Girven, M, Dugdale, HF, Owens, DJ, Hughes, DC, Stewart, CE and Sharples, AP

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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

Matthew Girven[1+]
Hannah F. Dugdale[1+]
Daniel J. Owens [1,2]
David C. Hughes[1,3]
Claire E. Stewart[1]
Adam P. Sharples [1*]

[1] Stem Cells, Ageing and Molecular Physiology Research Unit, Exercise Metabolism and Adaptation Research Group, Research Institute for Sport and Exercise Sciences (RISES), Liverpool John Moores University, Liverpool, UK.

[2] Sorbonne Universités, UPMC University of Paris 06, INSERM UMRS974, CNRS FRE3617, Centre de Recherche en Myologie (CRM), GH Pitié Salpêtrière, Paris 13, France.

[3] Department of Neurobiology, Physiology and Behavior, University of California Davis, Davis, USA.

Primary Authors [+]

* Senior Corresponding Author

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 *Foxo3*, *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 *Foxo3* 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; Greiwe et al., 2001) and the local production by the muscle is also elevated (Greiwe 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

baseline, albeit not completely restoring them to control levels as well as enabling normal myotube growth (Bonetto et al., 2011).

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 (Lagranha 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

C_2 and C_2C_{12} mouse skeletal myoblasts (Blau et al., 1985; Yaffe and Saxel, 1977), below passage 12, were employed in these studies. Cells were seeded at 1×10^6 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, 8×10^4 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 L-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 C_2 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, C_2 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 C_2 cells (Foulstone et al., 2001; Saini et al., 2008; Sharples et al., 2010; Stewart et al., 2004) and human cells (Foulstone et al., 2004). Similar doses have also been used to inhibit muscle cell differentiation in C_2C_{12} 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; Wernerman, 2008), therefore the high dose of LG investigated in the present study is relevant to receiving iv. administration

of glutamine. C_2C_{12} 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 0hrs) 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 (0hrs) 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 × 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 (μm^2) 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 OligoPerfectTM 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

sequences were designed to exclude hairpins, self-dimer and cross-dimers. All primers designed were amplified a product of between 76 - 280 bp% (Table 1.).

Reverse transcription real-time polymerase chain reaction (rt-RT-PCR) for gene expression and relative data analysis

Rt-RT-PCR reactions for DM vs. TNF-α conditions in C2C12 myoblast differentiation were performed using Power SYBR Green RNA-to-C_T 1 step kit (Applied Biosystems, Carlsbad, CA, USA) on a Chromo4 DNA engine supported by Opticon Monitor version 3.1.32, MJ Geneworks Inc., Bio-Rad Laboratories, Inc., (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-RT-PCR for both studies was performed as follows: 10min, 50°C (reverse transcription), 5 min 95°C (initial denaturation and reverse transcriptase inactivation), followed by 40 cycles of: 10secs, 95°C (denaturation), 30secs, 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 ($^{\Delta\Delta}C_t$) equation (Schmittgen & Livak, 2008). Polymerase (RNA) II polypeptide B (*Polr2β/Rp-IIb*) was used as the reference gene (this was highly stable across all conditions with a mean ± SD C_t 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 C_t 16.69 ± 0.72 (variation 4.3%) for rt-RT-PCR on the Rotorgene 3000Q in myotube 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 ($^{\Delta\Delta}C_t$) 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 $\mu g.ml^{-1}$) and incubated at RT for 30 mins 10 µl propidium iodide labeling buffer (50 $\mu g.ml^{-1}$ 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 detailed 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 Phycoerythrin (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 analyate 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 ANOVE (3 x2) 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 (5×2) 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 $P \leq 0.05$.

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, $P \leq 0.001$; 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 $P \leq 0.001$; 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; $P \leq 0.001$, 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 $P \leq 0.001$; 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, $P \leq 0.001$; 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).

TNF-α evokes a loss of myotubes and myotube atrophy in existing differentiated myotubes.

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 myotubes 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 ± 2810 μm^2, 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 administration for 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, Mafbx, Nf- κb, Tnf-α, both Foxo1 & Foxo3*), apoptosis/survival (*Tnfrsf1a & b, Cfos, Cmyc, p53, Bid, Bad, Bax, Adra1d, 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-κb (*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), *Mafbx* (DM 0.78 ± 0.4, TNF-α 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 11.43 ± 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 (Tnfrsf1b and Adrad1) 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 ± 3.98, 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, Murf-1, Mafbx, Nf- κb, Tnf-α, Foxo1, Foxo3*), apoptosis and survival (*Tnfrsf1a & b, Cfos, Cmyc, p53, Bid, Bad, Bax, Adra1d, Sirt1*) 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 (Figure5A) (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 = 0.006; 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 *Tnfrsf1b* (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 *Adrad1* (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 *Foxa3a* 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 ± .047, 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 howver *Foxo3* significantly returned back to baseline levels. Furthermore, in the presence of TNF-α, L-glutamine drove large increases in genes associated with myotoube formation (*myogenin*) muscle growth (*Igf-Ir*), myotube maturation (*Myhc 2 & 7*) and survival (*Tnfrsf1b* and *Adrad1*). 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-glutamines 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 *Foxo3a* back to baseline post TNF-α exposure, as well driving extensive upregulation of *Myogenin, Igf-Ir, Myhc2 & 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 C_2C_{12} cells (Li et al., 2000), and more recently we have reported that the MEK inhibitor PD98059 (that blocks ERK1/2) enhances C_2 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 if 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 Deactylases (Hdac 1-3 and 8) have been associated with increases in Fox03 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 Fox03 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 Fox03 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 *Tnfrsf1b* 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 *Tnfrsf1b* 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* knockdown 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

Al-Shanti N, Saini A, Faulkner SH, Stewart CE. 2008. Beneficial synergistic interactions of TNF-alpha and IL-6 in C2 skeletal myoblasts--potential cross-talk with IGF system. Growth factors (Chur, Switzerland) 26(2):61-73.

Al-Shanti N, Stewart CE. 2008. PD98059 enhances C2 myoblast differentiation through p38 MAPK activation: a novel role for PD98059. J Endocrinol 198(1):243-252.

Alvarez B, Quinn LS, Busquets S, Lopez-Soriano FJ, Argiles JM. 2001. Direct effects of tumor necrosis factor alpha (TNF-alpha) on murine skeletal muscle cell lines. Bimodal effects on protein metabolism. Eur Cytokine Netw 12(3):399-410.

Beharry AW, Sandesara PB, Roberts BM, Ferreira LF, Senf SM, Judge AR. 2014. HDAC1 activates FoxO and is both sufficient and required for skeletal muscle atrophy. J Cell Sci 127(Pt 7):1441-1453.

Bemelmans MH, Greve JW, Gouma DJ, Buurman WA. 1996a. Increased concentrations of tumour necrosis factor (TNF) and soluble TNF receptors in biliary obstruction in mice; soluble TNF receptors as prognostic factors for mortality. Gut 38(3):447-453.

Bemelmans MH, van Tits LJ, Buurman WA. 1996b. Tumor necrosis factor: function, release and clearance. Critical reviews in immunology 16(1):1-11.

Berg A, Forsberg E, Wernerman J. 2002. The local vascular tolerance to an intravenous infusion of a concentrated glutamine solution in ICU patients. Clinical Nutrition 21(2):135-139.

Berkes CA, Tapscott SJ. 2005. MyoD and the transcriptional control of myogenesis. Seminars in cell & developmental biology 16(4-5):585-595.

Blau HM, Pavlath GK, Hardeman EC, Chiu CP, Silberstein L, Webster SG, Miller SC, Webster C. 1985. Plasticity of the differentiated state. Science 230(4727):758-766.

Bode B, Fischer C, Abcouwer S, Wasa M, Souba W. 1996. Glutamine and Cancer Cachexia. Protein and Amino Acid Metabolism in Cancer Cachexia: Springer Berlin Heidelberg. p 139-170.

Bonetto A, Penna F, Minero VG, Reffo P, Costamagna D, Bonelli G, Baccino FM, Costelli P. 2011. Glutamine prevents myostatin hyperexpression and protein hypercatabolism induced in C2C12 myotubes by tumor necrosis factor-alpha. Amino acids 40(2):585-594.

Boza JJ, Turini M, Moennoz D, Montigon F, Vuichoud J, Gueissaz N, Gremaud G, Pouteau E, Piguet-Welsch C, Finot PA, Ballevre O. 2001. Effect of glutamine supplementation of the diet on tissue protein synthesis rate of glucocorticoid-treated rats. Nutrition 17(1):35-40.

Brunet A, Sweeney LB, Sturgill JF, Chua KF, Greer PL, Lin Y, Tran H, Ross SE, Mostoslavsky R, Cohen HY. 2004. Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science 303(5666):2011-2015.

Bruunsgaard H, Andersen-Ranberg K, Hjelmborg JB, Pedersen BK, Jeune B. 2003a. Elevated levels of tumor necrosis factor alpha and mortality in centenarians. The American journal of medicine 115(4):278-283.

Bruunsgaard H, Ladelund S, Pedersen AN, Schroll M, Jorgensen T, Pedersen BK. 2003b. Predicting death from tumour necrosis factor-alpha and interleukin-6 in 80-year-old people. Clinical and experimental immunology 132(1):24-31.

Bruunsgaard H, Pedersen BK. 2003. Age-related inflammatory cytokines and disease. Immunology and allergy clinics of North America 23(1):15-39.

Calder PC, Yaqoob P. 1999. Glutamine and the immune system. Amino acids 17(3):227-241.

Chen SE, Jin B, Li YP. 2007. TNF-alpha regulates myogenesis and muscle regeneration by activating p38 MAPK. Am J Physiol Cell Physiol 292(5):C1660-1671.

Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. 1997. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. J Biol Chem 272(10):6653-6662.

Curthoys NP, Watford M. 1995. Regulation of glutaminase activity and glutamine metabolism. Annu Rev Nutr 15:133-159.

633 Edstrom E, Altun M, Hagglund M, Ulfhake B. 2006. Atrogin-1/MAFbx and MuRF1 are downregulated in aging-
634 related loss of skeletal muscle. J Gerontol A Biol Sci Med Sci 61(7):663-674.
635 Foulstone EJ, Huser C, Crown AL, Holly JM, Stewart CE. 2004. Differential signalling mechanisms predisposing
636 primary human skeletal muscle cells to altered proliferation and differentiation: roles of IGF-I and
637 TNFalpha. Exp Cell Res 294(1):223-235.
638 Foulstone EJ, Meadows KA, Holly JM, Stewart CE. 2001. Insulin-like growth factors (IGF-I and IGF-II) inhibit
639 C2 skeletal myoblast differentiation and enhance TNF alpha-induced apoptosis. J Cell Physiol
640 189(2):207-215.
641 Foulstone EJ, Savage PB, Crown AL, Holly JM, Stewart CE. 2003. Role of insulin-like growth factor binding
642 protein-3 (IGFBP-3) in the differentiation of primary human adult skeletal myoblasts. J Cell Physiol
643 195(1):70-79.
644 Gillespie MA, Le Grand F, Scime A, Kuang S, von Maltzahn J, Seale V, Cuenda A, Ranish JA, Rudnicki MA.
645 2009. p38-{gamma}-dependent gene silencing restricts entry into the myogenic differentiation program.
646 J Cell Biol 187(7):991-1005.
647 Greer EL, Brunet A. 2005. FOXO transcription factors at the interface between longevity and tumor suppression.
648 Oncogene 24(50):7410-7425.
649 Greiwe JS, Cheng B, Rubin DC, Yarasheski KE, Semenkovich CF. 2001. Resistance exercise decreases skeletal
650 muscle tumor necrosis factor alpha in frail elderly humans. FASEB journal : official publication of the
651 Federation of American Societies for Experimental Biology 15(2):475-482.
652 Grohmann M, Foulstone E, Welsh G, Holly J, Shield J, Crowne E, Stewart C. 2005a. Isolation and validation of
653 human prepubertal skeletal muscle cells: maturation and metabolic effects of IGF-I, IGFBP-3 and
654 TNFalpha. J Physiol 568(Pt 1):229-242.
655 Grohmann M, Sabin M, Holly J, Shield J, Crowne E, Stewart C. 2005b. Characterization of differentiated
656 subcutaneous and visceral adipose tissue from children the influences of TNF-α and IGF-I. Journal of
657 lipid research 46(1):93-103.
658 Jejurikar SS, Henkelman EA, Cederna PS, Marcelo CL, Urbanchek MG, Kuzon WM, Jr. 2006. Aging increases
659 the susceptibility of skeletal muscle derived satellite cells to apoptosis. Exp Gerontol 41(9):828-836.
660 Karinch AM, Pan M, Lin CM, Strange R, Souba WW. 2001. Glutamine metabolism in sepsis and infection. J Nutr
661 131(9 Suppl):2535S-2538S; discussion 2550S-2531S.
662 Keren A, Tamir Y, Bengal E. 2006. The p38 MAPK signaling pathway: a major regulator of skeletal muscle
663 development. Mol Cell Endocrinol 252(1-2):224-230.
664 Kim J, Won KJ, Lee HM, Hwang BY, Bae YM, Choi WS, Song H, Lim KW, Lee CK, Kim B. 2009. p38 MAPK
665 Participates in Muscle-Specific RING Finger 1-Mediated Atrophy in Cast-Immobilized Rat
666 Gastrocnemius Muscle. Korean J Physiol Pharmacol 13(6):491-496.
667 Ko HM, Oh SH, Bang HS, Kang NI, Cho BH, Im SY, Lee HK. 2009. Glutamine protects mice from lethal
668 endotoxic shock via a rapid induction of MAPK phosphatase-1. J Immunol 182(12):7957-7962.
669 Labow BI, Souba WW. 2000. Glutamine. World J Surg 24(12):1503-1513.
670 Lacey JM, Wilmore DW. 1990. Is glutamine a conditionally essential amino acid? Nutrition reviews 48(8):297-
671 309.
672 Lagranha CJ, Hirabara SM, Curi R, Pithon-Curi TC. 2007. Glutamine supplementation prevents exercise-
673 induced neutrophil apoptosis and reduces p38 MAPK and JNK phosphorylation and p53 and caspase 3
674 expression. Cell Biochemistry and Function 25(5):563-569.
675 Lassar AB. 2009. The p38 MAPK family, a pushmi-pullyu of skeletal muscle differentiation. J Cell Biol
676 187(7):941-943.
677 Leger B, Derave W, De Bock K, Hespel P, Russell AP. 2008. Human sarcopenia reveals an increase in SOCS-3
678 and myostatin and a reduced efficiency of Akt phosphorylation. Rejuvenation research 11(1):163-175B.
679 Li Y, Jiang B, Ensign WY, Vogt PK, Han J. 2000. Myogenic differentiation requires signalling through both
680 phosphatidylinositol 3-kinase and p38 MAP kinase. Cellular signalling 12(11-12):751-757.
681 Li YP. 2003. TNF-alpha is a mitogen in skeletal muscle. Am J Physiol Cell Physiol 285(2):C370-376.
682 Li YP, Chen Y, John J, Moylan J, Jin B, Mann DL, Reid MB. 2005. TNF-alpha acts via p38 MAPK to stimulate
683 expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. Faseb J 19(3):362-370.
684 Li YP, Lecker SH, Chen Y, Waddell ID, Goldberg AL, Reid MB. 2003. TNF-alpha increases ubiquitin-conjugating
685 activity in skeletal muscle by up-regulating UbcH2/E220k. Faseb J 17(9):1048-1057.
686 Li YP, Reid MB. 2000. NF-kappaB mediates the protein loss induced by TNF-alpha in differentiated skeletal
687 muscle myotubes. Am J Physiol Regul Integr Comp Physiol 279(4):R1165-1170.
688 Lluis F, Ballestar E, Suelves M, Esteller M, Munoz-Canoves P. 2005. E47 phosphorylation by p38 MAPK
689 promotes MyoD/E47 association and muscle-specific gene transcription. Embo J 24(5):974-984.
690 Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SO, Jensen CH,
691 Schroder HD, Langberg H. 2007. The influence of anti-inflammatory medication on exercise-induced
692 myogenic precursor cell responses in humans. J Appl Physiol (1985) 103(2):425-431.
693 Manjavachi MN, Motta EM, Marotta DM, Leite DF, Calixto JB. 2010. Mechanisms involved in IL-6-induced
694 muscular mechanical hyperalgesia in mice. Pain 151(2):345-355.

May PE, Barber A, D'Olimpio JT, Hourihane A, Abumrad NN. 2002. Reversal of cancer-related wasting using oral supplementation with a combination of beta-hydroxy-beta-methylbutyrate, arginine, and glutamine. American journal of surgery 183(4):471-479.
Meadows KA, Holly JM, Stewart CE. 2000. Tumor necrosis factor-alpha-induced apoptosis is associated with suppression of insulin-like growth factor binding protein-5 secretion in differentiating murine skeletal myoblasts. J Cell Physiol 183(3):330-337.
Mikkelsen UR, Langberg H, Helmark IC, Skovgaard D, Andersen LL, Kjaer M, Mackey AL. 2009. Local NSAID infusion inhibits satellite cell proliferation in human skeletal muscle after eccentric exercise. J Appl Physiol (1985) 107(5):1600-1611.
Molkentin JD, Black BL, Martin JF, Olson EN. 1995. Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell 83(7):1125-1136.
Palacios D, Mozzetta C, Consalvi S, Caretti G, Saccone V, Proserpio V, Marquez VE, Valente S, Mai A, Forcales SV, Sartorelli V, Puri PL. 2010. TNF/p38alpha/polycomb signaling to Pax7 locus in satellite cells links inflammation to the epigenetic control of muscle regeneration. Cell stem cell 7(4):455-469.
Parry-Billings M, Evans J, Calder PC, Newsholme EA. 1990. Does glutamine contribute to immunosuppression after major burns? Lancet 336(8714):523-525.
Philip B, Lu Z, Gao Y. 2005. Regulation of GDF-8 signaling by the p38 MAPK. Cellular signalling 17(3):365-375.
Reid MB, Li YP. 2001. Cytokines and oxidative signalling in skeletal muscle. Acta Physiol Scand 171(3):225-232.
Roth E, Funovics J, Muhlbacher F, Schemper M, Mauritz W, Sporn P, Fritsch A. 1982. Metabolic disorders in severe abdominal sepsis: glutamine deficiency in skeletal muscle. Clin Nutr 1(1):25-41.
Saini A, Al-Shanti N, Faulkner SH, Stewart CE. 2008. Pro- and anti-apoptotic roles for IGF-I in TNF-alpha-induced apoptosis: a MAP kinase mediated mechanism. Growth factors (Chur, Switzerland) 26(5):239-253.
Saini A, Al-Shanti N, Sharples AP, Stewart CE. 2012. Sirtuin 1 regulates skeletal myoblast survival and enhances differentiation in the presence of resveratrol. Exp Physiol 97(3):400-418.
Saini A, Al-Shanti N, Stewart C. 2010. C2 skeletal myoblast survival, death, proliferation and differentiation: regulation by Adra1d. Cell Physiol Biochem 25(2-3):253-262.
Saini A, Al-Shanti N, Stewart CE. 2006. Waste management - cytokines, growth factors and cachexia. Cytokine and Growth Factor Reviews 17(6):475-486.
Salehian B, Mahabadi V, Bilas J, Taylor WE, Ma K. 2006. The effect of glutamine on prevention of glucocorticoid-induced skeletal muscle atrophy is associated with myostatin suppression. Metabolism 55(9):1239-1247.
Sandri M. 2008. Signaling in muscle atrophy and hypertrophy. Physiology 23(3):160-170.
Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. 2004. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell 117(3):399-412.
Schubert R, Geiger H, Zielen S, Baer PC. 2009. Simultaneous detection of ERK-, p38-, and JNK-MAPK phosphorylation in human adipose-derived stem cells using the Cytometric Bead Array technology. J Immunol Methods 350(1-2):200-204.
Serrano AL, Baeza-Raja B, Perdiguero E, Jardi M, Munoz-Canoves P. 2008. Interleukin-6 is an essential regulator of satellite cell-mediated skeletal muscle hypertrophy. Cell Metab 7(1):33-44.
Sharples AP, Al-Shanti N, Hughes DC, Lewis MP, Stewart CE. 2013. The role of insulin-like-growth factor binding protein 2 (IGFBP2) and phosphatase and tensin homologue (PTEN) in the regulation of myoblast differentiation and hypertrophy. Growth Horm IGF Res 23(3):53-61.
Sharples AP, Al-Shanti N, Stewart CE. 2010. C2 and C2C12 murine skeletal myoblast models of atrophic and hypertrophic potential: relevance to disease and ageing? J Cell Physiol 225(1):240-250.
Sharples AP, Hughes DC, Deane CS, Saini A, Selman C, Stewart CE. 2015a. Longevity and skeletal muscle mass: the role of IGF signalling, the sirtuins, dietary restriction and protein intake. Aging Cell 14(4):511-523.
Sharples AP, Polydorou I, Hughes DC, Owens DJ, Hughes TM, Stewart CE. 2015b. Skeletal muscle cells possess a 'memory' of acute early life TNF-alpha exposure: role of epigenetic adaptation. Biogerontology. Epub ahead of print: https://dx.doi.org/10.1007/s10522-015-9604-x
Singleton KD, Beckey VE, Wischmeyer PE. 2005. GLUTAMINE PREVENTS ACTIVATION OF NF-kappaB AND STRESS KINASE PATHWAYS, ATTENUATES INFLAMMATORY CYTOKINE RELEASE, AND PREVENTS ACUTE RESPIRATORY DISTRESS SYNDROME (ARDS) FOLLOWING SEPSIS. Shock (Augusta, Ga) 24(6):583-589.
Stewart CE, Newcomb PV, Holly JM. 2004. Multifaceted roles of TNF-alpha in myoblast destruction: a multitude of signal transduction pathways. J Cell Physiol 198(2):237-247.
Stewart CE, Newcomb PV, Savage PB, Dickens M, Tavare J, Holly JM. 1999. Increased, not decreased activation of the insulin-like growth factor (IGF) receptor signalling pathway during ceramide-induced apoptosis. Growth Horm IGF Res 9(2):131-142.
Tollefsen SE, Sadow JL, Rotwein P. 1989. Coordinate expression of insulin-like growth factor II and its receptor during muscle differentiation. Proc Natl Acad Sci U S A 86(5):1543-1547.

Tolosa L, Morla M, Iglesias A, Busquets X, Llado J, Olmos G. 2005. IFN-gamma prevents TNF-alpha-induced apoptosis in C2C12 myotubes through down-regulation of TNF-R2 and increased NF-kappaB activity. Cellular signalling 17(11):1333-1342.
van de Vyver M, Myburgh KH. 2012. Cytokine and satellite cell responses to muscle damage: interpretation and possible confounding factors in human studies. J Muscle Res Cell Motil 33(3-4):177-185.
Wang F, Nguyen M, Qin F, Tong Q. 2007. SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging cell 6(4):505-514.
Watford M. 2015. Glutamine and glutamate: Nonessential or essential amino acids? Animal Nutrition 1(3):119-122.
Wernerman J. 2008. Clinical use of glutamine supplementation. J Nutr 138(10):2040s-2044s.
Wischmeyer PE, Riehm J, Singleton KD, Ren H, Musch MW, Kahana M, Chang EB. 2003. Glutamine attenuates tumor necrosis factor-alpha release and enhances heat shock protein 72 in human peripheral blood mononuclear cells. Nutrition 19(1):1-6.
Yaffe D, Saxel O. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. Nature 270(5639):725-727.
Yang S, Alnaqeeb M, Simpson H, Goldspink G. 1996. Cloning and characterization of an IGF-1 isoform expressed in skeletal muscle subjected to stretch. J Muscle Res Cell Motil 17(4):487-495.
Zhan M, Jin B, Chen S-E, Reecy JM, Li Y-P. 2007. TACE release of TNF-α mediates mechanotransduction-induced activation of p38 MAPK and myogenesis. Journal of Cell Science 120(4):692-701.

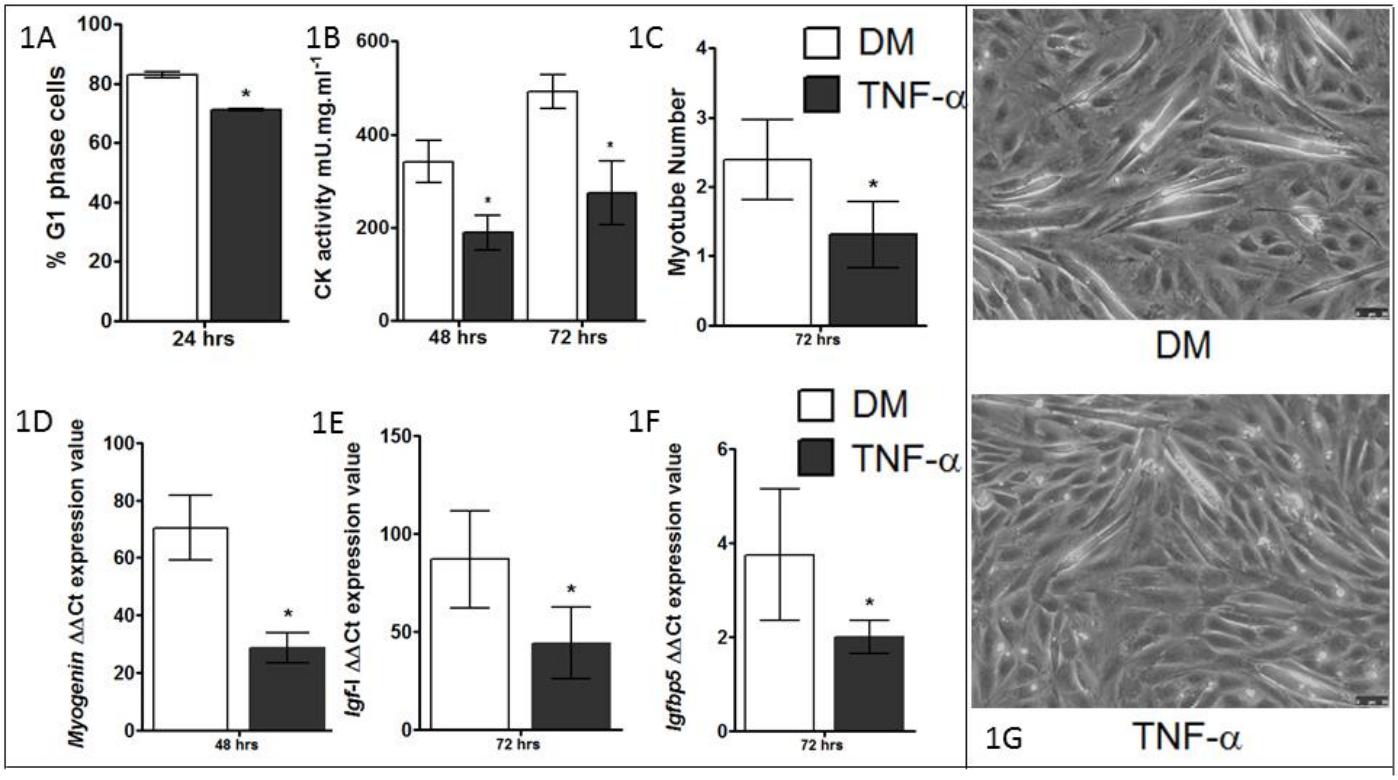

Figure 1: Three days post induction of differentiation via serum withdrawal, TNF-α (20 ng.ml^{-1}) administration reduced the proportion of cells in the G1 phase of the cell cycle at 24hrs (**1A**), a prerequisite for myoblast differentiation, as well as later (48 & 72hrs) reductions in biochemical (CK 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.

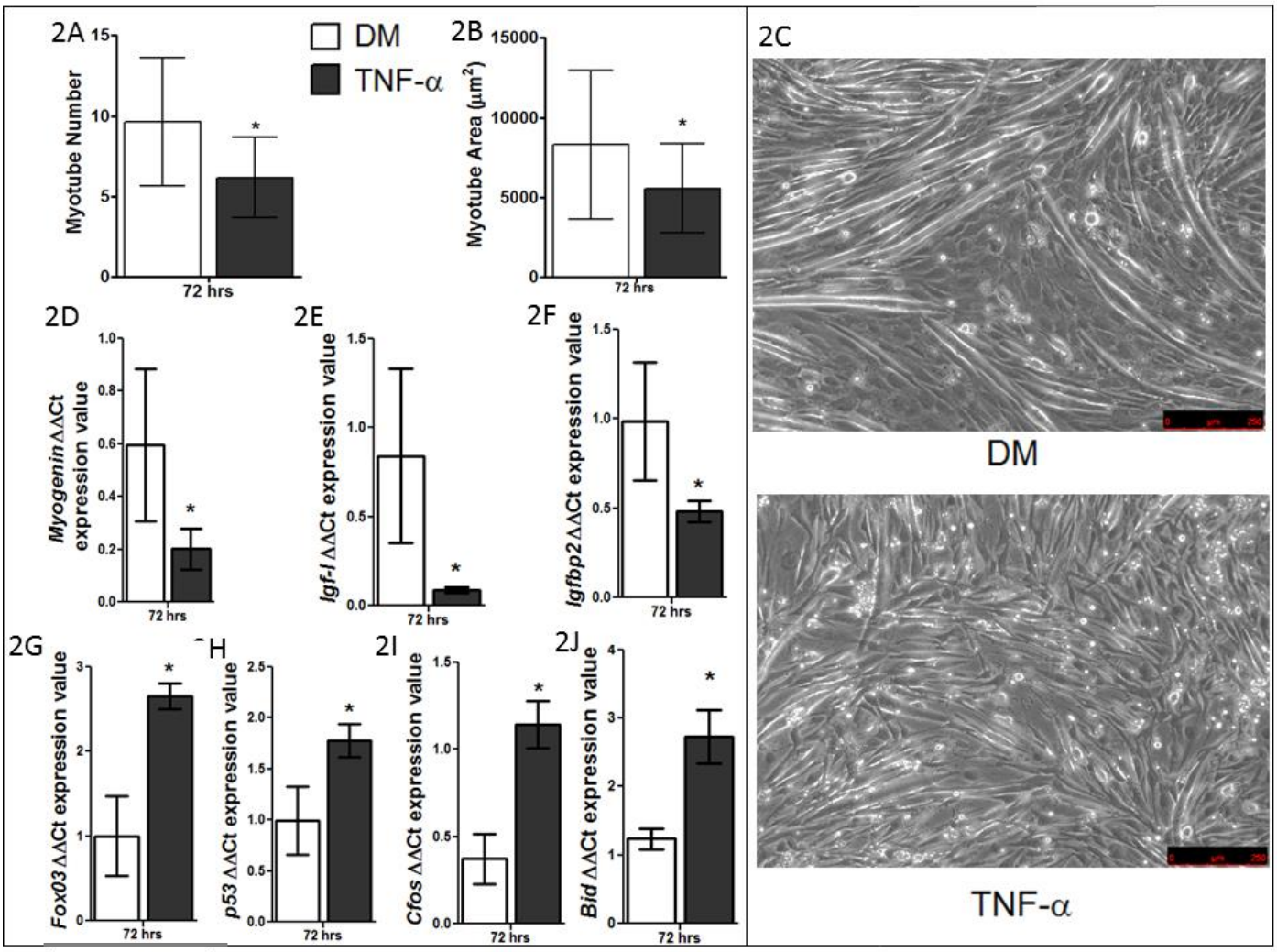

Figure 2: After 3 days of TNF-α administration to existing myotubes, TNF-α induced myotube loss and atrophy demonstrated via a loss in myotube number (**2A**) and area (**2B**). Representative morphological images (10x) of DM vs. TNF-α can been seen in figure **2C**. This was underpinned by reductions 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.

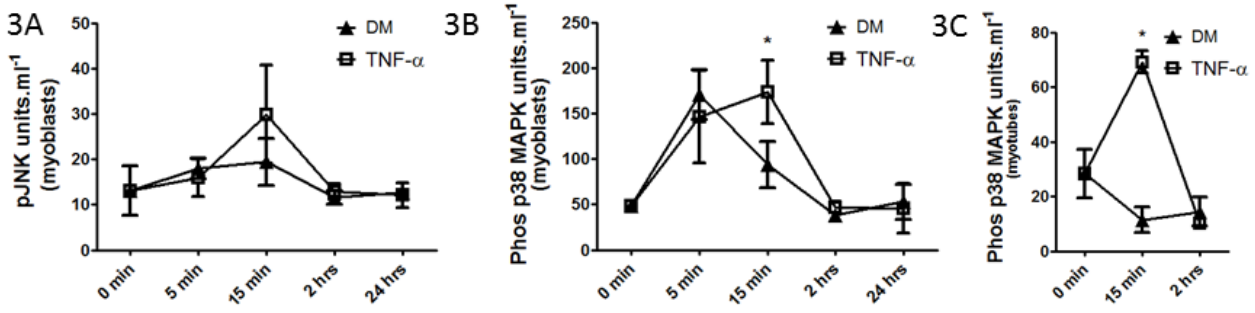

Figure 3: In order to investigate the underlying mechanisms of TNF-α's detrimental impact on myoblast/myotube morphology (observed in Figure 1 & 2), we investigated the earlier signaling responses. JNK activity was non-significant (**3A**), whereas P38 MAPK substantially and significantly increased its 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.

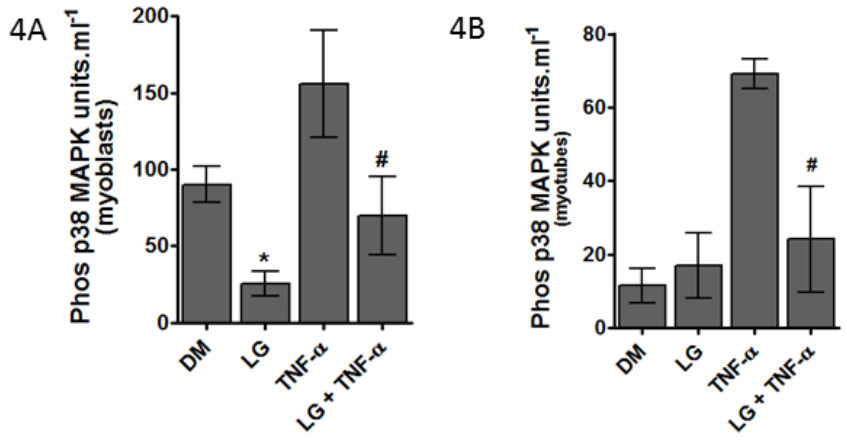

Figure 4: L-glutamine reduced p38 MAPK activity in TNF-α conditions back to control levels in both differentiating myoblasts (**4A**) and differentiated myotubes (**4B**). All experiments were conducted on 3 separate occasions (n = 3). CBA array assays were conducted in duplicate. * Significantly different to DM. # Significantly different vs. TNF-α.

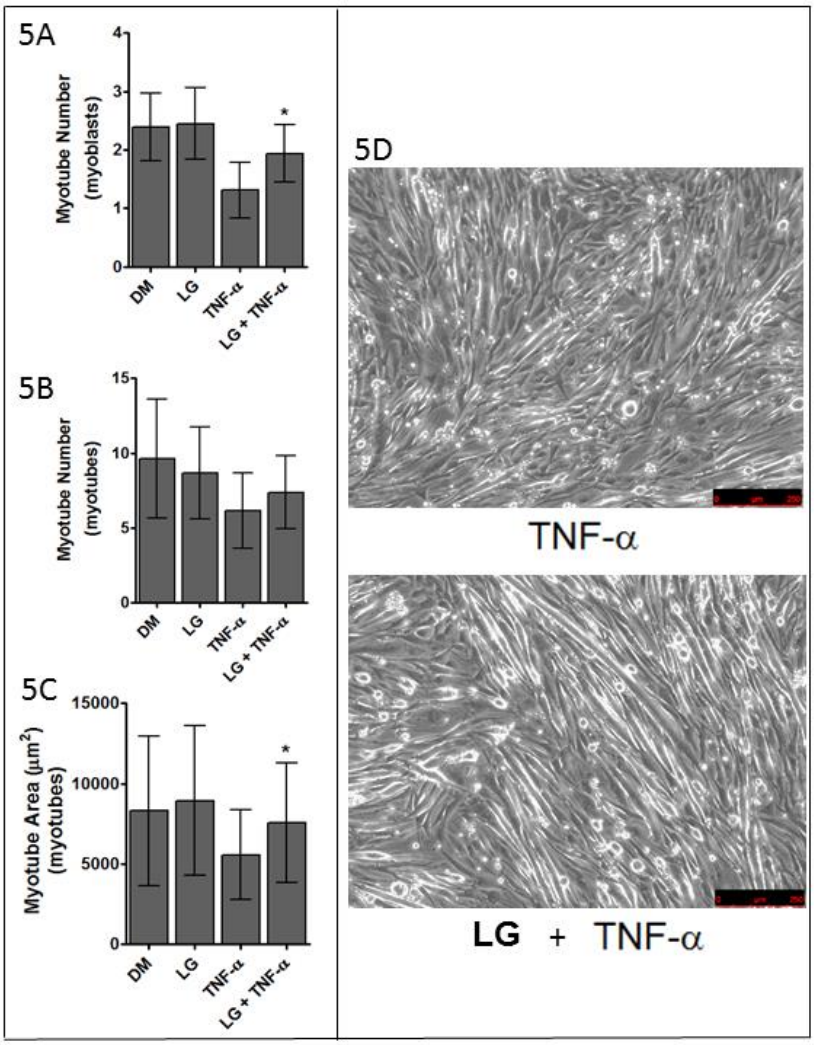

Figure 5: L-glutamine restored morphological measures of myotube formation in differentiating myoblasts (myotube number **5A**), helped somewhat retain myotube number (although non-significant) (**5B**) and prevented myotube area losses (**5C**) in myotubes following TNF-α administration. Representative 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.

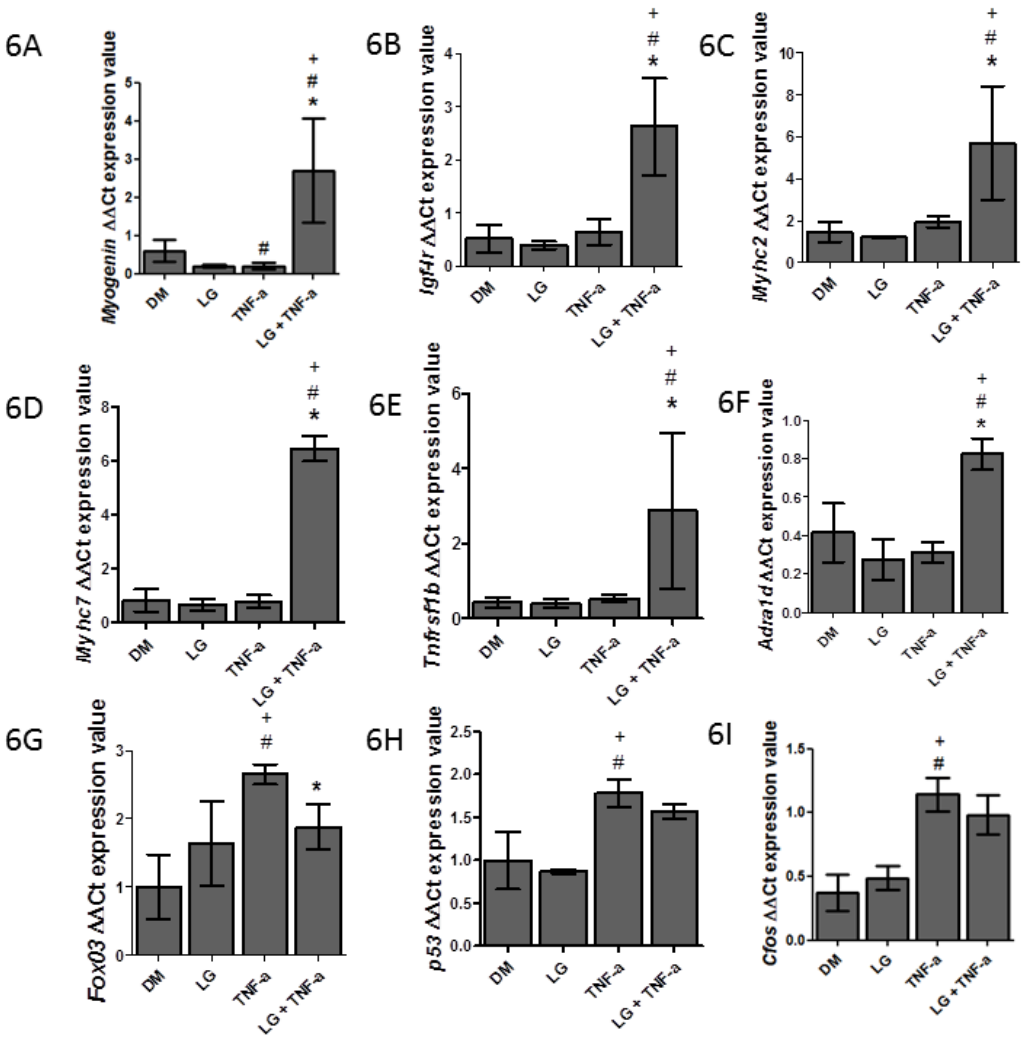

Figure 6: The signaling events (figure 4) and morphological adaptations (figure 5) in response to L-glutamine on a background of elevated TNF-α were driven by extensive upregulation of genes associated with growth and survival including; *Myogenin* (**6A**), *Igf-Ir* (**6B**), *Myhc2 (**6C**) & 7* (**6D**), *Tnfsfr1b (***6E***) Adra1d* (**6F**) and transcriptionally restoring some of the important genes associated with cell death and myotube atrophy, particularly *Foxo3* 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.