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L-glutamine improves skeletal muscle cell differentiation and prevents myotube atrophy after cytokine (TNF-α) stress via reduced p38 MAPK signal transduction

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Abstract

Tumour Necrosis Factor- Alpha (TNF-α) is chronically elevated in conditions where skeletal muscle loss occurs. As L-glutamine can dampen the effects of inflamed environments, we investigated the role of L-glutamine in both differentiating C2C12 myoblasts and existing myotubes in the absence/presence of TNF-α (20 ng.ml⁻¹) ± L-glutamine (20 mM). TNF-α reduced the proportion of cells in G1 phase, as well as biochemical (CK activity) and morphological differentiation (myotube number), with corresponding reductions in transcript expression of: Myogenin, Igf-I and Igfbp5. Furthermore, when administered to mature myotubes, TNF-α induced myotube loss and atrophy underpinned by reductions in Myogenin, Igf-I, Igfbp2 and glutamine synthetase and parallel increases in Fox03, Cfos, p53 and Bid gene expression. Investigation of signaling activity suggested that Akt and ERK1/2 were unchanged, JNK increased (non-significantly) whereas P38 MAPK substantially and significantly increased in both myoblasts and myotubes in the presence of TNF-α. Importantly, 20 mM L-glutamine reduced p38 MAPK activity in TNF-α conditions back to control levels, with a corresponding rescue of myoblast differentiation and a reversal of atrophy in myotubes. L-glutamine resulted in upregulation of genes associated with growth and survival including; Myogenin, Igf-Ir, Myhc2 & 7, Tnfsfr1b, Adra1d and restored atrophic gene expression of Fox03 back to baseline in TNF-α conditions. In conclusion, L-glutamine supplementation rescued suppressed muscle cell differentiation and prevented myotube atrophy in an inflamed environment via regulation of p38 MAPK. L-glutamine administration could represent an important therapeutic strategy for reducing muscle loss in catabolic diseases and inflamed ageing.
### Introduction

In skeletal muscle tissue, the production of the pleiotropic inflammatory cytokine Tumour Necrosis Factor-Alpha (TNF-α) via both immune and skeletal muscle cells improves surrounding vascular permeability, aids infiltration of immune cells from the circulation and facilitates the removal of necrotic tissue after injury and damaging exercise. Ourselves and others have demonstrated early acute increases in TNF-α are important in skeletal muscle cell proliferation via activation of the MAPK’s (Al-Shanti et al., 2008; Foulstone et al., 2004; Li, 2003; Serrano et al., 2008; Sharples et al., 2010; Stewart et al., 2004). Others have also suggested that the acute production of TNF-α correlates positively with satellite cell activation in-vivo after damaging exercise (Mackey et al., 2007; Mikkelsen et al., 2009; van de Vyver and Myburgh, 2012). Importantly, chronic TNF-α exposure is conversely associated with severe muscle cell apoptosis (Foulstone et al., 2001; Grohmann et al., 2005b; Meadows et al., 2000; Saini et al., 2008; Saini et al., 2012; Saini et al., 2010; Sharples et al., 2010; Stewart et al., 2004), muscle wasting in-vivo (Li et al., 2005; Li and Reid, 2000) and the pathology of cachexia, reviewed in (Saini et al., 2006). TNF-α is also chronically increased systemically in aged humans (Bruunsgaard et al., 2003a; Bruunsgaard et al., 2003b; Bruunsgaard and Pedersen, 2003; Grewe et al., 2001) and the local production by the muscle is also elevated (Grewe et al., 2001; Leger et al., 2008), an aetiology that strongly correlates with the incidence of muscle loss with age (sarcopenia), reviewed in (Saini et al., 2006; Sharples et al., 2015a). This reduction in muscle mass due to chronically elevated TNF-α can be somewhat attributed to inhibition of muscle cell differentiation and promotion of myofibre atrophy observed in-vitro and during muscle loss in-vivo (Al-Shanti et al., 2008; Foulstone et al., 2004; Foulstone et al., 2001; Foulstone et al., 2003; Grohmann et al., 2005a; Grohmann et al., 2005b; Jejurikar et al., 2006; Li et al., 2005; Li et al., 2003; Li and Reid, 2000; Meadows et al., 2000; Saini et al., 2008; Saini et al., 2012; Saini et al., 2010; Sharples et al., 2010; Stewart et al., 2004). Most recent studies by our group also show that skeletal muscle has a memory of acute early life exposure to TNF-α underpinned by epigenetic retention of DNA methylation over its proliferative lifespan, rendering skeletal muscle more susceptible to inflamed muscle loss events in later life (Sharples et al., 2015b). Therefore, understanding how to dampen the impact of chronically elevated inflammatory cytokines in skeletal muscle across the lifespan is important for future therapies to ameliorate muscle loss.

Importantly, high TNF-α as a result of surgery, sepsis, burns injury and cancer cachexia (Bode et al., 1996; Calder and Yaqoob, 1999; Karinch et al., 2001; Labow and Souba, 2000; Parry-Billings et al., 1990; Roth et al., 1982) are associated with large reductions in the amino acid, glutamine, both in the circulation and from skeletal muscle stores. Glutamine is predominantly synthesized endogenously by skeletal muscle and therefore traditionally considered a ‘non-essential’ amino acid (Curthoys and Watford, 1995; Watford, 2015). However, due to its considerable depletion after inflammatory stress, glutamine has been termed a ‘conditionally essential’ amino acid, as reviewed in (Lacey and Wilmore, 1990). Supplementary glutamine increases muscle protein synthesis by approximately 10% in rodents after administration of atrophy inducing glucocorticoid, dexamethasone (Boza et al., 2001), and can help prevent loss of fat free mass in cancer cachexia (May et al., 2002). These data suggest glutamine could be a promising therapy to ameliorate muscle loss in conditions that are driven by chronic increases in inflammatory cytokines such as TNF-α.

There are however limited studies into the molecular mechanisms of glutamines action, particularly the intracellular signaling mechanisms underpinning its positive action in inflamed skeletal muscle. At the cellular level, glutamine administration has been reported to significantly reduce TNF-α protein levels post lipopolysaccharide (LPS) administration in human peripheral blood mononuclear cells (PBMCs) (Wischmeyer et al., 2003). Specifically, in skeletal muscle it has been reported that glutamine was able to restore glucocorticoid (dexamethasone) induced skeletal muscle atrophy in rat tissue (Salehian et al., 2006). This positive adaptation with glutamine supplementation was associated with the suppression of myostatin transcription back towards levels seen in control and glutamine alone groups, as well as a corresponding return of myostatin protein abundance back towards glutamine only conditions (Salehian et al., 2006). In the same study, these findings were confirmed in C2C12 myoblasts where in this model the authors demonstrated that myostatin promoter activity was also suppressed towards baseline in glutamine conditions following an increased activity in the presence of the catabolic glucocorticoid (Salehian et al., 2006). More recently the findings suggesting a role for glutamine in modulating myostatin have been translated from glucocorticoids to the inflammatory cytokine TNF-α; where the treatment of C2C12 myotubes with this cytokine led to increased myostatin protein levels and myotube atrophy. Importantly again, glutamine was able to return myostatin protein levels back to towards
Despite the reported regulation of myostatin by L-glutamine in models of atrophic conditions, there are limited investigations into the intracellular signaling responses underlying the role of glutamine in the suppression of muscle loss following inflammation. TNF-α has been reported to strongly up-regulate p38 mitogen-activated protein kinases (p38 MAPK) demonstrated by ourselves (Grohmann et al., 2005a) and other groups (Alvarez et al., 2001; Chen et al., 2007; Li et al., 2005; Palacios et al., 2010; Zhan et al., 2007). Importantly, glutamine has also been shown to regulate p38 MAPK in other models, including; following exercise induced apoptosis in leukocytes isolated from rats (Lagarhna et al., 2007), in PBMC’s derived from a rat model of sepsis (Singleton et al., 2005), in-vivo (in the lungs of mice) and in alveolar macrophages post LPS administration in vitro (Ko et al., 2009). Combined, this evidence suggests that p38 MAPK could play an important role in reducing skeletal muscle loss on a background of TNF-α induced inflammation.

In the present study our objectives were therefore: 1) To assess the effect of glutamine in restoring impaired differentiation and reducing myotube atrophy observed in conditions of TNF-α induced inflammation and to; 2) investigate the underlying role of the stress related MAPKs, p38 and JNK (as well as other important associated kinases ERK1/2 and Akt) and their downstream modulation of gene transcription in controlling skeletal muscle differentiation and hypertrophy/atrophy in the presence of TNF-α. We hypothesised that: 1) Glutamine would rescue TNF-α induced impairments in myoblast differentiation in myoblasts, reduce myotube atrophy and improve myotube survival in existing myotubes and; 2) the stress related MAPK kinase family of signaling proteins would be partly responsible for co-coordinating these morphological responses via transcriptionally restoring genes associated with muscle differentiation, survival and myotube hypertrophy/atrophy that were otherwise impaired in TNF-α conditions.

**Methods**

**Cell Culture and treatments**

C2 and C2C12 mouse skeletal myoblasts (Blau et al., 1985; Yaffe and Saxel, 1977), below passage 12, were employed in these studies. Cells were seeded at 1 × 10⁶ cells in gelatinized T75 flasks in growth medium (GM) composed of: DMEM plus 10% hi (heat-inactivated) fetal bovine serum, 10% hi newborn calf serum, 2 mM L-glutamine, and 1% penicillin-streptomycin solution and grown to approximately 80% confluency. Following trypsinisation, 8 × 10⁴ cells.ml⁻¹ in 2 ml GM/well were plated into pre-gelatinized wells of a six well plate and incubated for 24 hrs. To assess the impact on early differentiation/fusion, C2C12 myoblasts were washed twice with PBS and transferred to one of four different dosing conditions: 1) Low serum media/differentiation media (DM) alone composed of: DMEM plus 2% heat inactivated horse serum, 2 mM L-glutamine, and 1% penicillin-streptomycin solution; 2) DM + TNF-α (TNF-α) at 20 ng.ml⁻¹; 3) DM+ L-glutamine (LG) at 20 mM and finally 4) DM + 20 ng.ml⁻¹ TNF-α + 20 mM LG-glutamine (TNF-α + LG) for up to 72hrs (TNF-α: Merck Millipore, UK).

To further assess the impact on established myotube cultures, C2C12 cells were cultured in DM for 7 days to induce myotube formation then dosed as above (DM, TNF-α, LG and TNF-α + LG) and cultured for a further 72 hrs (up to a total of 10 days post low serum transfer). We have shown previously that C2C12 myoblasts are resistant to early cell death following TNF-α administration, whereas parental C2 cells are susceptible to cell death in these conditions (Sharples et al., 2010). Therefore, in order to assess the impact on early myoblast cell death, C2 myoblasts were dosed with TNF-α in the absence and presence of LG for 48 hrs and dead cell percentage was assessed (methods as described below). We have previously used the above doses of TNF-α to study skeletal muscle apoptosis in murine C2 cells (Foulstone et al., 2001; Saini et al., 2009; Sharple et al., 2010; Stewart et al., 2010) and human cells (Foulstone et al., 2004). Similar doses have also been used to inhibit muscle cell differentiation in C2C12 by ourselves and others (Sharples et al., 2010; Tolosa et al., 2005). It is also worth noting that for most experiments using myoblasts, 2 mM L-glutamine is already present in the differentiation medium (used in DM control conditions) therefore reductions in differentiation observed with TNF-α administration occur even in these lower 2 mM L-glutamine concentrations. Therefore, for the purposes of these experiments LG conditions described above are administered at an additional bolus of 20 mM. Finally, intravenous (iv.) glutamine administration (0.5 g/kg/bw over 4 hours for three consecutive days) in critically ill patients has been suggested to elevate circulating levels between 10-50 mM (Berg et al., 2002; Werner, 2008), therefore the high dose of LG investigated in the present study is relevant to receiving iv. administration
of glutamine. C2C12 myoblasts undergo spontaneous differentiation into myotubes on serum withdrawal, and do not require growth factor addition to stimulate the process (Blau et al., 1985; Tollefsen et al., 1989). Time point zero (DM 0 hrs) was defined as 30 minutes subsequent to transferring into DM for transcript and morphological assessment. For signaling studies in myoblasts/differentiating cells, time point 0 (ohrs) was defined as immediately post GM removal (and washing with PBS) before dosing conditions were applied. For signaling studies in differentiated myotubes, time point 0 was defined as immediately post removal of DM at 7 days (and washing with PBS) before fresh dosing conditions were applied as described above. For p38 MAPK inhibitor studies SB203580 (Merck Millipore, UK) was administered at concentrations of 5 and 10 µM.

**Cell extractions**

Cells were extracted for total protein assays and CK (creatine kinase) activity (a biochemical marker of myoblast differentiation) at 0, 48 and 72 hrs in differentiating C2C12 cells. Briefly, cells were washed twice in PBS and lysed in 200 µl.well⁻¹ of 0.05 M Tris/MES Triton lysis buffer (TMT: 50 mM Tris-MES, pH 7.8, 1% Triton X-100) and assayed using commercially available BCA™ (Pierce, Rockford, IL, U.S.A) and CK activity (Catachem Inc., Connecticut, N.E, U.S.A) assay kits according to manufacturer’s instructions. The enzymatic activity for CK was normalised to total protein content. For rt-RT-PCR experiments cells were lysed in 250 - 300 µl/well TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA), the RNA isolated and quantified (see below). Cytometric bead arrays (CBAs) for cell signaling analysis, cells were extracted at 0, 5 min, 15 min, 2 hrs and 24 hrs post transfer into dosing conditions above. All cell culture experiments were performed on 3 separate occasions and all assays were performed in duplicate (refer to figure legends for specific information on the different analyses).

**Morphological assessment: Myotube number, diameter and area**

Morphological differentiation was assessed using a cell imaging system at 10 or 20 x magnification for representative images and morphological counts/analysis respectively (Leica, DMI 6000 B). Images were subsequently used for quantitative measures of myotube number and area. Light microscope images were imported into Image J software (Java soft-ware, National Institutes of Health, USA) for analyses. Myotube numbers were counted per image and global mean ± SD was determined across all images per experimental condition (a myotube was defined as containing 3+ nuclei encapsulated within cellular structures, to avoid counting of cells undergoing mitosis). Myotube area (µm²) was determined by carefully tracing around myotube structures after converting pixel length to µm using Image J software.

**Cell death**

Adherent cells were collected following trypsinisation and pooled with detached cells in the supernatant. The cell suspension was prepared as a 1:1 dilution in 0.4% trypan blue stain. Samples were loaded into a Neubauer haemocytometer for viable and dead cell counting. Dead cell percentage (%) was calculated as a proportion of total cell number (viable and dead).

**RNA extraction and analysis**

Extraction of RNA was performed using the TRIZOL method, according to the manufacturer’s instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration and quality were assessed by UV spectroscopy at 260 and 280 nM using a Biotech Photometer (WPA UV1101, Biochrom, Cambridge, UK) or Nanodrop spectrophotometer 2000c (Fisher, Rosklide, Denmark). 70 ng RNA was used for each RT-PCR reaction.

**Primer Design**

Primer sequences (Table 1) were identified using Gene (NCBI, www.ncbi.nlm.nih.gov/gene) and designed using both web-based OligoPerfect™ Designer (Invitrogen, Carlsbad, CA, USA) and Primer-BLAST (NCBI, http://www.ncbi.nlm.nih.gov/tools/primer-blast). With the exception of IGF-I mature peptide mRNA primers that were used in (Yang et al., 1996). Primers were purchased from Sigma (Suffolk, UK) without the requirement of further purification. Sequence homology (BLAST) searches ensured specificity to ensure the primers matched the sequence and therefore gene that they were designed for. Three or more GC bases in the last 5 bases at the 3’ end of the primer were avoided as stronger bonding of G and C bases can cause nonspecific amplification. Primer
our inson, Franklin Lakes, NJ, USA) or data were collected using Cell Quest (Becton Dickinson, Oxford, England) or QuantiFast SYBR Green RT-PCR one-step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA). RT-PCR reactions for TNF-α ± L-glutamine comparisons in differentiated myotubes were performed using QuantiFast SYBR Green RT-PCR one-step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA). RT-PCR for both studies was performed as follows: 10 min, 50°C (reverse transcription), 5 min 95°C (initial denaturation and reverse transcriptase inactivation), followed by 40 cycles of: 10 seconds, 95°C (denaturation), 30 seconds, 60°C (annealing and extension). Melt-curve analysis was used to determine and exclude samples with any non-specific amplification or primer/dimer issues. All melt analysis for the genes of interest produced single peaks indicating amplification of one gene product with no primer dimer issues. Relative gene expression levels were calculated using the delta delta Ct (ΔΔCt) equation (Schmittgen & Livak, 2008). Polymerase (RNA) II polypeptide B (Polr2β/Rp-IIIb) was used as the reference gene (this was highly stable across all conditions with a mean ± SD Ct of 22.5 ± 0.59 (variation 2.69%) for rt-RT-PCR using Chromo4 for comparisons of DM vs. TNF-α in differentiating myoblasts and with a mean ± SD Ct 16.69 ± 0.72 (variation 4.3%) for rt-RT-PCR on the Rotogene 3000Q in myobute studies). To directly compare fold changes between conditions, the 0 hr time point (as defined above for differentiating myoblast and myotube studies) for C2C12 cells was used as the calibrator condition in the Ct (ΔΔCt) equation.

Flow cytometry: Cell cycle analysis

In differentiating myoblasts at 0, 24 & 48 hrs following transfer into DM or DM + TNF-α cells were trypsinised (200 µl, 0.5% trypsin/0.02% EDTA solution/well) and pooled with detached cells from the conditioned media. Following centrifugation (10 mins, 300 g at 4°C), the supernatant was removed, and the pelleted cells vortexed and fixed in ice cold 75% ethanol (dropwise) and stored for 24 hrs at -20°C. Cells were again centrifuged for 10 minutes at 300 g at 4°C. The supernatant was removed, and 2 ml PBS added to wash the cells, prior to vortexing and centrifuging as above (twice). 1.5 ml of the supernatant was removed and the cell pellet vortexed prior to addition of 50 µl of ribonuclease A (20 µg/ml) and incubated at RT for 30 mins 10 µl propidium iodide labeling buffer (50 µg/ml propidium iodide, 0.1% sodium citrate, 0.3% Nonidet P-40, pH 8.3) was added to each sample prior to incubation in the dark at 4°C for 24 hrs before analysis using a Becton Dickinson FACSCalibur flow cytometer. Data were collected using Cell Quest (Becton Dickinson, Oxford, England) and analysed using Modfit™ software (Verity Software House, Topsham, ME, USA).

Flow cytometry: Cytometric Bead Array (CBA) for quantification of phosphorylated proteins

BD Cytometric Bead Array (CBA) enables simultaneous quantification of intracellular phosphorylated signalling proteins (Manjavachi et al., 2010; Schubert et al., 2009). Assays were performed according to manufacturer’s instructions. Briefly, cells were washed and lysed on ice (1x lysis buffer provided in the Cell Signaling Master Buffer Kit (BD™ Cytometric Bead Array (CBA)) prior to boiling. Protein concentrations were determined as described above. Samples were added to the assay diluent provided (15 µg/sample). Standards were prepared by serially diluting a stock of recombinant protein (50,000 Units/ml) contained in each BD CBA Cell Signaling Flex Set (separate flex set for phosphorylated proteins of interest: Akt (T308), ERK1/2 (T202/Y204), p38 MAPK (T180/Y182) and JNK (T183/Y185). For DM vs. TNF-α comparison for Akt, ERK, JNK and p38 four phosphorylated proteins per sample were analysed simultaneously in the presence of test capture beads. For L-glutamine ± TNF-α comparisons in both myoblasts and myotubes p38 MAPK was performed alone. All samples were incubated with the test capture beads (3 hrs) and Phycocerythrin (PE, -1 hr). The samples were washed and centrifuged at 300 g for 5 mins. Supernatant was removed and 300 µl of fresh wash buffer was added prior to resuspension and analyses on a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) or a BD Accuri flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). 300 events were captured per analyte per sample according to manufacturer’s instructions. Data were analyzed using FCAP array software (Hungary Software Ltd., for BD Biosciences, San Jose, CA, USA). Although changes in total p38 MAPK protein would be unlikely to change at the time points studied for p38 MAPK activity (i.e. minutes – hours), it can be confirmed that there
were no changes in p38 MAPK (alpha or beta) gene expression (primer details in table 1) in any of the above 
culture dosing conditions.

**Statistical Analyses**

Statistical analyses and the significance of the data were determined using Minitab version 17. Results are 
presented as mean ± standard deviation (SD). For initial TNF-α dosing experiments statistical significance for 
interactions between time (varied depending on the analysis undertaken- detailed above) and dose (DM vs. TNF- 
α) were determined using a two-way Factorial ANOVA. For cell cycle a two-way ANOVA (3 x 2) with time (0, 24, 
48 hrs) and dose (DM vs. TNF-α). For CK activity (3 x 2 ANOVA) for time (0, 48, 72 hrs) and dose (DM vs. TNF- 
α). For gene expression in differentiating myoblasts a two-way (2 x 2) factorial ANOVA for time (0, 48 hrs & 72 
hrs) and dose (DM vs. TNF-α) was undertaken. Interactions for phosphorylated proteins in differentiating 
myoblasts were determined for time (0, 5, 15 mins, 2, 24 hrs) and dose (DM and TNF-α) also using a two-way 
(5x2) factorial ANOVA. Post hoc analyses (with Bonferroni correction) were conducted on data where main 
effects for time and dose occurred, without a significant interaction between time and dose. If there were 
significant interactions present, t-tests were conducted to confirm statistical significance between the variable of 
interest e.g. between doses and/or time. For glutamine experiments; phosphorylated protein analysis was 
conducted using a 2 way (2 x 4) Factorial ANOVA for time (15 mins vs. 2hrs) and dose (DM, TNF-α, LG, LG + 
TNF-α) and post hoc (with Bonferroni correction) tests conducted for pairwise comparisons. Morphological 
analysis for glutamine experiments (myotube number and area) were performed at either 72 hrs in 
differentiating myoblasts or at +72 hrs (10 days total as described above) in differentiated myotubes using a one- 
way ANOVA for dose (DM, TNF-α, LG, LG + TNF-α). Glutamine experiments for gene expression in myotube 
cultures were analyzed using a one-way ANOVA for dose (DM, TNF-α, LG, LG + TNF-α). Fisher LSD post hoc 
tests were conducted for pairwise comparisons following one-way ANOVA’s. For all statistical analyses, 
significance was accepted at P ≤ 0.05.

**Results**

**TNF-α reduces early myoblast fusion and myotube formation in differentiating myoblasts**

Fusion and myotube number were reduced in early differentiating C2C12 cells in the presence of TNF-α (Figure 
1). As G1 cell cycle exit is a prerequisite for myoblast differentiation we observed that there was a significant 
decrease in the proportion of cells in the G1 phase at 24 hrs following the induction of differentiation following 
serum withdrawal and TNF-α administration (CON DM 82.96 ± 1.01 vs. TNF-α 71.23 ± 0.3% at 24 hrs, P ≤ 
0.001; Figure 1A) and a relative increase in S/G2 phase (data not included), suggesting a shift from 
differentiation to continued cellular division. Biochemical analyses of differentiation at the later time-points 
of 48 and 72 hrs following serum withdrawal subsequently confirmed the cell cycle differences, where it was 
observed that compared with control, there were significant reductions in CK activity in TNF-α conditions (DM 
342 ± 46 vs. TNF-α 189 ± 38 mU.mg.ml⁻¹ at 48 hrs; DM 492 ± 36 vs. TNF-α 274 ± 68.3 mU.mg.ml⁻¹ at 72 hrs; 
both P ≤ 0.001; Figure 1B). Overall this resulted in a significant impact on cellular morphology shown by a 
reduction in myotube number (DM 2.39 ± 0.58 vs. TNF-α 1.31 ± 0.48; P ≤ 0.001, Figure 1C and 1G) at 72 hrs in 
the presence of TNF-α. To confirm cell cycle, biochemical and morphological data at the molecular level we 
investigated gene transcription of Myogenin (an important myogenic regulator factor) and insulin-like growth 
factor family members (Igf-I, Igf-Ir, Igfbp5, Igfbp2) involved in fusion and myotube formation in differentiating 
myoblasts. Myogenin decreased in myoblasts in the presence of TNF-α at 48 hrs by 2.45 fold (DM 70.4 ± 11.2 vs. 
TNF-α 28.69 ± 5.35 P ≤ 0.001; Figure 1D). A similar trend was observed at 72 hrs for Igf-I expression with a near 
2 fold average reduction following TNF-α administration (DM 86.9 ± 24.7 vs. TNF-α 44 ± 18.3, P ≤ 0.001; Figure 
1E). Igf-Ir was unchanged with the addition of TNF-α (data not shown). Due to reductions in Igf-I, IGF binding 
proteins 2 and 5 that modulate the binding of IGFs to their receptors and regulate myoblast differentiation 
(Foulstone et al., 2001; Meadows et al., 2000; Sharples et al., 2013) were assessed. Indeed, in the presence of 
TNF-α there was a decline in IGFBP5 expression at 72 hrs by 1.87 fold (DM 3.74 ± 1.39 vs. TNF-α 2 ± 0.35, P = 
0.03; Figure 1F), there were however no significant differences in IGFBP2 expression following TNF-α exposure 
(data not shown).
When TNF-α was administered to existing myotubes the cytokine significantly reduced myotube survival shown via reductions in myotube number (DM 9.63 ± 3.98 vs. TNF-α 6.15 ± 2.5; P ≤ 0.05; Figure 2A). Furthermore, in the myotube area that did survive, compared with controls, atrophy occurred as evidenced by significant reductions in myotube area in the presence of TNF-α (DM 8305 ± 4676 vs. TNF-α 5557 ± 2816 μm²; P ≤ 0.001; Figure 2B).

In order to investigate the molecular regulators of these processes in differentiated myotubes we investigated the transcript expression in response to TNF-α as a range of genes important in myotube maturation and growth (Myogenin, Myomaker, Mrf4, Myhc’s 1, 2, 4 & 7, Igf-I, Igf-Ir, Igfbp2), atrophy (Myostatin, Musa1, Murf-1, Mafb, Nf-κb, TNF-α, both Foxo1 & Foxo3), apoptosis/survival (Tnfrsf1α & b, Cfos, Cmyc, p53, Bid, Bad, Bax, A3raf, Sirt1) and glutamine synthesis (glutamine synthetase). Seventy two hrs post TNF-α administration to existing myotubes, there were significant reductions in genes associated with myotube maturation and growth. There was an average 2.95 fold reduction in myogenin (DM 0.59 ± 0.29 vs. TNF-α 0.2 ± 0.04; P = N.S, Figure 2C), as well as a significant, almost 10 fold reduction in Igf-I (DM 0.84 ± 0.49 vs. TNF-α 0.09 ± 0.02; P = 0.02, Figure 2D), with corresponding reductions for Igfbp2 (2 fold) (DM 0.98 ± 0.33 vs. TNF-α 0.48 ± 0.06, P = 0.007, Figure 2E). Furthermore, TNF-α significantly increased transcription of genes involved in skeletal muscle atrophy including a 2.65 fold increase in Foxo3 (DM 1.0 ± 0.47 vs. TNF-α 2.65 ± 0.15, P = 0.002, Figure 2F), as well as corresponding increases in cell death/survival genes; p53 by 1.78 fold (DM 1.0 ± 0.334 vs. TNF-α 1.78 ± 0.16, P = 0.001, Figure 2H), and Cfos by 3 fold (DM 0.37 ± 0.14 vs. TNF-α 1.14 ± 0.13 P ≤ 0.001, Figure 2I). A 2.2 fold increase was observed for Bid mRNA (DM 1.24 ± 0.15 vs. TNF-α 2.73 ± 0.39, P = 0.003, Figure 2J) in the presence of TNF-α. Finally, glutamine synthetase reduced 2 fold in the present of TNF-α (DM 0.96 ± 0.09 vs. TNF-α 0.45 ± 0.03, P = 0.002, Figure not shown). It is worth noting that we observed no significant differences in the remaining genes investigated (above) including those previously associated with elevated TNF-α e.g. Nf-kb (DM 0.24 ± 0.18 vs. TNF-α 0.48 ± 0.06), TNf-α (DM 0.96 ± 0.44, TNF-α 0.72 ± 0.17), myostatin (DM 1.53 ± 0.51, TNF-α 1.54 ± 0.07), Mafb (DM 0.78 ± 0.4, TNF-1 1.2 ± 0.50), Murf-1 (DM 0.97 ± 0.16, TNF-α 1.07 ± 0.09) and Foxo1 (DM 0.73 ± 0.24, TNF-α 0.72 ± 0.04), all comparisons, P = N.S.

Early increases in phosphorylated p38 MAPK but not JNK, AKT or ERK1/2 in the presence of TNF-α are associated with reductions in myoblast differentiation, myotube formation and increased atrophy.

It has previously been observed that the MAPK and PI3K pathways regulate proliferation and differentiation (Coolican et al., 1997) as well as survival basally (Stewart et al., 1999) and in the presence of TNF-α (Al-Shanti et al., 2008; Foulstone et al., 2001). We therefore hypothesized that these pathways were important for the morphological adaptation and gene expression patterns described above. There was however, no impact of TNF-α on ERK1/2 or Akt activity over 24 hours (analyses conducted at 0, 5, 15 mins, 2hrs, 24 hrs) in myoblasts (data not shown). While there was a mean increase in JNK1/2 activity at 15 mins this did not reach significance (Figure 3A), however p38 MAPK activation over 24 hrs (analysis conducted at 0, 5, 15 mins, 2hrs, 24 hrs; Figure 3B) was significantly increased at 15 mins after transferring into DM vs. DM + TNF-α (DM 93.5 ± 25.6 vs. TNF-α 173.8 ± 35 units.ml⁻¹, P = 0.014; Figure 3B). Overall suggesting early p38 MAPK activity may be important in the TNF-α induced reduction in early differentiation. We subsequently confirmed that p38 MAPK increased at the same time point 15 mins in differentiated myotubes (DM 1.43 ± 4.69 vs. TNF-α 69.18 ± 3.99 units.ml⁻¹, P ≤ 0.001; Figure 3C), indicating that p38 MAPK activity was also important in the TNF-induced myotube atrophy.

L-glutamine reduced p38 MAPK activity, rescued myotube atrophy in the presence of TNF-α via reduced atrophic (Foxo3) and increased muscle growth (Igf-Ir), myotube maturation (Myhc 2 & 7) and survival (Tnfrsfib and A3raf) gene expression.

In differentiating myoblasts at 15 mins, L-glutamine alone was sufficient to significantly reduce the activity of p38 MAPK vs. control DM conditions (DM 90.13 ± 11.85 vs. LG 25.46 ± 7.96, P ≤ 0.05, Figure 4A), importantly L-glutamine also reduced p38 MAPK activity in TNF-α conditions at this time point in differentiating myoblasts (LG + TNF-α 69.93 ± 25.71 vs. TNF-α 155.88 ± 34.98, P ≤ 0.01; vs. DM 90.13 ± 11.85, P = N.S; vs. LG alone 25.46 ± 7.96, P = N.S, Figure 4A). In differentiated myotubes on a background of TNF-α, L-glutamine was also able to return the high activity levels observed in TNF-α conditions back to baseline DM values (LG + TNF-α 24.14 ± 14.4 vs. TNF-α 69.18 ± 3.99, P ≤ 0.001; vs. DM 11.42 ± 4.69, P = N.S; vs. LG alone 16.89 ± 8.83 units.ml⁻¹, P = N.S, Figure 4B). It is worth noting that p38 MAPK inhibitor (SB203580) was unable to attenuate the negative alterations in myotube morphology following TNF-α administration (data not shown).
L-glutamine was also able to improve myotube number back towards baseline in the presence of TNF-α (LG + TNF-α 1.94 ± 0.49 vs. TNF-α 1.31 ± 0.48, P ≤ 0.03; vs. DM 2.39 ± 0.58, P = N.S; vs. LG alone 2.45 ± 0.61, P = N.S, Figure 5A) in existing myotubes L-glutamine on average reduced the myotube number observed in TNF-α conditions although this was not significant (LG + TNF-α 7.37 ± 2.45 vs. TNF-α 6.15 ± 2.52, P = 0.147; vs. DM 9.63 ± 3.08, P = 0.008; vs. LG alone 8.67 ± 3.076, P = N.S, Figure 5B) and importantly helped prevent myotube atrophy in existing myotubes in the presence of TNF-α (area LG + TNF-α 7576 ± 3792 vs. TNF-α 5557 ± 2810, P ≤ 0.001; vs. DM 7949 ± 4730, P = N.S; vs. LG alone 9063 ± 4669 µm², P ≤ 0.003, Figure 5C). Glutamine was however unable to significantly reduce dead cell percentage in the presence of TNF-α in parental C2 myoblasts at 48hrs (TNF-α 27 ± 9% vs. TNF + LG 23 ± 10%, P = N.S.). As there was extensive impact of TNF-α on myotube atrophy when administered to existing myotubes, we investigated the downstream transcriptional targets of increased p38 MAPK signaling post rescue with L-glutamine for an array of genes involved in myotube maturation and growth (Myogenin, Myomaker, Mrf4, Myhc’s 1, 2, 4 & 7, Igf-I, Igf-IR, Igfbp2), atrophy (Myostatin, Musa1, Mafbx, Nf-κb, Tnfα, Foxo1, Foxo3), apoptosis and survival (Tnfrsfα & b, Cfos, Cmyc, p53, Bid, Bad, Bax, Adraid, Sirti) and glutamine synthesis (glutamine synthetase). Importantly, L-glutamine administration in response to TNF-α administration led to a dramatic 10 fold upregulation of Myogenin versus TNF-α alone (Figure 5A) (LG + TNF-α 2.7 ± 1.36 vs. TNF-α 0.2 ± 0.04, P = 0.002; Figure 6A) resulting in significantly higher expression versus baseline (LG + TNF-α 2.7 ± 1.36 vs. DM 0.56 ± 0.29, P = 0.006; Figure 6A) and L-glutamine alone conditions (LG + TNF-α 2.7 ± 1.36 vs. LG 0.2 ± 0.08, P = 0.002).

Similar trends were observed for Igf-Ir gene expression where a 4 fold increase was observed in LG + TNF-α vs. TNF-α alone (LG + TNF-α 2.62 ± 0.91 vs. TNF-α 0.64 ± 0.24, P ≤ 0.001; Figure 6B) with significantly higher expression than that seen at baseline and vs. L-glutamine alone treatments (LG + TNF-α 2.62 ± 0.91 ± vs. DM 0.51 ± 0.26, P = 0.001; vs. LG 0.39 ± 0.07, P = 0.001; Figure 6B). The same trend was mirrored for: Myhc2 with an almost 3 fold increase (LG + TNF-α 5.69 ± 2.71 vs. TNF-α 1.94 ± 0.264, P = 0.01; vs. DM 1.458 ± 0.473, P = 0.066; vs. LG alone 1.22 ± 0.06, P = 0.004; Figure 6C), an 8.2 fold increase in Myhc7 (LG + TNF-α 6.45 ± 0.47 vs. TNF-α 0.79 ± 0.23, P ≤ 0.001; vs. DM 0.82 ± 0.41, P ≤ 0.001; vs. LG alone 0.66 ± 0.2, P ≤ 0.001; Figure 6D), as well as a 5.4 fold increase in Tnfrsfα (LG + TNF-α 2.88 ± 2.09 vs. TNF-α 0.53 ± 0.09, P = 0.025; vs. DM 0.43 ± 0.14, P = 0.021; vs. LG alone 0.4 ± 0.11, P = 0.02; Figure 6E), and finally an 2.7 fold increase in Adradi (LG + TNF-α 0.83 ± 0.08 vs. TNF-α 0.31 ± 0.05, P ≤ 0.001; vs. DM 0.42 ± 0.15, P = 0.002; vs. LG alone 0.28 ± 0.11, P ≤ 0.001; Figure 6F). Importantly, in the presence of TNF-α L-glutamine was also able to significantly downregulate transcriptional target Foxo3a from 2.65 fold in TNF-α conditions (described above) down to 1.87 fold (LG + TNF-α 1.87 ± 0.33 vs. TNF-α 2.65 ± 0.15, P = 0.05 Figure 6G) returning them back to baseline and LG alone levels (LG + TNF-α 1.87 ± 0.33 vs. DM 1.0 ± 0.47, P = 0.038, vs. LG alone 1.64 ± 0.62, P = N.S). Results for p53 were somewhat similar, where L-glutamine was able to return 1.78 fold increases above baseline observed in TNF-α conditions to 1.53 fold increases above baseline, despite this LG + TNF-α was not significantly different to TNF-α alone (LG + TNF-α 1.56 ± 0.08 vs. TNF-α 1.78 ± 0.16, P = N.S; Figure 6H) and was still significantly elevated above baseline/DM (LG + TNF-α 1.56 ± 0.08 vs. DM 1.0 ± 0.33, P = 0.001; Figure 6H) Finally, following a 3 fold increase in Cfos post TNF administration, L-glutamine was able to reduce this increase vs. baseline to 2.64 fold, however this did not result in significance vs. TNF-α alone conditions (LG + TNF-α 0.98 ± 0.16 vs. TNF-α 1.14 ± 0.13, P = N.S, vs. baseline DM 0.37 ± 0.14, P ≤ 0.001; Figure 6I). L-glutamine was unable to return the 2.2 fold increase in Bid mRNA described above post TNF-α administration, where levels were the same when dosed with LG + TNF-α (2.85 ± 0.4 vs. TNF-α alone 2.73 ± 0.4, P = N.S; Figure not shown). Finally, LG alone conditions resulted in no change in glutamine synthetase (DM 0.96 ± 0.09 vs. LG alone 1.07 ± 0.27, P = N.S; figure not shown), furthermore, LG was unable to return the reduced levels of glutamine synthetase in TNF-α conditions back to baseline (0.38 ± 0.12 vs. TNF-α alone 0.45 ± 0.03, P = N.S; figure not shown). Overall L-glutamine reduced transcript expression of genes that were otherwise increased post TNF-α administration alone, where p53 and Cfos where partly returned back towards baseline (e.g p53, cfos) yet were non-significant, importantly however Foxo3 significantly returned back to baseline levels. Furthermore, in the presence of TNF-α, L-glutamine drove large increases in genes associated with myotube formation (myogenin) muscle growth (Igf-Ir), myotube maturation (Myhc 2 & 7) and survival (Tnfrsfα and Adradi). As these were not significantly reduced with TNF-α vs. baseline, it suggests L-glutamine was driving these increases in transcription rather than as simply a compensatory mechanism following TNF-α induced reductions. It is worth noting that we observed no significant differences in the remaining genes investigated (above) including those previously associated with elevated L-glutamine e.g. Tnf-α (DM 0.96 ± 0.44, LG 0.55 ± 0.06, TNF-α 0.72 ± 0.17, LG + TNF-α 1.2 ± 0.67) and myostatin (DM 1.53 ± 0.51, LG 1.42 ± 0.08, TNF-α 1.54 ± 0.07, LG + TNF-α 1.47 ± 0.12). All comparisons P = N.S.
Discussion

In the present study we aimed to assess the effect of L-glutaminates role in restoring the impaired fusion and myotube atrophy observed in the presence of TNF-α; as well as the underlying role of the MAPK's (ERK1/2, p38 and JNK) and associated signalling (Akt) in their downstream modulation of gene transcription involved in skeletal muscle cell differentiation, myotube growth and survival. We were able to accept our original hypotheses whereby we demonstrated that; 1) L-glutamine rescued myoblast differentiation and reduced myotube atrophy in the presence of TNF-α, and that; 2) p38 MAPK kinase (but not JNK, ERK1/2 or Akt) was responsible for coordinating these morphological responses via transcriptionally restoring some of the important genes associated with survival and myotube atrophy, particularly Foxo3α back to baseline post TNF-α exposure, as well driving extensive upregulation of Myogenin, Igf-Ir, Myhcs 2 & 7, Tnfsfr1b, Adrad1 to enable normal differentiation, myotube growth and survival.

The findings in the present study investigating the impact of TNF-α on skeletal muscle cell differentiation and hypertrophy/atrophy correspond with previous studies, whereby after 3 days post serum withdrawal, TNF-α administration reduces differentiation in myoblasts and results in myotube atrophy when administered to existing myotubes (Al-Shanti et al., 2008; Foulstone et al., 2004; Foulstone et al., 2001; Foulstone et al., 2003; Grohmann et al., 2005a; Grohmann et al., 2005b; Jejurikar et al., 2006; Li et al., 2005; Li et al., 2003; Li and Reid, 2000; Meadows et al., 2000; Saini et al., 2008; Saini et al., 2012; Saini et al., 2010; Sharples et al., 2010; Stewart et al., 2004). Importantly, we report for the first time that L-glutamine rescued differentiation in myoblasts in the presence of TNF-α and confirmed previous findings for the role of L-glutamine in rescuing myotube atrophy in existing myotubes in the presence of TNF-α (Karinch et al., 2001). Interestingly, Akt and ERK1/2 activity were unchanged in the presence of TNF-α, although JNK activation increased on average with TNF-α exposure at 15 mins this did not attain significance. These signaling studies confirmed previous findings where the presence of ERK (PD98059) and JNK (SP60012) inhibitors were unable to ameliorate the negative impact of TNF-α on protein degradative pathways in skeletal muscle cells (Li et al., 2005). Importantly however, stress related p38 MAPK activity substantially and significantly increased after 15 mins in the presence of TNF-α in both differentiating myoblasts and differentiated myotubes, a finding supported in previous studies (Alvarez et al., 2001; Chen et al., 2007; Grohmann et al., 2005b; Li et al., 2005; Palacios et al., 2010; Zhan et al., 2007). Importantly, we report for the first time that a bolus (20 mM) of L-glutamine reduced p38 MAPK activity in TNF-α conditions in both differentiating myoblasts and differentiated myotubes; a signaling response that corresponded with the ability of L-glutamine to improve fusion of myoblasts and also retain myotube area in the presence of TNF-α in already differentiated myotubes. Therefore, the results from this study point to an important role for L-glutamine in regulating p38 MAPK in the presence of TNF-α.

In inflammatory conditions p38 MAPK has previously been shown to be activated and involved in culminating in protein degradation in skeletal muscle (Kim et al., 2009; Li et al., 2003; Li and Reid, 2000; Philip et al., 2005). However, in basal or healthy conditions p38 MAPK has also been shown to have a positive role in skeletal muscle cell differentiation, whereby studies adding p38 MAPK inhibitor (SB203580) to myoblasts inhibited differentiation of C2C12 cells (Li et al., 2000), and more recently we have reported that the MEK inhibitor PD98059 (that blocks ERK1/2) enhances C2 myoblast differentiation with corresponding increases in phosphorylated p38 MAPK (Al-Shanti and Stewart, 2008). Therefore, p38 MAPK may have positive or negative roles in myoblast differentiation depending upon the inflammatory context of the cells. p38-α MAPK has also been linked to myoD, whereby it phosphorylates MEF2 proteins (Molkentin et al., 1995) which in turn phosphorylate E47 promoting its heterodimerisation with myoD (Lluis et al., 2005), this then results in muscle specific transcription such as myogenin gene expression (Keren et al., 2006). It has also been demonstrated that a different p38 MAPK family member, p38-γ, also plays a crucial role in regulating skeletal muscle differentiation (Gillespie et al., 2009; Lassar, 2009). In the present study, p38 MAPK phosphorylation (specific isoforms not distinguished) was elevated in TNF-α treated conditions where impaired differentiation and myotube atrophy were observed, whereas P38 activity was suppressed in L-glutamine conditions where differentiation and myotube atrophy were restored in the presence of TNF-α. This suggests that p38 MAPK is activated via stress related inflammatory mechanisms in the present study. However, it is worth noting that L-glutamine drove significant 10 fold increases in myogenin gene expression even in the presence of TNF-α, despite reductions in p38 MAPK, that while perhaps enabling a compensatory drive in differentiation following lack of fusion at earlier time points in the differentiation program (Berkes and Tapscott, 2005), suggests that the increase in myogenin expression was not regulated by the p38 MAPK/MEF2/myoD/myogenin mechanism.
described above. Furthermore, inhibition of p38 MAPK using chemical inhibitor SB203580 in the present study was unable to rescue the TNF-α induced reductions myotube morphology, this may be due to its apparent bimodal functions described above as it may be difficult to recover differentiation in the presence of TNF-α without affecting its role in positively regulating differentiation.

Unlike previous studies, showing that TNF-α in C2C12 myotubes led to increased myostatin protein levels, that were also returned following L-glutamine administration (Karinch et al., 2001), the present study saw no change in myostatin, albeit at the mRNA level, in both TNF-α alone conditions and when the cytokine was co-administered with L-glutamine. Following TNF-α induced increases in p38 MAPK signaling, we did however see a large increase in Foxo3 gene expression, that at the protein level is an important transcription factor in regulating muscle protein degradation via upregulation of the ubiquitin ligases (Edstrom et al., 2006; Sandri, 2008; Sandri et al., 2004). Despite this, we found no changes in gene expression of the ubiquitin ligases/protein degradative markers; Atrogin-1 or Mafbx in the presence of TNF-α in myotubes, previously reported to be transcriptionally regulated by p38 MAPK and correspond with muscle protein degradation (Kim et al., 2009; Li et al., 2005); perhaps suggesting that future studies should investigate the temporal regulation of these genes to fully elucidate their role. Importantly however, we were able to demonstrate novel findings that L-glutamine reduced a TNF-α induced increase in Foxo3 back to control conditions. It worth mentioning here that class I Histone Deacetylases (Hdac 1-3 and 8) have been associated with increases in Foxo3 and atrophy in skeletal muscle during nutrient deprivation and disuse induced atrophy (Beharry et al., 2014), and therefore require further investigation with TNF-α in the current model. Foxo’s have also been reported to promote cell apoptosis, shifting cellular function towards oxidative stress resistance/detoxification and DNA repair (Brunet et al., 2004; Greer and Brunet, 2005; Wang et al., 2007). We observed average increases in survival of myotubes and a a restoration of myotube size in L-glutamine conditions on a background of elevated myotube loss and atrophy, post TNF-α exposure, perhaps suggesting a key role for Foxo3 in this process. Further, as TNF-α has been shown to increase oxidative stress via elevating reactive oxygen species in skeletal muscle (Reid and Li, 2001), although requiring confirmation, L-glutamine could also be serving to reduce Foxo3 in order to provide reactive oxygen species detoxification to encourage myotube survival and growth. Another noteworthy finding is that L-glutamine extensively upregulated Myhc 2 & 7 gene expression, coding for slow type I and intermediate type IIA myofibrillar proteins respectively involved in laying down contractile proteins to prevent myotube atrophy.

Furthermore, the rescue of myotube atrophy post TNF-α exposure by L-glutamine administration substantially increased the gene expression of Myogenin, Igf-Ir, Tnfsfr1b and Adra1d. As mentioned above the 10 fold induced increase in Myogenin following L-glutamine administration suggests that the non-essential amino acid may enable a compensatory drive in differentiation following for lack of fusion at earlier time points (Berkes and Tapscott, 2005) despite the presence of a potent inhibitor of these processes (TNF-α). In addition to increased Myhc 2 & 7 and myogenin, following L-glutamine supplementation in the presence of inflammation, an increase in Igf-Ir was observed and could therefore serve as a feedback mechanism following low Igf-I expression in TNF-α alone conditions (that were not rescued with L-glutamine). Where an increase in Igf-Ir may act as a drive to enable IGF-I binding to its receptor on a background of low IGF-I ligand; a process we have previously observed in the presence of TNF-α in C2C12 cells, where a background of impaired Igf-I expression led to compensatory elevations in Igf-Ir expression (Sharples et al., 2010). Supporting these processes further, the large increase in Tnfsfr1b gene expression in L-glutamine conditions with exogenous TNF-α may serve to dampen the impact of TNF-α. This is because this TNF receptor can be liberated from the cell surface and represents an important mechanism of negative regulation for the biological activity of soluble TNF-α (Bemelmans et al., 1996a; Bemelmans et al., 1996b), via inactivating and clearing TNF-α. This process of liberated vs. membrane bound receptor is controlled by TNF converting enzyme (TACE). TACE would increase abundance of cleaved soluble receptor in the medium and would therefore enable the increase in Tnfsfr1b observed in the present study post L-glutamine administration to liberate and clear the excess TNF-α, warranting further investigation. Finally, we have previously seen that Adra1d was elevated following incubations that induced myoblast survival (co-incubations of IGF-I with TNF-α) (Saini et al., 2010). Subsequently, Adra1d knockout resulted in significantly higher levels of cell death under TNF-α administration suggesting Adra1d expression is essential for skeletal muscle cell survival (Saini et al., 2010). A similar role here could be extrapolated to myotubes in the present study, where with TNF-α exposure Adra1d is elevated to evoke myotube survival when elevated L-glutamine is available.

In conclusion for the first time we indicate that L-glutamine suppressed a TNF-α induced increase in stress related p38 MAPK activity and enabled a restoration of Foxo3 gene expression levels back toward baseline post
TNF-α addition, as well as driving extensive upregulation of Myogenin, Igf-Ir, Myhc2 & 7, Tnfsfr1b, Adrad1 to enable normal myotube growth and survival. Overall, L-glutamine supplementation is important in reducing the suppression of muscle differentiation and in restoring atrophic myotube phenotypes in inflamed environments.

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References


Figure 1: Three days post induction of differentiation via serum withdrawal, TNF-α (20 ng.ml⁻¹) administration reduced the proportion of cells in the G1 phase of the cell cycle at 24hrs (1A), a prerequisite for myoblast differentiation, as well as later (48 & 72hrs) reductions in biochemical (CK activity- 1B) and morphological differentiation (myotube number at 72hrs- 1C). This corresponded with significant reductions in the transcript expression of important myogenic and growth related genes; Myogenin (1D), Igf-I(1E) and Igfbp5 (1F). Representative morphological images (20X) for DM vs. TNF-α conditions can be observed in figure 1G. All experiments were conducted on 3 separate occasions (n = 3); 4-5 morphological images per condition in duplicate were analyzed for each n. RT-PCR assays were performed in duplicate. * Significantly different vs. DM.
Figure 2: After 3 days of TNF-α administration to existing myotubes, TNF-α induced myotube loss and atrophy demonstrated via a loss in myotube number (2A) and area (2B). Representative morphological images (10x) of DM vs. TNF-α can been seen in figure 2C. This was underpinned by reductions in transcript expression of genes involved in myotube growth; Myogenin (2D), Igf-I (2E), and Igfbp2 (2F) and an increase in those related to atrophy (Foxo3- 2G) and apoptosis/survival; p53 (2H), cfos (2I), Bid (2J). All experiments were conducted on 3 separate occasions (n = 3); 4-5 morphological images per condition in duplicate were analyzed for each n. RT-PCR assays were performed in duplicate. * Significantly different vs. DM.
**Figure 3:** In order to investigate the underlying mechanisms of TNF-α's detrimental impact on myoblast/myotube morphology (observed in Figure 1 & 2), we investigated the earlier signaling responses. JNK activity was non-significant (3A), whereas P38 MAPK substantially and significantly increased its activity after 15 minutes in the presence of TNF-α in differentiating myoblasts (3B) and differentiated myotubes (3C) vs. baseline. All experiments were conducted on 3 separate occasions (n = 3). CBA array assays were conducted in duplicate. * Significantly different vs. DM.
**Figure 4:** L-glutamine reduced p38 MAPK activity in TNF-α conditions back to control levels in both differentiating myoblasts (4A) and differentiated myotubes (4B). All experiments were conducted on 3 separate occasions ($n = 3$). CBA array assays were conducted in duplicate. * Significantly different to DM. # Significantly different vs. TNF-α.
Figure 5: L-glutamine restored morphological measures of myotube formation in differentiating myoblasts (myotube number 5A), helped somewhat retain myotube number (although non-significant) (5B) and prevented myotube area losses (5C) in myotubes following TNF-α administration. Representative morphological images (10X) can be seen for TNF-α vs. L-glutamine (LG) + TNF-α conditions. All experiments were conducted on 3 separate occasions (n = 3); 4-5 morphological images per condition in duplicate were analyzed for each n. * Significantly different vs. TNF-α conditions.
Figure 6: The signaling events (figure 4) and morphological adaptations (figure 5) in response to L-glutamine on a background of elevated TNF-α were driven by extensive upregulation of genes associated with growth and survival including; Myogenin (6A), Igf-Ir (6B), Myhc2 (6C) & 7 (6D), Tnfsfr1b (6E) Adra1d (6F) and transcriptionally restoring some of the important genes associated with cell death and myotube atrophy, particularly Fox03 back to baseline (6G) as well as average reductions (yet non-significant) for p53 (6H) and Cfos (6I), that were otherwise increased post TNF-α administration. All experiments were conducted on 3 separate occasions (n = 3), RT-PCR assays were performed in duplicate. *Significantly different vs. TNF-α. # Significantly different vs. DM. + Significantly different vs. LG.