and without MCC950, a pharmacological inhibitor of NLRP3-induced IL-1β production. We assessed markers of the NLRP3 inflammasome, cell diameter, reactive oxygen species, and mitochondrial function.

Results NLRP3 gene expression and protein concentrations increased in a time-dependent and dose-dependent manner. Intracellular IL-1β concentration significantly increased (P < 0.0001), but significantly less with MCC950 (P = 0.03), suggestive of moderate activation of the NLRP3 inflammasome in cultured myotubes upon LPS stimulation. LPS suppressed myotube growth after 24 h (P = 0.03), and myotubes remained smaller up to 72 h (P = 0.0009). Exposure of myotubes to IL-1β caused similar alterations in cell morphology, and MCC950 mitigated these LPS-induced differences in cell diameter. NLRP3 appeared to co-localize with mitochondria, more so upon exposure to LPS. Mitochondrial reactive oxygen species were higher after LPS (P = 0.03), but not after addition of MCC950. Myotubes had higher glycolytic rates, and mitochondria were more fragmented upon LPS exposure, which was not altered by MCC950 supplementation.

Conclusions LPS-induced activation of the NLRP3 inflammasome in cultured myotubes contributes to morphological and metabolic alterations, likely due to its mitochondrial association.

Keywords Systemic inflammation; NLRP3 inflammasome; Skeletal muscle; Metabolism; Morphology

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Introduction

Systemic inflammation represents a hallmark of various chronic and metabolic diseases and contributes to muscle wasting in cancer cachexia, ageing, and cardiovascular and lung diseases. Eventually, systemic inflammation can cause mitochondrial dysfunction and muscle atrophy. This process is mediated by immune cells that secrete inflammatory mediators, including chemokines and cytokines, partly via the activation of the nucleotide-binding oligomerization-domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasome.

NLRP3 inflammasome activation in monocytes and macrophages represents a crucial part of the innate immune system. This specific inflammasome is a multi-protein complex consisting of the NOD-like receptor 3 (NLRP3) protein, the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) and the effector protein (pro-)caspase-1. Assembly of this protein complex in macrophages takes place upon the presentation of various endogenous pathogen-associated or danger-associated molecular patterns, which induces cleavage and activation of caspase-1, ultimately resulting in the secretion of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18.

Although recent preliminary work suggests that the NLRP3 inflammasome is also expressed in myoblasts, it is currently unknown whether NLRP3 is activated and produces endogenous caspase-1 and IL-1β upon systemic inflammation in skeletal muscle cells. Also, a role of mitochondria in the activation of the NLRP3 inflammasome has been suggested, but it remains to be studied whether a structural and functional link exists between NLRP3 and skeletal muscle mitochondria.

Studying the involvement of the NLRP3 inflammasome in skeletal muscle in vivo is complicated by factors such as immune cell function, circulating chemokines and cytokines, as well as local immune cell infiltration. Therefore, in the present study, we used the endotoxin lipopolysaccharide (LPS) to mechanistically study how inflammation affects NLRP3 inflammasome activation in differentiated C2C12 myotubes in vitro and its potential morphological and metabolic consequences. LPS, as a constituent of Escherichia coli bacteria, is present in the human gut and in the circulation of septic patients or after surgery. Circulatory LPS concentrations are increased in several endogenous diseases such as diabetes or chronic heart failure. With these diseases being associated with skeletal muscle wasting, LPS represents a physiological model for studying inflammation-induced skeletal muscle alterations.

The endotoxin LPS binds to the Toll-like receptor 4 (TLR4) at the cell membrane and induces an intracellular cascade, ultimately inhibiting protein synthesis via Akt/mammalian target of rapamycin (mTOR), mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) 1/2, and p38 MAPK. Binding of LPS to TLR4 is also the common inducer of inflammation or the NLRP3 inflammasome in macrophages. Therefore, we hypothesized LPS exposure of myotubes to result in NLRP3 inflammasome activation, contributing to inflammation-induced alterations in myotube morphology, which can be mitigated by a pharmacological blocker of NLRP3 activation (MCC950). Further, we hypothesized that the NLRP3 inflammasome causes mitochondrial dysfunction, ultimately contributing to myotube growth impairments.

Methods

Cell culture

C2C12 mouse myoblast cells (ATCC CRL-1772, Wesel, Germany) were cultured in growth medium consisting of high-glucose Dulbecco’s Modified Eagle’s Medium (Gibco, 11995, Waltham, MA, USA), supplemented with 10% fetal bovine serum (Biowest, S181B, Nuaillé, France), 100 μg/mL carbenicillin/ampicillin (Sigma-Aldrich C1389, A0166), and 0.5% fungizone (Gibco, A2942, Waltham, MA, USA). Streptomycin was avoided as this likely affects mitochondrial function. Unless stated otherwise, 12 × 10³ cells were seeded on a 12-well cell culture plate, which resulted in 70–80% confluency on day 4. Myotube differentiation was induced by switching from growth to differentiation medium (DMEM supplemented with 2% horse serum [HyClone, 10407223, Marlborough, MA, USA], 100 μg/mL carbenicillin/ampicillin and 0.5% fungizone), refreshed daily, for a period of 96 h. Myotubes were kept in a humidified incubator at 37°C with 5% CO₂. All data points represent individual independent replicates (with ≥ 3 technical replicates) from different cell experiments with freshly prepared medium. The maximal passage of cells was kept below nine.

Cell treatments

Following 96 h of differentiation, mature myotubes were treated with varying concentrations of LPS (10 and 100–200 ng/mL; Sigma-Aldrich L2630) for 24–72 h. These concentrations were based on previous work in monocytes and macrophages. The pharmacological NLRP3 inhibitor MCC950 (10 μM; CRID3 sodium salt; TOCRIS 5479) was dissolved in water as vehicle and used to block NLRP3 inflammasome activation. This small-molecule inhibitor acts via blocking oligomerization of the NLRP3 inflammasome, thus preventing caspase-1 cleavage and downstream IL-1β production. MCC950 has proven its effectiveness in vitro, ex vivo, and in vivo in various inflammatory diseases. Myotubes were exposed to IL-1β (50 ng/mL; PeproTech 200-01B) as a positive control of inflammasome activation. A pre-treatment
of myotubes with the cardiolipin stabilizer SS31 (1 µM) was added before the LPS exposure to study whether lowering mitochondrial reactive oxygen species (mtROS) production affected cell diameter. Results were compared with vehicle-treated control.

**Myotube diameter**

Myotube diameters were measured at each treatment timepoint, as previously described²³ (see Supporting Information for more details).

**Real-time quantitative polymerase chain reaction**

To study the initial response of LPS on gene expression, we performed real-time quantitative polymerase chain reaction.²⁴ The Supporting Information provides an in-depth description. Primers of the following genes are listed in Table S1: Nlrp3, Casp1, Il1b, Il18, Il6, Fbxo32, and Trim63. Gene expression levels were normalized to 18S, as this housekeeping rRNA shows less variability than other commonly used methods, using the delta C_T method.

**Western blotting**

Western immunoblotting was performed to assess protein concentration of markers of the NLRP3 inflammasome (see Supporting Information). Primary antibodies used were NLRP3 (rabbit polyclonal antibody, d1:500, NB2-12446SS, Novus Biologicals), caspase-1 (rabbit polyclonal antibody, d1:1000, NBP1-45433SS, Novus Biologicals), and pan-actin (rabbit polyclonal antibody, d1:4000, D18C11, Cell Signaling Technology, Bioké, 4968s) as loading control. Quantification was performed using ImageJ, and results were normalized with control.

**Enzyme-linked immunosorbent assay**

Intracellular interleukin-1β (IL-1β) protein concentration in myotubes was assessed using the Quantikine Mouse IL-1β enzyme-linked immunosorbent assay (ELISA) (MLB00C, R&D systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Cells were washed, centrifuged, and then lysed in Cell Lysis Buffer 2 (895347, R&D systems, Minneapolis, MN, USA). Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, C10640, USA) according to manufacturer’s instructions. EdU staining was performed using the Click-it EdU Imaging Kit (Thermo Fisher, C10640, USA) according to manufacturer’s instructions. EdU-positive cells were stained in red, and cell nuclei visualized by DAPI (VECTASHIELD, H-1200, Vector Laboratories, USA). Images were taken at five distinct locations per well. The percentage of EdU-positive cells was determined by the number of EdU-positive cells/nuclei using ImageJ.

**Immunofluorescence**

To assess the spatio-temporal location of NLRP3 in relation to mitochondria, myotubes were cultured on matrigel-coated glass-cover slips (see Supporting Information). Myotubes were incubated overnight with the primary mouse monoclonal anti-succinate dehydrogenase subunit A antibody (SDHA, 1:500, ab14715, Abcam) and rabbit polyclonal anti-NLRP3 antibody (1:50, NB2-12446SS, Novus Biologicals) at 4°C. Subsequently, myotubes were incubated with secondary antibodies (goat anti-mouse; 1:100; Abberior 580 and goat anti-rabbit; 1:100; Abberior 635P), and DAPI (0.2 µg/mL). Mander’s co-localization coefficient was used to assess the fraction of NLRP3 overlapping with SDHA (JACoP ImageJ plug-in). For myosin heavy chain (MHC) staining, myotubes were stained using a mouse monoclonal anti-MHC antibody (MF-20, 1:50, DSHB, USA) overnight at 4°C, after which cells were incubated with secondary antibody (goat anti-mouse; 1:1000, Thermo Fisher, A21141). Nuclei were stained by DAPI (VECTASHIELD, H-1200, Vector Laboratories, USA). Differentiation index was defined as number of MHC-positive cells (more than two nuclei)/total number of cells and fusion index as the average number of nuclei in MHC-positive cells, per region of interest.

**Proliferation**

After 24 h of 100 ng/mL LPS treatment, myoblasts were incubated for 2 h with 10 µM 5-ethynyl-2′-deoxyuridine (EdU). EdU staining was performed using the Click-iT EdU Imaging Kit (Thermo Fisher, C10640, USA) according to manufacturer’s instructions. EdU-positive cells were stained in red, and cell nuclei visualized by DAPI (VECTASHIELD, H-1200, Vector Laboratories, USA). Images were taken at five distinct locations per well. The percentage of EdU-positive cells was determined by the number of EdU-positive cells/nuclei using ImageJ.

**Mitochondrial respiration and glycolytic rate**

The Seahorse XFe96 analyser (Seahorse Bioscience) was used to assess mitochondrial respiration, as described before ²¹ (see Supporting Information). Briefly, routine respiration was assessed before addition of oligomycin (2 µM; leak respiration). Maximal uncoupled mitochondrial respiration was measured after addition of 1 µM FCCP. Background respiration was measured after addition of 0.5 µM rotenone and 0.5 µM antimycin A and subtracted from all values. Cell count was performed with the Harmony Operetta CLS high-content analysis system (PerkinElmer). No differences in cell or nuclei count between conditions were observed in these micro-
well. Adenosine triphosphate (ATP) production rate was calculated as routine minus leak respiration, and glycolysis rates were derived from the extracellular acidification rates (ECAR), presented in mP/mn.

**Live-cell imaging of mitochondrial morphology**

To assess mitochondrial morphology by live-cell imaging, C2C12 myoblasts were processed as described before. 10 x 10^3 myoblasts were seeded on matrigel-coated 8-well Ibidi plates and differentiated into myotubes as described above. Post-treatment, myotubes were rinsed with PBS and incubated in phenol-red-free DMEM (Gibco) with 250 nM tetramethylrhodamine methyl ester, which accumulates in polarized mitochondria, for 20 min. After wash-out, images were obtained with the Leica TCS SP8 gated STED 3X microscope (Leica Microsystems GmbH, Wetzlar, Germany), at 37°C, 5% CO₂. Images were processed using Leica LAS-X software, and ImageJ, using the Feature plug-in. Particles were analysed for area, aspect ratio, and circularity.

**Live-cell imaging of mtROS production**

mtROS production was assessed as described before (see Supporting Information). Briefly, myotubes were incubated for 30 min with 5 μM of the Mitochondrial Superoxide indicator MitoSOX (Thermo Fisher Scientific, M36008), which targets mitochondria in live cells and produces a fluorescent signal when oxidized by superoxide. Images were acquired with the Leica TCS SP8 gated STED 3X microscope (Leica Microsystems GmbH, Wetzlar, Germany), at 37°C and 5% CO₂. Images were processed using Leica LAS-X software and ImageJ. For fluorescence quantification, at least five myotubes per image were analysed for mean grey value of the area directly next to the nucleus, with all values being corrected for background. Rotenone (1 μM) treatment served as positive control.

**Statistics**

For experimental conditions in which there were two independent factors and multiple comparisons, a factorial ANOVA with subsequent post hoc analysis was performed. Where appropriate, a one-way ANOVA with post hoc analysis or t-tests were performed. All statistical analyses were performed using GraphPad Prism (9.1.2, San Diego, CA, USA). Results are reported as means ± standard error of the mean. Statistical significance was set to P < 0.05.

**Results**

**Induction of the NLRP3 inflammasome**

We first determined the gene expression of NLRP3 and downstream activators of the NLRP3 inflammasome in myotubes upon 24-h stimulation with LPS. LPS exposure resulted in a dose-dependent increase in NLRP3 gene expression (by 45 ± 9% at 200 ng/mL LPS, P = 0.009) and tended to increase caspase-1 gene expression (by 74 ± 33% at 200 ng/mL LPS, P = 0.059) compared to control (Figure 1A and 1B). Next, we tested for gene expression levels of the inflammasome targets IL-1β and IL-18. Although the mRNA level of IL-1β in the control samples was below detection level, we did observe a trend for a higher mRNA level in the LPS-treated myotubes (10 vs. 200 ng/mL LPS, P = 0.089), albeit Ct values between 32 and 34 (Figure 1C). IL-18 mRNA increased by 198 ± 41% in response to 200 ng/mL LPS compared with vehicle (P = 0.001; Figure S1). IL-6 gene expression and protein content was higher with LPS (P < 0.05; Figure S1).

Oligomerization of the NLRP3 inflammasome results in phosphorylation and cleavage of caspase-1 into its mature, bioactive (p20) form. NLRP3 protein levels increased after 48 and 72 h in a time-dependent and dose-dependent manner compared with respective controls (Figure 1D). There was a trend towards increased caspase-1 protein cleavage (p20) upon myotube exposure to higher LPS concentrations compared with unstimulated controls (72 h: 0 vs. 100 ng/mL LPS, P = 0.0774; Figure 1E).

Mature IL-1β protein levels in C2C12 myotubes increased after LPS stimulation (72 h: 0 vs. 100 ng/mL LPS, P < 0.0001), indicating NLRP3 inflammasome activation. Addition of the NLRP3 inflammasome specific inhibitor MCC950 resulted in significantly lower IL-1β protein levels compared with LPS supplementation alone (P = 0.03; Figure 1F), confirming that IL-1β expression by myotubes following exposure to LPS is dependent on NLRP3 inflammasome activation.

These data suggest that exposure of the clinically relevant endotoxin LPS increases NLRP3 receptor and caspase-1 effector gene expression and protein concentration and activates the NLRP3 inflammasome in myotubes, independent of the involvement of the immune system.

**The activation of the NLRP3 inflammasome impairs myotube growth**

Next, we determined whether the activated NLRP3 inflammasome contributes to a lower myotube diameter upon LPS exposure (Figure 2A). LPS exposure resulted in a lower myotube diameter after 24 h (−1.3 ± 0.6 μm for 100 ng/mL LPS vs. control, P = 0.04, Figure 2B). Although untreated myotubes
increased in diameter after 24 h (1.2 ± 0.6 μm/day), this growth response was blunted for LPS-exposed myotubes (0.3 ± 0.3 μm/day for 100 ng/mL LPS, P = 0.03; Figure 2B).

Myotubes remained smaller compared with controls from 24 to 72 h of LPS exposure (-2.2 ± 0.6 μm for 100 ng/mL LPS vs. control, P = 0.0009), due to a significantly impeded growth rate (P = 0.034, Figure 2B). Gene expression of the E3 ligases MAFbx and MuRF1 (Fbxo32 and Trim63, respectively) was not different in LPS-exposed myotubes (Figure S2A and S2B), suggesting that the suppressed myotube growth was not mediated by an increase in the ubiquitin–proteasome system.

Myotube differentiation index was significantly decreased after 24 h, but not 72 h, of LPS exposure (Figure S3). LPS did not affect fusion index after 24 nor 72 h (Figure S3), indicating that the lower myotube diameter upon LPS was due to a loss of contractile protein rather than cell fusion (Figure S3). Proliferation rate of myoblasts was unaffected.

Figure 1 LPS induces NLRP3 inflammasome activation in cultured myotubes. (A and B) Myotube exposure to LPS resulted in increased NLRP3 and caspase-1 gene expression. (C) IL-1β mRNA was below detection level for the control condition. Albeit qPCR results of CTs in the range of 32–34, IL-1β mRNA dose-dependently increased upon 24 h of LPS exposure. (D) Typical Western blot examples of NLRP3 and cleaved (p20) caspase-1 relative to actin. NLRP3 protein levels dose-dependently increased after LPS exposure. (E) Caspase-1 protein cleavage into its mature (p20) form tended to increase upon LPS (P = 0.0774 for LPS 100 ng/mL vs. control). (F) IL-1β protein concentrations significantly increased upon 72 h of LPS stimulation. Addition of MCC950 resulted in significantly decreased IL-1β protein levels compared with LPS treatment alone (F). Data: Mean ± SEM. *P < 0.05 vs. control, #P < 0.05 vs. 100 ng/mL LPS; n.d., not detectable.
MCC950 mitigated the LPS-induced myotube growth impairments for the first 24 h ($P = 0.0475$; Figure 2C) and increased the growth rate back to control levels from 24 to 72 h ($P = 0.005$) compared with LPS-exposed myotubes (Figure 2C). Supplementing control cells with MCC950 had no effect on myotube growth (Figure S4).

To further elucidate whether the downstream IL-1β production upon NLRP3 inflammasome activation was responsible for the impaired myotube growth, we treated differentiated myotubes with 50 ng/mL IL-1β. Exposure to IL-1β suppressed myotube growth for the first 24 h of treatment ($P = 0.017$), and the absolute myotube diameter of IL-1β-treated cells tended to be smaller ($-1.45 \pm 0.6 \mu m$, $P = 0.051$). Following 72 h of treatment with IL-1β, myotubes were significantly smaller compared with untreated cells ($-1.6 \pm 0.6 \mu m$, $P = 0.03$; Figure 2D), although growth rate was not different.

Taken together, these data suggest the NLRP3 inflammasome to mechanistically contribute to LPS-induced changes in myotube diameter, partly via the production of IL-1β.

**NLRP3 localizes at myotube mitochondria**

We next studied the association between NLRP3 and mitochondria. We performed a double immunostaining for succinate dehydrogenase subunit A (SDHA), a subunit of mitochondrial complex II, and NLRP3, to identify mitochondrial localization of NLRP3 (Figure 3A). On average, $55 \pm 4\%$ of the signal of NLRP3 overlapped with that of SDHA in vehicle-treated myotubes (Figure 3B). Following 72 h of LPS exposure (100 ng/mL), more NLRP3 was associated with mitochondria, shown by a significant increase in

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**Figure 2** LPS impairs myotube growth. (A) Images of differentiated C2C12 myotubes after 72 h of treatment. (B) LPS significantly reduced myotube diameter and growth rate after 24 h, which remained smaller up to 72 h. (C) Addition of the pharmacological inhibitor MCC950 significantly mitigated diameter reductions after 72 h. (D) Myotube exposure to IL-1β tended to reduce cell diameter after 24 h, but significantly after 72 h. Data: Mean ± SEM. *$P < 0.05$ vs. control, †$P < 0.05$ vs. 100 ng/mL LPS + MCC950, ‡$P < 0.05$ vs. 100 ng/mL LPS.
co-localization between NLRP3 and SDHA of 13 ± 6% \( (P = 0.03) \). LPS treatment did not affect the overlap of SDHA in NLRP3 (Figure 3C). These results indicate an association between NLRP3 and mitochondria in C2C12 myotubes, which increased upon LPS stimulation.

**Inflammation-induced mitochondrial fragmentation**

A fragmented mitochondrial network may explain the LPS-induced metabolic stress. To study whether LPS affects mitochondrial morphology we performed live-cell imaging using tetramethylrhodamine methyl ester, a marker for mitochondrial membrane potential (Figure 5A–5C). Myotube exposure to LPS (100 ng/mL) significantly decreased mitochondrial size after 24 and 72 h compared with control (\( P = 0.02 \) and \( P = 0.03 \), respectively; Figure 5D). Aspect ratio significantly decreased after 24 h (\( P = 0.03 \)), but not after 72 h (Figure 5E), whereas mitochondria had an increased circular shape after 24 and up to 72 h of LPS exposure (\( P = 0.04 \) and \( P = 0.03 \) vs. control, respectively; Figure 5D and 5F), suggesting no role for NLRP3 in the LPS-induced changes in mitochondrial morphology.

**IL-1β does not affect mitochondrial morphology or function**

We reasoned that exposure to IL-1β would also not cause significant alterations in mitochondrial structure or function.
Indeed, myotubes exposed to IL-1β showed no significant changes in mitochondrial size, aspect ratio, or circularity compared with controls (Figure 6A and 6B–6D). Hence, IL-1β did not alter the mitochondrial network, nor mitochondrial respiration (Figure 6E) or ECAR (Figure 6F) in myotubes after 24 h. Likewise, exposure to IL-1β did not alter leak respiration, oxygen utilization rates for ATP production, and maximal respiration (Figure S6). Taken together, the LPS-induced shift towards glycolysis, metabolic stress, and altered mitochondrial shape after 24 h developed independently of IL-1β.

**Inflammation-induced mitochondrial oxidative stress**

The observed inflammation-induced changes in cell metabolism and mitochondrial morphology may be driven by oxidative stress. Therefore, we measured live-cell mtROS production with MitoSOX (Figure 7A). Rotenone treatment served as positive control and increased mtROS by 98 ± 4%. Myotube exposure to LPS (100 ng/mL, 24 h) induced a 51 ± 12% increase in mtROS production compared with controls (P = 0.03; Figure 7B), which was blunted by addition of the pharmacological NLRP3 inhibitor MCC950 (P > 0.05 vs. control and P = 0.04 vs. 100 ng/mL LPS; Figure 7B). With IL-1β being the downstream target of the NLRP3 inflammasome, we also tested the potential effects of the pro-inflammatory cytokine on mtROS production. Similarly, IL-1β exposure resulted in a 44 ± 8% increase in mtROS compared with controls (P = 0.03; Figure 7C). Therefore, these findings suggest an involvement of the NLRP3 inflammasome in the development of mitochondrial oxidative stress in myotubes under inflammatory conditions.

Because IL-1β was only partially able to explain the observed effects of NLPR3 at the mitochondria, we tested the hypothesis that LPS-induced mtROS production contributed to the initially observed myotube growth impairments. To test this, we treated myotubes with SS31, a cardiolipin stabilizer reducing mtROS production. Pre-treatment with SS31 prior to LPS exposure mitigated the inflammation-induced blunted myotube growth compared with LPS treatment alone (P = 0.04, 72 h; Figure 7D). This preservation of myotube growth in response to SS31 was only apparent in conjunction with a high LPS concentration (100 ng/mL), but did not show in control myotubes or at a low LPS concentration (10 ng/mL, data not shown). Overall, these data suggest the involvement of the NLRP3 inflammasome in mtROS production to partly account for the observed impaired growth in myotubes exposed to LPS.
**Discussion**

This study aimed to establish the mechanistic function of the NLRP3 inflammasome in skeletal muscle. By exposing cultured myotubes to the endotoxin LPS, we circumvented the contribution of the immune system and observed that low-grade and high-grade inflammation induced moderate activation of the NLRP3 inflammasome. Upon inflammation, mitochondrial localization of NLRP3 increased, where it contributed to an enhanced production of mtROS. The NLRP3 inhibitor MCC950 mitigated LPS-induced impaired growth, but did not alleviate mitochondrial dysfunction, suggesting a role for the downstream acting IL-1β in blunting myotube growth, but not in affecting mitochondrial function. Both mtROS production and IL-1β appear to drive the observed NLRP3-induced growth impairments in cultured myotubes.

**Induction of the NLRP3 inflammasome in skeletal muscle**

NLRP3 inflammasomes are expressed in various cell types, such as in fibroblasts, myoblasts, renal cells, and cardiomyocytes. Recent evidence suggests a role of the NLRP3 inflammasome on skeletal muscle function, such as in dysferlin deficiency. A deletion of the inflammasome contributed to attenuated atrophy in sepsis and protection from age-related muscle loss in mice, whereas its inhibition resulted in a diminished disease pattern in Duchenne muscular dystrophy. Here, we observed a direct effect of inflammation on NLRP3 expression and activation in skeletal muscle, independent of the confounding role of circulating and infiltrating immune cells and their downstream produced IL-1β and IL-18. By circumventing the contribution of the...
immune system, our model of low-grade and high-grade inflammation confirmed activation of the NLRP3 inflammasome and subsequent IL-1β production in myotubes to be implicated in morphological alterations and mtROS production.

NLRP3 inflammasome activation is marked by an autocleavage of its pro-caspase-1 component into its mature, biologically active caspase-1 form. Caspase-1 cleavage tended to be higher, and together with increased intracellular IL-1β protein levels, this confirmed LPS-induced activation of the NLRP3 inflammasome. IL-1β protein levels were lower upon treatment with the NLRP3 specific pharmacological inhibitor MCC950, confirming the activation of the NLRP3 inflammasome. Therefore, our findings present the NLRP3 inflammasome as a new potential therapeutic target for the treatment in muscle wasting disorders.

Our observed increased protein levels of the pro-inflammatory cytokine IL-1β after 72 h of LPS exposure were of the same magnitude as those seen in the circulation of the septic patient. IL-1β has been identified as a key regulator of muscle fibre size. In particular, the bioactive form of IL-1β possesses the ability of inducing specific E3 ligases in the atrophying muscle, such as atrogin-1 or MuRF1. However, the blunted myotube growth in our study was not mediated by an increase in the protein degradation pathway. Crosstalk between the NLRP3 inflammasome and endoplasmic reticulum (ER) may cause stress, in turn triggering the unfolded protein response. Though,

Figure 6 IL-β does not affect mitochondrial morphology or function. (A) Typical images of differentiated myotubes incubated with tetramethylrhodamine methyl ester showing mitochondrial morphology after IL-1β exposure (72 h). Myotube exposure to IL-1β (50 ng/mL) did not alter mitochondrial size (B), aspect ratio (C), or circularity (D). (E and F) Seahorse respirometry showed no significant effects of IL-1β on routine respiration (E) and extracellular acidification rates (F). Data: Mean ± SEM.
this deserves future research in the context of skeletal muscle protein synthesis.

**Link between NLRP3 and intracellular metabolism**

In immune cells, NLRP3 is structurally linked with the mitochondrial outer membrane, an association that was also found in cardiac fibroblasts. We confirmed this link between NLRP3 and mitochondria in skeletal muscle myotubes. Additionally, we observed an increased association between NLRP3 and mitochondria upon LPS stimulation, suggesting a potential role of NLRP3 to translocate to the mitochondria during conditions of inflammation.

With the present study, we confirmed LPS-induced alterations in cellular metabolism. Our findings of higher oxygen consumption and ECAR upon LPS suggest that cells required substantially more energy for normal cell homoeostasis, indicative of inflammation-induced metabolic stress and/or lower mitochondrial efficiency. We observed fragmented mitochondrial network dynamics in response to LPS, likely mediating these metabolic changes. Blocking of the NLRP3 inflammasome with MCC950 did not alleviate these mitochondrial morphological changes, nor the metabolic alterations, but did reduce mtROS production. Exposing myotubes to the downstream acting pro-inflammatory cytokine IL-1β did not alter mitochondrial form nor function.

A role of mitochondrial function in NLRP3 inflammasome activation has been postulated before. Enhanced mitochondrial fusion by dynamin-related protein 1 knockdown was related to increased NLRP3-dependent cleavage of caspase-1 and subsequent IL-1β production. Meanwhile, mitochondrial fission led to decreased assembly of the inflammasome complex and lowered its activation. Because we observed mitochondrial fission upon LPS treatment, this may be a compensatory mechanism to reduce chronic ROS production and the downstream effects of IL-1β. Taken together, these findings suggest an intricate interplay between mitochondrial...

**Figure 7** LPS-induced mitochondrial reactive oxygen species (mtROS) production is mitigated by MCC950. (A) Typical images of differentiated myotubes incubated with MitoSOX showing mtROS production. (B) mtROS production significantly increased in response to LPS exposure (100 ng/mL, 24 h). Addition of MCC950 (10 μM) blunted the mtROS increases elicited by LPS. (C) Myotube exposure to the NLRP3 inflammasome target IL-1β also increased mtROS. (D) Treatment of myotubes with SS31 (1 μM), which preserved bioenergetic function, mitigated myotube atrophy with LPS exposure (72 h). Data: Mean ± SEM. *P < 0.05, **P < 0.05 vs. LPS (100 ng/mL).
morphology and network dynamics, activation of the NLRP3 inflammasome, and ROS production.

ROS has frequently been proposed to be a stimulant of NLRP3 inflammasome activation by inducing a re-localization of NLRP3 from the ER to mitochondrial-ER organelle clusters.\(^{36}\) Our results of increased mtROS production after 24-h LPS are in line with observations by Bracey et al.,\(^ {14}\) who reported NLRP3 to produce ROS independent of the inflammasome complex at the mitochondrial membrane in primary cardiac fibroblasts. We found that the NLRP3 inhibitor MCC950 blunted ROS production and mitigated LPS-induced myotube growth impairments after 24 h. ROS has been associated with skeletal muscle wasting by interfering with the anabolic Akt/mTOR signalling pathway as well as increasing proteolysis.\(^ {37}\) Because E3 ligases were unaffected in our study, NLRP3-priming-induced mtROS production contributed to the lower myotube size during the acute phase by impeding protein synthesis.

The increase in mtROS production in myotubes likely leads to oxidized proteins and lipids, including cardiolipin.\(^ {38}\) Previously, SS31, a cardiolipin stabilizer, was reported to reduce ROS formation.\(^ {39}\) Pre-treatment of myotubes with SS31 before LPS exposure mitigated the LPS-induced myotube growth impairments, suggesting sustained mitochondrial function to contribute to myotube growth. Therefore, SS31 can mitigate the NLRP3-induced morphological changes upon inflammation, likely by preserving mitochondrial bioenergetic function.

**Limitations**

Whereas the induction of the inflammasome in immune cells has been studied with the same concentrations of LPS as applied in the present study,\(^ {20,22}\) inflammasome activation was less pronounced for our cultured C2C12 myotubes compared with that in macrophages. Consequently, muscle cells are less responsive to LPS or NLRP3 compared with immune cells, likely due to protein concentration differences of key signalling molecules. Despite this, physiological LPS concentrations caused NLRP3 inflammasome activation, which was sufficient in eliciting both morphological and metabolic adaptations.

The selective NLRP3 inhibitor MCC950 caused similar results compared with a shRNA-mediated knockdown of Nlrp3 in atrial fibrillation,\(^ {40}\) but it is unknown whether NLRP3 itself or the downstream production of IL-1\(\beta\) underlies this

![Diagram](image-url)

**Figure 8** Schematic diagram of the links between the NLRP3 inflammasome with metabolic alterations and skeletal muscle growth. LPS induces activation of the NLRP3 inflammasome and its downstream pro-inflammatory cytokine IL-1\(\beta\), which impairs muscle growth. MCC950 mitigates these inflammasome-induced growth impairments. NLRP3 localizes at mitochondria and causes mitochondrial reactive oxygen species production, which blunts muscle growth, however this is alleviated by MCC950 and SS31. An impaired protein synthesis rate likely contributes to the impaired muscle growth. IL-1\(\beta\), interleukin-1\(\beta\); LPS, lipopolysaccharides; mtROS, mitochondrial ROS; NLRP3, nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3; TLR4, Toll-like receptor 4.
response. Nlrp3 knockout led to significantly decreased serum levels of IL-1β in septic mice, which mitigated muscle atrophy,29 confirming our observation that the pro-inflammatory cytokine IL-1β blunts myotube growth. Genetically blocking the expression of IL-1β in the presence of LPS with or without supplementation of MCC950 would provide additional information whether IL-1β in response to NLRP3 activation is responsible for the observed myotube growth impairments in this study. It is unknown if certain cytokines, such as tumour necrosis factor alpha or IL-6 as common markers for inflammation in chronic diseases,2,2 are also inducers of the NLRP3 inflammasome in skeletal muscle, similar to LPS used here.

Conclusion

This study provides evidence for an association between an innate immune component, the NLRP3 inflammasome, and inflammation-induced morphological and metabolic alterations in skeletal muscle (see Figure 8). LPS-induced activation of the NLRP3 inflammasome is present in myotubes. NLRP3 was localized at muscle mitochondria, an association that increased upon LPS. Overall, the effects of the NLRP3 inflammasome in skeletal muscle appear to be twofold: NLRP3 induces an acute mitochondrial response upon an inflammatory stimulus contributing to a blunted growth, although after chronic LPS exposure, NLRP3 contributes to growth impairments via additional production of IL-1β, likely via an impaired protein synthesis rate. Inhibition of NLRP3 by MCC950 alleviated the impaired inflammation-induced muscle growth, which may provide new avenues to treat inflammation-mediated skeletal muscle wasting during acute and chronic diseases.

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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.41 The authors would like to acknowledge Peter J.M. Wejs for insightful discussions.

Conflict of interest

Moritz Eggelbusch, Andi Shi, Bonnie C. Broeksma, Mariana Vázquez-Cruz, Madu N. Soares, Gerard M.J. de Wit, Bart Everts, Richard T. Jaspers, and Rob C.I. Wüst declare that they have no conflict of interest.

Online supplementary material

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

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