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7 L-glutamine improves skeletal muscle cell differentiation and 8 prevents myotube atrophy after cytokine (TNF-α) stress via 9 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 *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

126 baseline, albeit not completely restoring them to control levels as well as enabling normal myotube growth
127 (Bonetto et al., 2011).
128
129 Despite the reported regulation of myostatin by L-glutamine in models of atrophic conditions, there are limited
130 investigations into the intracellular signaling responses underlying the role of glutamine in the suppression of
131 muscle loss following inflammation. TNF-α has been reported to strongly up-regulate p38 mitogen-activated
132 protein kinases (p38 MAPK) demonstrated by ourselves (Grohmann et al., 2005a) and other groups (Alvarez et
133 al., 2001; Chen et al., 2007; Li et al., 2005; Palacios et al., 2010; Zhan et al., 2007). Importantly, glutamine has
134 also been shown to regulate p38 MAPK in other models, including; following exercise induced apoptosis in
135 leukocytes isolated from rats (Lagranha et al., 2007), in PBMC's derived from a rat model of sepsis (Singleton et
136 al., 2005), *in-vivo* (in the lungs of mice) and in alveolar macrophages post LPS administration *in vitro* (Ko et al.,
137 2009). Combined, this evidence suggests that p38 MAPK could play an important role in reducing skeletal
138 muscle loss on a background of TNF-α induced inflammation.
139
140 In the present study our objectives were therefore: 1) To assess the effect of glutamine in restoring impaired
141 differentiation and reducing myotube atrophy observed in conditions of TNF-α induced inflammation and to; 2)
142 investigate the underlying role of the stress related MAPKs, p38 and JNK (as well as other important associated
143 kinases ERK1/2 and Akt) and their downstream modulation of gene transcription in controlling skeletal muscle
144 differentiation and hypertrophy/atrophy in the presence of TNF-α. We hypothesised that: 1) Glutamine would
145 rescue TNF-α induced impairments in myoblast differentiation in myoblasts, reduce myotube atrophy and
146 improve myotube survival in existing myotubes and; 2) the stress related MAPK kinase family of signaling
147 proteins would be partly responsible for co-coordinating these morphological responses via transcriptionally
148 restoring genes associated with muscle differentiation, survival and myotube hypertrophy/atrophy that were
149 otherwise impaired in TNF-α conditions.
150

151 **Methods**

152

153 *Cell Culture and treatments*

154

155 C_2 and C_2C_{12} mouse skeletal myoblasts (Blau et al., 1985; Yaffe and Saxel, 1977), below passage 12, were
156 employed in these studies. Cells were seeded at 1 × 10^6 cells in gelatinized T75 flasks in growth medium (GM)
157 composed of: DMEM plus 10% hi (heat-inactivated) fetal bovine serum, 10% hi newborn calf serum, 2 mM L-
158 glutamine, and 1% penicillin-streptomycin solution and grown to approximately 80% confluency. Following
159 trypsinisation, 8 × 10^4 cells.ml^{-1} in 2 ml GM/well were plated into pre-gelatinized wells of a six well plate and
160 incubated for 24 hrs. To assess the impact on early differentiation/fusion, C2C12 myoblasts were washed twice
161 with PBS and transferred to one of four different dosing conditions: 1) Low serum media/differentiation media
162 **(DM)** alone composed of: DMEM plus 2% heat inactivated horse serum, 2 mM L –glutamine, and 1% penicillin-
163 streptomycin solution; 2) DM + TNF-α **(TNF-α)** at 20 ng.ml^{-1}; 3) DM+ L-glutamine **(LG)** at 20 mM and finally
164 4) DM + 20 ng.ml^{-1} TNF-α + 20 mM L-glutamine (**TNF-α + LG**) for up to 72hrs (TNF-α: Merck Millipore, UK).
165 To further assess the impact on established myotube cultures, C2C12 cells were cultured in DM for 7 days to
166 induce myotube formation then dosed as above (DM, TNF-α, LG and TNF-α + LG) and cultured for a further 72
167 hrs (up to a total of 10 days post low serum transfer). We have shown previously that C2C12 myoblasts are
168 resistant to early cell death following TNF-α administration, whereas parental C_2 cells are susceptible to cell
169 death in these conditions (Sharples et al., 2010). Therefore, in order to assess the impact on early myoblast cell
170 death, C_2 myoblasts were dosed with TNF-α in the absence and presence of LG for 48 hrs and dead cell
171 percentage was assessed (methods as described below). We have previously used the above doses of TNF-α to
172 study skeletal muscle apoptosis in murine C_2 cells (Foulstone et al., 2001; Saini et al., 2008; Sharples et al., 2010;
173 Stewart et al., 2004) and human cells (Foulstone et al., 2004). Similar doses have also been used to inhibit
174 muscle cell differentiation in C_2C_{12} by ourselves and others (Sharples et al., 2010; Tolosa et al., 2005). It is also
175 worth noting that for most experiments using myoblasts, 2 mM L-glutamine is already present in the
176 differentiation medium (used in DM control conditions) therefore reductions in differentiation observed with
177 TNF-α administration occur even in these lower 2 mM L-glutamine concentrations. Therefore, for the purposes
178 of these experiments **LG** conditions described above are administered at an additional bolus of 20 mM. Finally,
179 intravenous (iv.) glutamine administration (0.5 g/kg/bw over 4 hours for three consecutive days) in critically ill
180 patients has been suggested to elevate circulating levels between 10-50 mM (Berg et al., 2002; Wernerman,
181 2008), therefore the high dose of LG investigated in the present study is relevant to receiving iv. administration

182 of glutamine. C_2C_{12} myoblasts undergo spontaneous differentiation into myotubes on serum withdrawal, and do
183 not require growth factor addition to stimulate the process (Blau et al., 1985; Tollefsen et al., 1989). Time point
184 zero (DM 0hrs) was defined as 30 minutes subsequent to transferring into DM for transcript and morphological
185 assessment. For signaling studies in myoblasts/differentiating cells, time point 0 (0hrs) was defined as
186 immediately post GM removal (and washing with PBS) before dosing conditions were applied. For signaling
187 studies in differentiated myotubes, time point 0 was defined as immediately post removal of DM at 7 days (and
188 washing with PBS) before fresh dosing conditions were applied as described above. For p38 MAPK inhibitor
189 studies SB203580 (Merck Millipore, UK) was administered at concentrations of 5 and 10 μM.
190
191 *Cell extractions*
192
193 Cells were extracted for total protein assays and CK (creatine kinase) activity (a biochemical marker of myoblast
194 differentiation) at 0, 48 and 72 hrs in differentiating C2C12 cells. Briefly, cells were washed twice in PBS and
195 lysed in 200 $\mu l.well^{-1}$ of 0.05 M Tris/MES Triton lysis buffer (TMT: 50 mM Tris-MES, pH 7.8, 1% Triton X-100)
196 and assayed using commercially available BCA™ (Pierce, Rockford, IL, U.S.A) and CK activity (Catachem Inc.,
197 Connecticut, N.E, U.S.A) assay kits according to manufacturer's instructions. The enzymatic activity for CK was
198 normalised to total protein content. For rt-RT-PCR experiments cells were lysed in 250 - 300 μl/well TRIZOL
199 reagent (Invitrogen Life Technologies, Carlsbad, CA), the RNA isolated and quantified (see below). Cytometric
200 Bead Arrays (CBAs) for cell signaling analysis, cells were extracted at 0, 5 min, 15 min, 2 hrs and 24 hrs post
201 transfer into dosing conditions above. All cell culture experiments were performed on 3 separate occasions and
202 all assays were performed in duplicate (refer to figure legends for specific information on the different analyses).
203
204 *Morphological assessment: Myotube number, diameter and area*
205
206 Morphological differentiation was assessed using a cell imaging system at 10 or 20 × magnification for
207 representative images and morphological counts/analysis respectively (Leica, DMI 6000 B). Images were
208 subsequently used for quantitative measures of myotube number and area. Light microscope images were
209 imported into Image J software (Java soft-ware, National Institutes of Health, USA) for analyses. Myotube
210 numbers were counted per image and global mean ± SD was determined across all images per experimental
211 condition (a myotube was defined as containing 3^+ nuclei encapsulated within cellular structures, to avoid
212 counting of cells undergoing mitosis). Myotube area (μm^2) was determined by carefully tracing around myotube
213 structures after converting pixel length to μm using Image J software.
214
215 *Cell death*
216
217 Adherent cells were collected following trypsinisation and pooled with detached cells in the supernatant. The cell
218 suspension was prepared as a 1:1 dilution in 0.4% trypan blue stain. Samples were loaded into a Neubauer
219 haemocytometer for viable and dead cell counting.' Dead cell percentage (%) was calculated as a proportion of
220 total cell number (viable and dead).
221
222 *RNA extraction and analysis*
223
224 Extraction of RNA was performed using the TRIzol method, according to the manufacturer's instructions
225 (Invitrogen Life Technologies, Carlsbad, CA, USA). RNA concentration and quality were assessed by UV
226 spectroscopy at 260 and 280 nM using a Biotech Photometer (WPA UV1101, Biochrom, Cambridge, UK) or
227 Nanodrop spectrophotometer 2000c (Fisher, Rosklide, Denmark). 70 ng RNA was used for each RT-PCR
228 reaction.
229
230 *Primer Design*
231
232 Primer sequences (Table 1) were identified using Gene (NCBI, www.ncbi.nlm.nih.gov/gene) and designed using
233 both web-based OligoPerfectTM Designer (Invitrogen, Carlsbad, CA, USA) and Primer-BLAST (NCBI,
234 http://www.ncbi.nlm.nih.gov/tools/primer-blast). With the exception of IGF-I mature peptide mRNA primers
235 that were used in (Yang et al., 1996). Primers were purchased from Sigma (Suffolk, UK) without the requirement
236 of further purification. Sequence homology (BLAST) searches ensured specificity to ensure the primers matched
237 the sequence and therefore gene that they were designed for. Three or more GC bases in the last 5 bases at the 3'
238 end of the primer were avoided as stronger bonding of G and C bases can cause nonspecific amplification. Primer

239 sequences were designed to exclude hairpins, self-dimer and cross-dimers. All primers designed were amplified a
240 product of between 76 - 280 bp% (Table 1.).
241
242 *Reverse transcription real-time polymerase chain reaction (rt-RT-PCR) for gene expression and relative data*
243 *analysis*
244
245 Rt-RT-PCR reactions for DM vs. TNF-α conditions in C2C12 myoblast differentiation were performed using
246 Power SYBR Green RNA-to-C_T 1 step kit (Applied Biosystems, Carlsbad, CA, USA) on a Chromo4 DNA engine
247 supported by Opticon Monitor version 3.1.32, MJ Geneworks Inc., Bio-Rad Laboratories, Inc., (Hercules, CA,
248 USA). RT-PCR reactions for TNF-α ± L-glutamine comparisons in differentiated myotubes were performed
249 using QuantiFast SYBR Green RT-PCR one-step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by
250 Rotogene software (Hercules, CA, USA). Rt-RT-PCR for both studies was performed as follows: 10min, 50°C
251 (reverse transcription), 5 min 95°C (initial denaturation and reverse transcriptase inactivation), followed by 40
252 cycles of: 10secs, 95°C (denaturation), 30secs, 60°C (annealing and extension). Melt-curve analysis was used to
253 determine and exclude samples with any non-specific amplification or primer/dimer issues. All melt analysis for
254 the genes of interest produced single peaks indicating amplification of one gene product with no primer dimer
255 issues. Relative gene expression levels were calculated using the delta delta Ct ($^{\Delta\Delta}C_t$) equation (Schmittgen &
256 Livak, 2008). Polymerase (RNA) II polypeptide B (*Polr2β/Rp-IIb*) was used as the reference gene (this was
257 highly stable across all conditions with a mean ± SD C_t of 22.5 ± 0.59 (variation 2.69%) for rt-RT-PCR using
258 Chromo4 for comparisons of DM vs. TNF- α in differentiating myoblasts and with a mean ± SD C_t 16.69 ± 0.72
259 (variation 4.3%) for rt-RT-PCR on the Rotorgene 3000Q in myotube studies). To directly compare fold changes
260 between conditions, the 0 hr time point (as defined above for differentiating myoblast and myotube studies) for
261 C2C12 cells was used as the calibrator condition in the Ct ($^{\Delta\Delta}C_t$) equation.
262
263 *Flow cytometry: Cell cycle analysis*
264
265 In differentiating myoblasts at 0, 24 & 48 hrs following transfer into DM or DM + TNF-α cells were trypsinised
266 (200 µl, 0.5% trypsin/0.02% EDTA solution/well) and pooled with detached cells from the conditioned media.
267 Following centrifugation (10 mins, 300 g at 4°C), the supernatant was removed, and the pelleted cells vortexed
268 and fixed in ice cold 75% ethanol (dropwise) and stored for 24 hrs at -20°C. Cells were again centrifuged for 10
269 minutes at 300 g at 4°C. The supernatant was removed, and 2 ml PBS added to wash the cells, prior to vortexing
270 and centrifuging as above (twice). 1.5 ml of the supernatant was removed and the cell pellet vortexed prior to
271 addition of 50 µl of ribonuclease A (20 $\mu g.ml^{-1}$) and incubated at RT for 30 mins 10 µl propidium iodide labeling
272 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
273 prior to incubation in the dark at 4°C for 24 hrs before analysis using a Becton Dickinson FACSCalibur flow
274 cytometer. Data were collected using Cell Quest (Becton Dickinson, Oxford, England) and analysed using
275 Modfit™ software (Verity Software House, Topsham, ME, USA).
276
277 *Flow cytometry: Cytometric Bead Array (CBA) for quantification of phosphorylated proteins*
278
279 BD™ Cytometric Bead Array (CBA) enables simultaneous quantification of intracellular phosphorylated
280 signalling proteins (Manjavachi et al., 2010; Schubert et al., 2009). Assays were performed according to
281 manufacturer's instructions. Briefly, cells were washed and lysed on ice (1x lysis buffer provided in the Cell
282 Signaling Master Buffer Kit (BD™ Cytometric Bead Array (CBA)) prior to boiling. Protein concentrations were
283 determined as detailed above. Samples were added to the assay diluent provided (15 µg/sample). Standards were
284 prepared by serially diluting a stock of recombinant protein (50,000 Units/ml) contained in each BD CBA Cell
285 Signaling Flex Set (separate flex set for phosphorylated proteins of interest: Akt (T308), ERK1/2 (T202/Y204),
286 p38 MAPK (T180/Y182) and JNK (T183/Y185). For DM vs. TNF-α comparison for Akt, ERK, JNK and p38 four
287 phosphorylated proteins per sample were analysed simultaneously in the presence of test capture beads. For L-
288 glutamine ± TNF-α comparisons in both myoblasts and myotubes p38 MAPK was performed alone. All samples
289 were incubated with the test capture beads (3 hrs) and Phycoerythrin (PE -1 hr). The samples were washed and
290 centrifuged at 300 *g* for 5 mins. Supernatant was removed and 300 µl of fresh wash buffer was added prior to
291 resuspension and analyses on a BD FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) or a BD Accuri
292 flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). 300 events were captured per analyate per sample
293 according to manufacturer's instructions. Data were analyzed using FCAP array software (Hungary Software
294 Ltd., for BD Biosciences, San Jose, CA, USA). Although changes in total p38 MAPK protein would be unlikely to
295 change at the time points studied for p38 MAPK activity (i.e. minutes – hours), it can be confirmed that there

296 were no changes in p38 MAPK (alpha or beta) gene expression (primer details in table 1) in any of the above
297 culture dosing conditions.
298
299 *Statistical Analyses*
300
301 Statistical analyses and the significance of the data were determined using Minitab version 17. Results are
302 presented as mean ± standard deviation (SD). For initial TNF-α dosing experiments statistical significance for
303 interactions between time (varied depending on the analysis undertaken- detailed above) and dose (DM vs. TNF-
304 α) were determined using a two-way Factorial ANOVA. For cell cycle a two-way ANOVE (3 x2) with time (0, 24,
305 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-
306 α). For gene expression in differentiating myoblasts a two-way (2 x 2) factorial ANOVA for time (0, 48 hrs & 72
307 hrs) and dose (DM vs. TNF-α) was undertaken. Interactions for phosphorylated proteins in differentiating
308 myoblasts were determined for time (0, 5, 15 mins, 2, 24 hrs) and dose (DM and TNF-α) also using a two-way
309 (5×2) factorial ANOVA. Post hoc analyses (with Bonferroni correction) were conducted on data where main
310 effects for time and dose occurred, without a significant interaction between time and dose. If there were
311 significant interactions present, t-tests were conducted to confirm statistical significance between the variable of
312 interest e.g. between doses and/or time. For glutamine experiments; phosphorylated protein analysis was
313 conducted using a 2 way (2 x 4) Factorial ANOVA for time (15 mins vs. 2hrs) and dose (DM, TNF-α, LG, LG +
314 TNF-α) and post hoc (with Bonferroni correction) tests conducted for pairwise comparisons. Morphological
315 analysis for glutamine experiments (myotube number and area) were performed at either 72 hrs in
316 differentiating myoblasts or at +72 hrs (10 days total as described above) in differentiated myotubes using a one-
317 way ANOVA for dose (DM, TNF-α, LG, LG + TNF-α). Glutamine experiments for gene expression in myotube
318 cultures were analyzed using a one-way ANOVA for dose (DM, TNF-α, LG, LG + TNF-α). Fisher LSD post hoc
319 tests were conducted for pairwise comparisons following one-way ANOVA's. For all statistical analyses,
320 significance was accepted at $P \leq 0.05$.
321
322 **Results**
323
324 *TNF-α reduces early myoblast fusion and myotube formation in differentiating myoblasts*
325
326 Fusion and myotube number were reduced in early differentiating C2C12 cells in the presence of TNF-α (Figure
327 1). As G1 cell cycle exit is a prerequisite for myoblast differentiation we observed that there was a significant
328 decrease in the proportion of cells in the G1 phase at 24 hrs following the induction of differentiation following
329 serum withdrawal and TNF-α administration (CON DM 82.96 ± 1.01 vs. TNF-α 71.23 ± 0.3% at 24 hrs, P ≤
330 0.001; Figure 1A) and a relative increase in S/G2 phase (data not included), suggesting a shift from
331 differentiation to continued cellular division. Biochemical analyses of differentiation at the later time-points of
332 48 and 72 hrs following serum withdrawal subsequently confirmed the cell cycle differences, where it was
333 observed that compared with control, there were significant reductions in CK activity in TNF-α conditions (DM
334 342 ± 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;
335 both P ≤ 0.001; Figure 1B). Overall this resulted in a significant impact on cellular morphology shown by a
336 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
337 the presence of TNF-α. To confirm cell cycle, biochemical and morphological data at the molecular level we
338 investigated gene transcription of *Myogenin* (an important myogenic regulator factor) and insulin-like growth
339 factor family members (*Igf-I, Igf-Ir, Igfbp5, Igfbp2*) involved in fusion and myotube formation in differentiating
340 myoblasts. *Myogenin* decreased in myoblasts in the presence of TNF-α at 48 hrs by 2.45 fold (DM 70.4 ± 11.2 vs.
341 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
342 2 fold average reduction following TNF-α administration (DM 86.9 ± 24.7 vs. TNF-α 44 ± 18.3, P ≤ 0.001; Figure
343 1E). *Igf-Ir* was unchanged with the addition of TNF-α (data not shown). Due to reductions in *Igf-I*, IGF binding
344 proteins 2 and 5 that modulate the binding of IGFs to their receptors and regulate myoblast differentiation
345 (Foulstone et al., 2001; Meadows et al., 2000; Sharples et al., 2013) were assessed. Indeed, in the presence of
346 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 =
347 0.03); Figure 1F), there were however no significant differences in IGFBP2 expression following TNF-α exposure
348 (data not shown).

349

350

351 *TNF-α evokes a loss of myotubes and myotube atrophy in existing differentiated myotubes.*
352
353 When TNF-α was administered to existing myotubes the cytokine significantly reduced myotube survival shown
354 via reductions in myotube number (DM 9.63 ± 3.98 vs. TNF-α 6.15 ± 2.5, P ≤ 0.05; Figure 2A). Furthermore, in
355 the myotubes that did survive, compared with controls, atrophy occurred as evidenced by significant reductions
356 in myotube area in the presence of TNF-α (DM 8305 ± 4676 vs. TNF-α 5557 ± 2810 μm^2, P ≤ 0.001; Figure 2B).
357 In order to investigate the molecular regulators of these processes in differentiated myotubes we investigated the
358 transcript expression in response to TNF-α administration for a range of genes important in myotube maturation
359 and growth (*Myogenin, Myomaker, Mrf4, Myhc's 1, 2, 4 & 7, Igf-I, Igf-Ir, Igfbp2*), atrophy (*Myostatin, Musa1,*
360 *Murf-1, Mafbx, Nf- κb, Tnf-α, both Foxo1 & Foxo3*), apoptosis/survival (*Tnfrsf1a & b, Cfos, Cmyc, p53, Bid, Bad,*
361 *Bax, Adra1d, Sirt1*) and glutamine synthesis (glutamine synthetase). Seventy two hrs post TNF-α administration
362 to existing myotubes, there were significant reductions in genes associated with myotube maturation and
363 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,
364 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 =
365 0.02, Figure 2D), with corresponding reductions for *Igfbp2* (2 fold) (DM 0.98 ± 0.33 vs. TNF-α 0.48 ± 0.06, P =
366 0.007, Figure 2E). Furthermore, TNF-α significantly increased transcription of genes involved in skeletal muscle
367 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
368 well as corresponding increases in cell death/survival genes; *p53* by 1.78 fold (DM 1.0 ± 0.334 vs. TNF-α 1.78 ±
369 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
370 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
371 presence of TNF-α. Finally, glutamine synthetase reduced 2 fold in the present of TNF-α (DM 0.96 ± 0.09 vs.
372 TNF-α 0.45 ± 0.03, P = 0.002, Figure not shown). It is worth noting that we observed no significant differences
373 in the remaining genes investigated (above) including those previously associated with elevated TNF-α e.g. *Nf-*
374 *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 ±
375 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 ±
376 0.09) and *Foxo1* (DM 0.73 ± 0.24, TNF-α 0.72 ± 0.04,) all comparisons, P = N.S.

377 *Early increases in phosphorylated p38 MAPK but not JNK, AKT or ERK1/2 in the presence of TNF- α are*
378 *associated with reductions in myoblast differentiation, myotube formation and increased atrophy.*
379
380 It has previously been observed that the MAPK and PI3K pathways regulate proliferation and differentiation
381 (Coolican et al., 1997) as well as survival basally (Stewart et al., 1999) and in the presence of TNF-α (Al-Shanti et
382 al., 2008; Foulstone et al., 2001). We therefore hypothesized that these pathways were important for the
383 morphological adaptation and gene expression patterns described above. There was however, no impact of TNF-
384 α on ERK1/2 or Akt activity over 24 hours (analyses conducted at 0, 5, 15 mins, 2hrs, 24 hrs) in myoblasts (data
385 not shown). While there was a mean increase in JNK1/2 activity at 15 mins this did not reach significance (Figure
386 3A), however p38 MAPK activation over 24 hrs (analysis conducted at 0, 5, 15 mins, 2hrs, 24 hrs; Figure 3B) was
387 significantly increased at 15 mins after transferring into DM vs. DM + TNF-α (DM 93.5 ± 25.6 vs. TNF-α 173.8 ±
388 35 $units.ml^{-1}$, P = 0.014; Figure 3B). Overall suggesting early p38 MAPK activity may be important in the TNF-α
389 induced reduction in early differentiation. We subsequently confirmed that p38 MAPK increased at the same
390 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;
391 Figure 3C), indicating that p38 MAPK activity was also important in the TNF-induced myotube atrophy.

392 *L-glutamine reduced p38 MAPK activity, rescued myotube atrophy in the presence of TNF-α via reduced*
393 *atrophic (Foxo3) and increased muscle growth (Igf-Ir), myotube maturation (Myhc 2 & 7) and survival*
394 *(Tnfrsf1b and Adrad1) gene expression.*

395 In differentiating myoblasts at 15 mins, L-glutamine alone was sufficient to significantly reduce the activity of
396 p38 MAPK vs. control DM conditions (DM 90.13 ± 11.85 vs. LG 25.46 ± 7.96, P ≤ 0.05, Figure 4A), importantly
397 L-glutamine also reduced p38 MAPK activity in TNF-α conditions at this time point in differentiating myoblasts
398 (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
399 25.46 ± 7.96, P = N.S, Figure 4A). In differentiated myotubes on a background of TNF-α, L-glutamine was also
400 able to return the high activity levels observed in TNF-α conditions back to baseline DM values (LG + TNF-α
401 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 $units.ml^{-}$
402 ^{1}, P = N.S, Figure 4B). It is worth noting that p38 MAPK inhibitor (SB203580) was unable to attenuate the
403 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 μm^2, 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.*

460 **Discussion**

461 In the present study we aimed to assess the effect of L-glutamines role in restoring the impaired fusion and
462 myotube atrophy observed in the presence of TNF-α; as well as the underlying role of the MAPK's (ERK1/2, p38
463 and JNK) and associated signalling (Akt) in their downstream modulation of gene transcription involved in
464 skeletal muscle cell differentiation, myotube growth and survival. We were able to accept our original hypotheses
465 whereby we demonstrated that; 1) L-glutamine rescued myoblast differentiation and reduced myotube atrophy
466 in the presence of TNF-α, and that; 2) p38 MAPK kinase (but not JNK, ERK1/2 or Akt) was responsible for
467 coordinating these morphological responses via transcriptionally restoring some of the important genes
468 associated with survival and myotube atrophy, particularly *Foxo3a* back to baseline post TNF-α exposure, as well
469 driving extensive upregulation of *Myogenin, Igf-Ir, Myhc2 & 7, Tnfsfr1b, Adrad1* to enable normal
470 differentiation, myotube growth and survival.
471
472 The findings in the present study investigating the impact of TNF-α on skeletal muscle cell differentiation and
473 hypertrophy/atrophy correspond with previous studies, whereby after 3 days post serum withdrawal, TNF-α
474 administration reduces differentiation in myoblasts and results in myotube atrophy when administered to
475 existing myotubes (Al-Shanti et al., 2008; Foulstone et al., 2004; Foulstone et al., 2001; Foulstone et al., 2003;
476 Grohmann et al., 2005a; Grohmann et al., 2005b; Jejurikar et al., 2006; Li et al., 2005; Li et al., 2003; Li and
477 Reid, 2000; Meadows et al., 2000; Saini et al., 2008; Saini et al., 2012; Saini et al., 2010; Sharples et al., 2010;
478 Stewart et al., 2004). Importantly, we report for the first time that L-glutamine rescued differentiation in
479 myoblasts in the presence of TNF-α and confirmed previous findings for the role of L-glutamine in rescuing
480 myotube atrophy in existing myotubes in the presence of TNF-α (Karinch et al., 2001). Interestingly, Akt and
481 ERK1/2 activity were unchanged in the presence of TNF-α, although JNK activation increased on average with
482 TNF-α exposure at 15 mins this did not attain significance. These signaling studies confirmed previous findings
483 where the presence of ERK (PD98059) and JNK (SP60012) inhibitors were unable to ameliorate the negative
484 impact of TNF-α on protein degradative pathways in skeletal muscle cells (Li et al., 2005). Importantly however,
485 stress related p38 MAPK activity substantially and significantly increased after 15 mins in the presence of TNF-α
486 in both differentiating myoblasts and differentiated myotubes, a finding supported in previous studies (Alvarez
487 et al., 2001; Chen et al., 2007; Grohmann et al., 2005b; Li et al., 2005; Palacios et al., 2010; Zhan et al., 2007).
488 Importantly, we report for the first time that a bolus (20 mM) of L-glutamine reduced p38 MAPK activity in
489 TNF-α conditions in both differentiating myoblasts and differentiated myotubes; a signaling response that
490 corresponded with the ability of L-glutamine to improve fusion of myoblasts and also retain myotube area in the
491 presence of TNF-α in already differentiated myotubes. Therefore, the results from this study point to an
492 important role for L-glutamine in regulating p38 MAPK in the presence of TNF-α.
493
494 In inflammatory conditions p38 MAPK has previously been shown to be activated and involved in culminating in
495 protein degradation in skeletal muscle (Kim et al., 2009; Li et al., 2003; Li and Reid, 2000; Philip et al., 2005).
496 However, in basal or healthy conditions p38 MAPK has also been shown to have a positive role in skeletal muscle
497 cell differentiation, whereby studies adding p38 MAPK inhibitor (SB203580) to myoblasts inhibited
498 differentiation of C_2C_{12} cells (Li et al., 2000), and more recently we have reported that the MEK inhibitor
499 PD98059 (that blocks ERK1/2) enhances C_2 myoblast differentiation with corresponding increases in
500 phosphorylated p38 MAPK (Al-Shanti and Stewart, 2008). Therefore, p38 MAPK may have positive or negative
501 roles in myoblast differentiation depending upon the inflammatory context of the cells. p38-α MAPK has also
502 been linked to myoD, whereby it phosphorylates MEF2 proteins (Molkentin et al., 1995) which in turn
503 phosphorylate E47 promoting its heterodimerisation with myoD (Lluis et al., 2005), this then results in muscle
504 specific transcription such as myogenin gene expression (Keren et al., 2006). It has also been demonstrated that
505 a different p38 MAPK family member, p38-γ, also plays a crucial role in regulating skeletal muscle
506 differentiation (Gillespie et al., 2009; Lassar, 2009). In the present study, p38 MAPK phosphorylation (specific
507 isoforms not distinguished) was elevated in TNF-α treated conditions where impaired differentiation and
508 myotube atrophy were observed, whereas P38 activity was suppressed in L-glutamine conditions where
509 differentiation and myotube atrophy were restored in the presence if TNF-α. This suggests that p38 MAPK is
510 activated via stress related inflammatory mechanisms in the present study. However, it is worth noting that L-
511 glutamine drove significant 10 fold increases in myogenin gene expression even in the presence of TNF-α,
512 despite reductions in p38 MAPK, that while perhaps enabling a compensatory drive in differentiation following
513 lack of fusion at earlier time points in the differentiation program (Berkes and Tapscott, 2005), suggests that the
514 increase in myogenin expression was not regulated by the p38 MAPK/MEF2/myoD/myogenin mechanism

515 described above. Furthermore, inhibition of p38 MAPK using chemical inhibitor SB203580 in the present study
516 was unable to rescue the TNF-α induced reductions myotube morphology, this may be due to its apparent
517 bimodal functions described above as it may be difficult to recover differentiation in the presence of TNF-α
518 without affecting its role in positively regulating differentiation.
519
520 Unlike previous studies, showing that TNF-α in C2C12 myotubes led to increased myostatin protein levels, that
521 were also returned following L-glutamine administration (Karinch et al., 2001), the present study saw no change
522 in myostatin, albeit at the mRNA level, in both TNF-α alone conditions and when the cytokine was co-
523 administered with L-glutamine. Following TNF-α induced increases in p38 MAPK signaling, we did however see
524 a large increase in *Foxo3* gene expression, that at the protein level is an important transcription factor in
525 regulating muscle protein degradation via upregulation of the ubiquitin ligases (Edstrom et al., 2006; Sandri,
526 2008; Sandri et al., 2004). Despite this, we found no changes in gene expression of the ubiquitin ligases/protein
527 degradative markers; Atrogin-1 or Mafbx in the presence of TNF-α in myotubes, previously reported to be
528 transcriptionally regulated by p38 MAPK and correspond with muscle protein degradation (Kim et al., 2009; Li
529 et al., 2005); perhaps suggesting that future studies should investigate the temporal regulation of these genes to
530 fully elucidate their role. Importantly however, we were able to demonstrate novel findings that L-glutamine
531 reduced a TNF-α induced increase in *Foxo3* back to control conditions. It worth mentioning here that class I
532 Histone Deactylases (Hdac 1-3 and 8) have been associated with increases in Foxo3 and atrophy in skeletal
533 muscle during nutrient deprivation and disuse induced atrophy (Beharry et al., 2014), and therefore require
534 further investigation with TNF-α in the current model. FoxO's have also been reported to promote cell apoptosis,
535 shifting cellular function towards oxidative stress resistance/detoxification and DNA repair (Brunet et al., 2004;
536 Greer and Brunet, 2005; Wang et al., 2007). We observed average increases in survival of myotubes and a a
537 restoration of myotube size in L-glutamine conditions on a background of elevated myotube loss and atrophy,
538 post TNF-α exposure, perhaps suggesting a key role for Foxo3 in this process. Further, as TNF-α has been shown
539 to increase oxidative stress via elevating reactive oxygen species in skeletal muscle (Reid and Li, 2001), although
540 requiring confirmation, L-glutamine could also be serving to reduce Foxo3 in order to provide reactive oxygen
541 species detoxification to encourage myotube survival and growth. Another noteworthy finding is that L-
542 glutamine extensively upregulated Myhc 2 & 7 gene expression, coding for slow type I and intermediate type IIa
543 myofibrillar proteins respectively involved in laying down contractile proteins to prevent myotube atrophy.
544 Furthermore, the rescue of myotube atrophy post TNF-α exposure by L-glutamine administration substantially
545 increased the gene expression of *Myogenin, Igf-Ir, Tnfsfr1b* and *Adra1d*. As mentioned above the 10 fold
546 induced increase in *Myogenin* following L-glutamine administration suggests that the non-essential amino acid
547 may enable a compensatory drive in differentiation following for lack of fusion at earlier time points (Berkes and
548 Tapscott, 2005) despite the presence of a potent inhibitor of these processes (TNF-α). In addition to increased
549 *Myhc 2 & 7* and *myogenin*, following L-glutamine supplementation in the presence of inflammation, an increase
550 in *Igf-Ir* was observed and could therefore serve as a feedback mechanism following low *Igf-I* expression in TNF-
551 α alone conditions (that were not rescued with L-glutamine). Where an increase in *Igf-Ir* may act as a drive to
552 enable IGF-I binding to its receptor on a background of low IGF-I ligand; a process we have previously observed
553 in the presence of TNF-α in C2C12 cells, where a background of impaired *Igf-I* expression led to compensatory
554 elevations in *Igf-Ir* expression (Sharples et al., 2010). Supporting these processes further, the large increase in
555 *Tnfrsf1b* gene expression in L-glutamine conditions with exogenous TNF-α may serve to dampen the impact of
556 TNF-α. This is because this TNF receptor can be liberated from the cell surface and represents an important
557 mechanism of negative regulation for the biological activity of soluble TNF-α (Bemelmans et al., 1996a;
558 Bemelmans et al., 1996b), via inactivating and clearing TNF-α. This process of liberated vs. membrane bound
559 receptor is controlled by TNF converting enzyme (TACE). TACE would increase abundance of cleaved soluble
560 receptor in the medium and would therefore enable the increase in *Tnfrsf1b* observed in the present study post
561 L-glutamine administration to liberate and clear the excess TNF-α, warranting further investigation. Finally, we
562 have previously seen that Adra1d was elevated following incubations that induced myoblast survival (co-
563 incubations of IGF-I with TNF-α) (Saini et al., 2010). Subsequently, *Adra1d* knockdown resulted in significantly
564 higher levels of cell death under TNF-α administration suggesting *Adra1d* expression is essential for skeletal
565 muscle cell survival (Saini et al., 2010). A similar role here could be extrapolated to myotubes in the present
566 study, where with TNF-α exposure *Adra1d* is elevated to evoke myotube survival when elevated L-glutamine is
567 available.
568
569 In conclusion for the first time we indicate that L-glutamine suppressed a TNF-α induced increase in stress
570 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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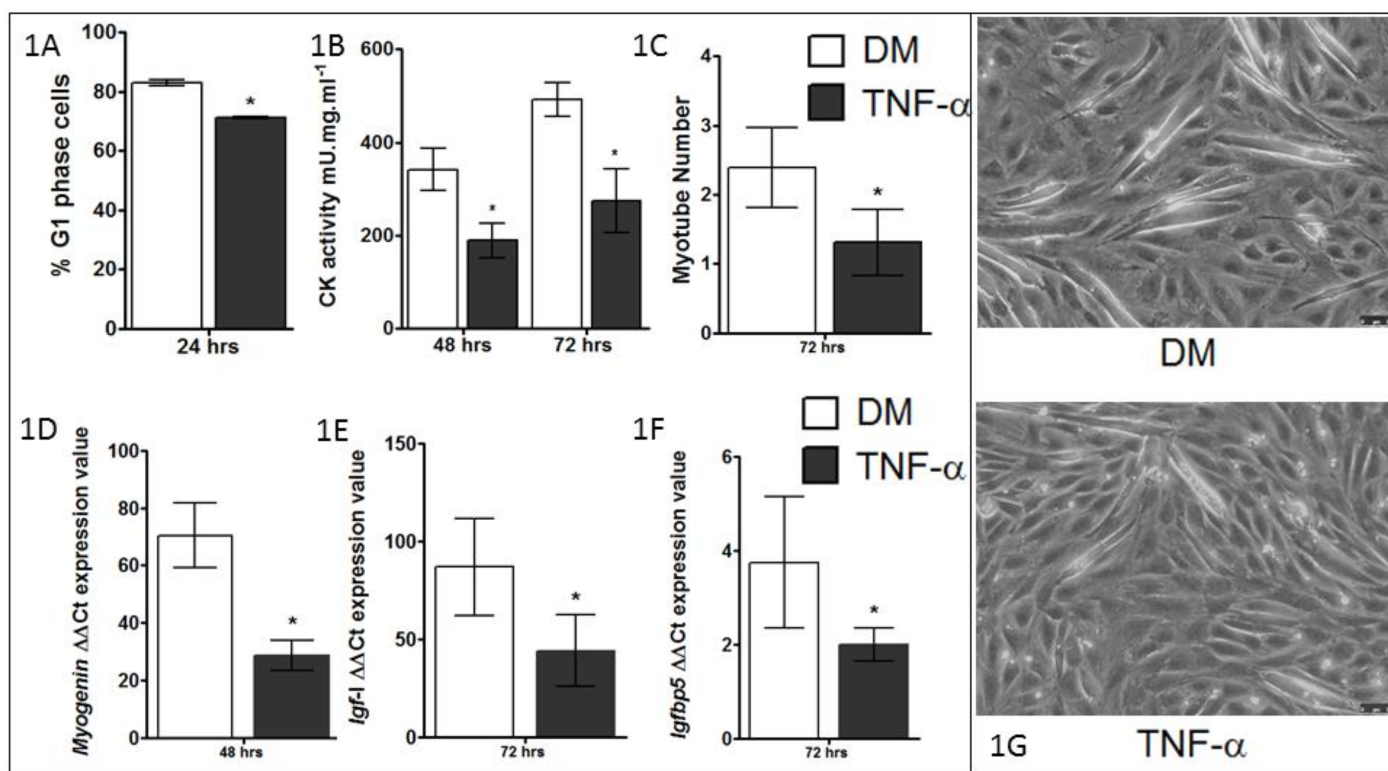

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829 **Figure 1:** Three days post induction of differentiation via serum withdrawal, TNF-α (20 $ng.ml^{-1}$)
830 administration reduced the proportion of cells in the G1 phase of the cell cycle at 24hrs (**1A**), a
831 prerequisite for myoblast differentiation, as well as later (48 & 72hrs) reductions in biochemical
832 (CK activity- **1B**) and morphological differentiation (myotube number at 72hrs- **1C**). This
833 corresponded with significant reductions in the transcript expression of important myogenic and
834 growth related genes; *Myogenin* (**1D**), *Igf-I*(**1E**) *and Igfbp5* (**1F**). Representative morphological
835 images (20X) for DM vs. TNF-α conditions can be observed in figure **1G**. All experiments were
836 conducted on 3 separate occasions (*n* = 3); 4-5 morphological images per condition in duplicate
837 were analyzed for each *n*. RT-PCR assays were performed in duplicate. * Significantly different vs.
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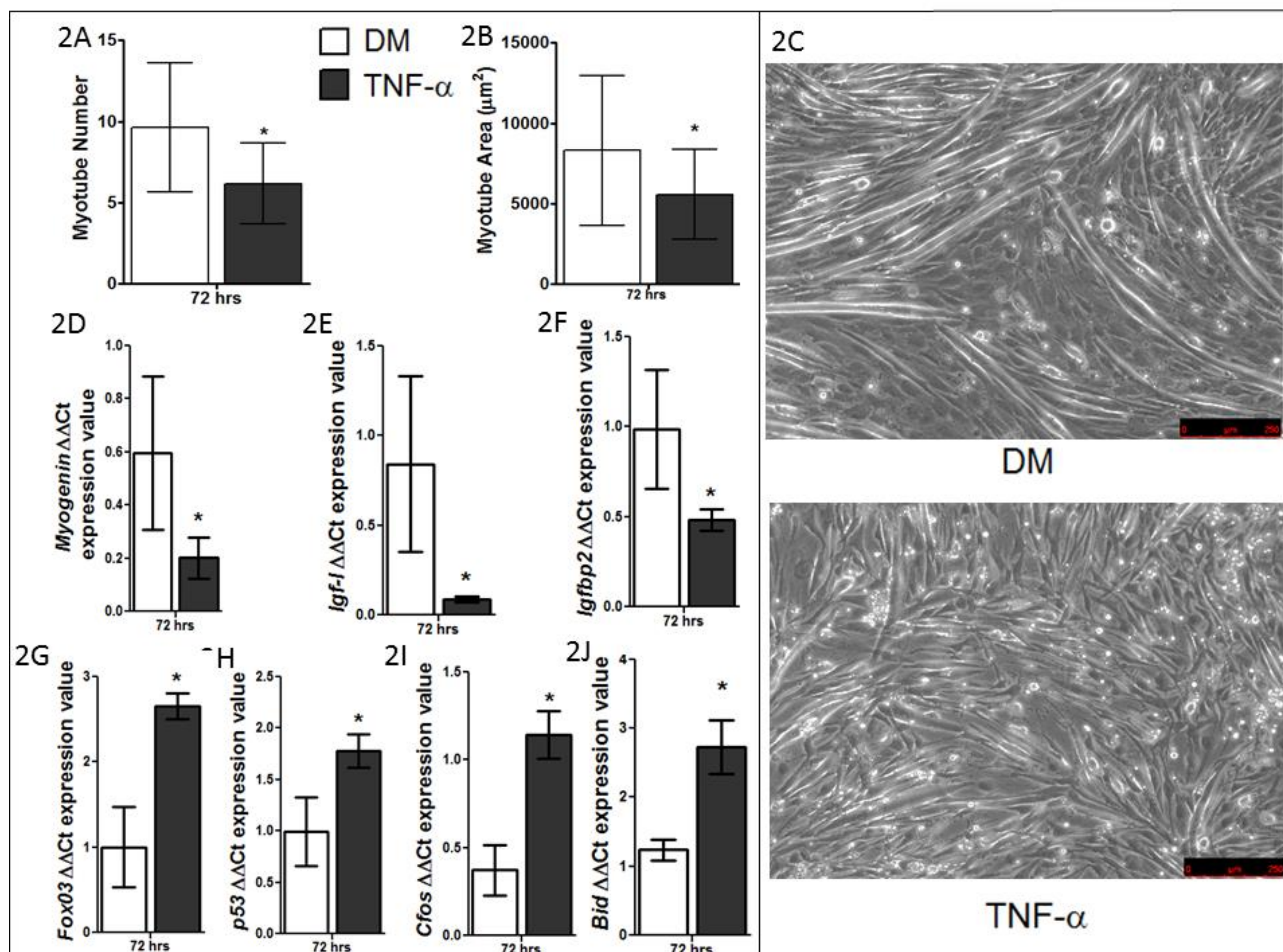

Figure 2: After 3 days of TNF-α administration to existing myotubes, TNF-α induced myotube loss and atrophy demonstrated via a loss in myotube number (**2A**) and area (**2B**). Representative morphological images (10x) of DM vs. TNF-α can been seen in figure **2C**. This was underpinned by reductions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

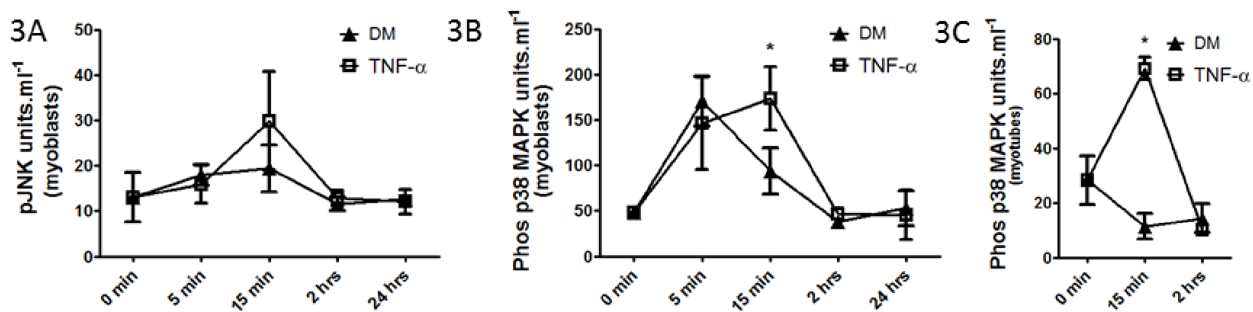

Figure 3: In order to investigate the underlying mechanisms of TNF-α's detrimental impact on myoblast/myotube morphology (observed in Figure 1 & 2), we investigated the earlier signaling responses. JNK activity was non-significant (**3A**), whereas P38 MAPK substantially and significantly increased its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

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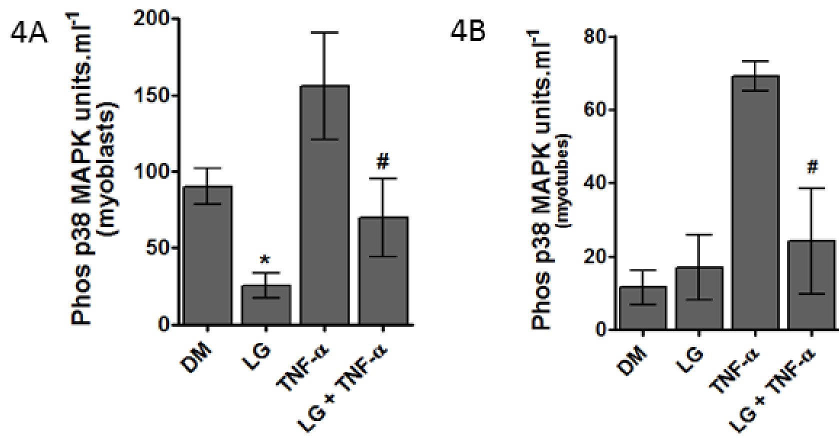

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928 **Figure 4:** L-glutamine reduced p38 MAPK activity in TNF-α conditions back to control levels in
929 both differentiating myoblasts (**4A**) and differentiated myotubes (**4B**). All experiments were
930 conducted on 3 separate occasions (*n*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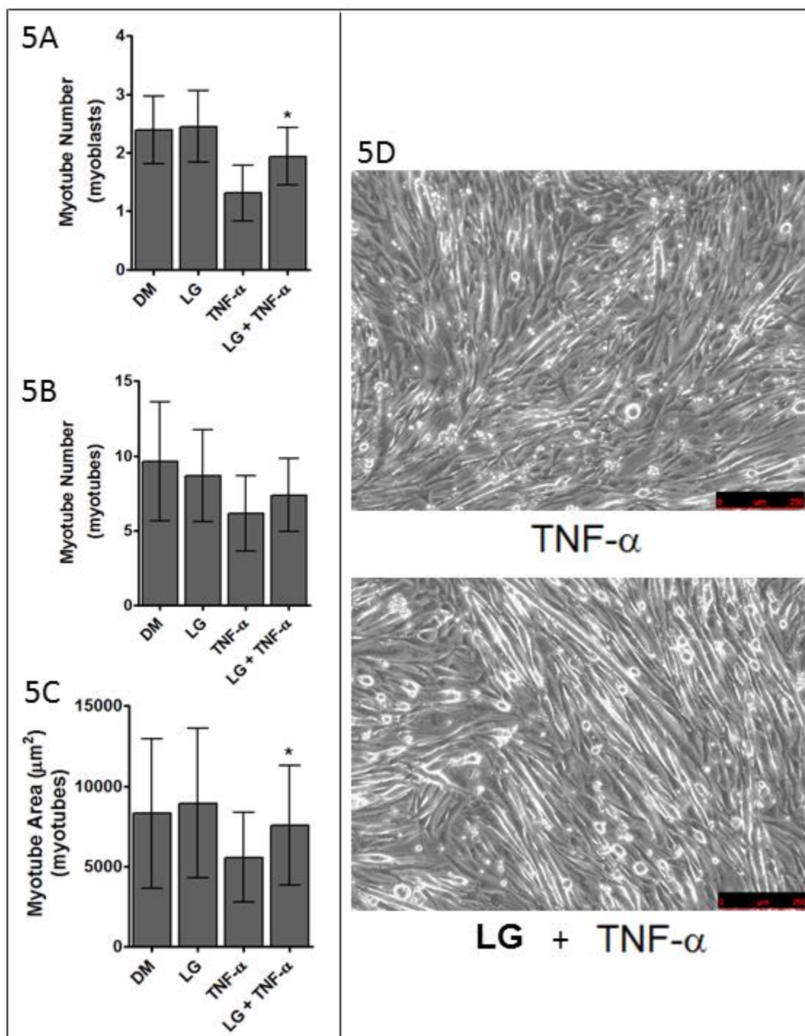

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951 **Figure 5:** L-glutamine restored morphological measures of myotube formation in differentiating
952 myoblasts (myotube number **5A**), helped somewhat retain myotube number (although non-
953 significant) (**5B**) and prevented myotube area losses (**5C**) in myotubes following TNF-α
954 administration. Representative morphological images (10X) can be seen for TNF-α vs. L-glutamine
955 (LG) + TNF-α conditions. All experiments were conducted on 3 separate occasions (n = 3); 4-5
956 morphological images per condition in duplicate were analyzed for each n. * Significantly different
957 vs. TNF-α conditions.
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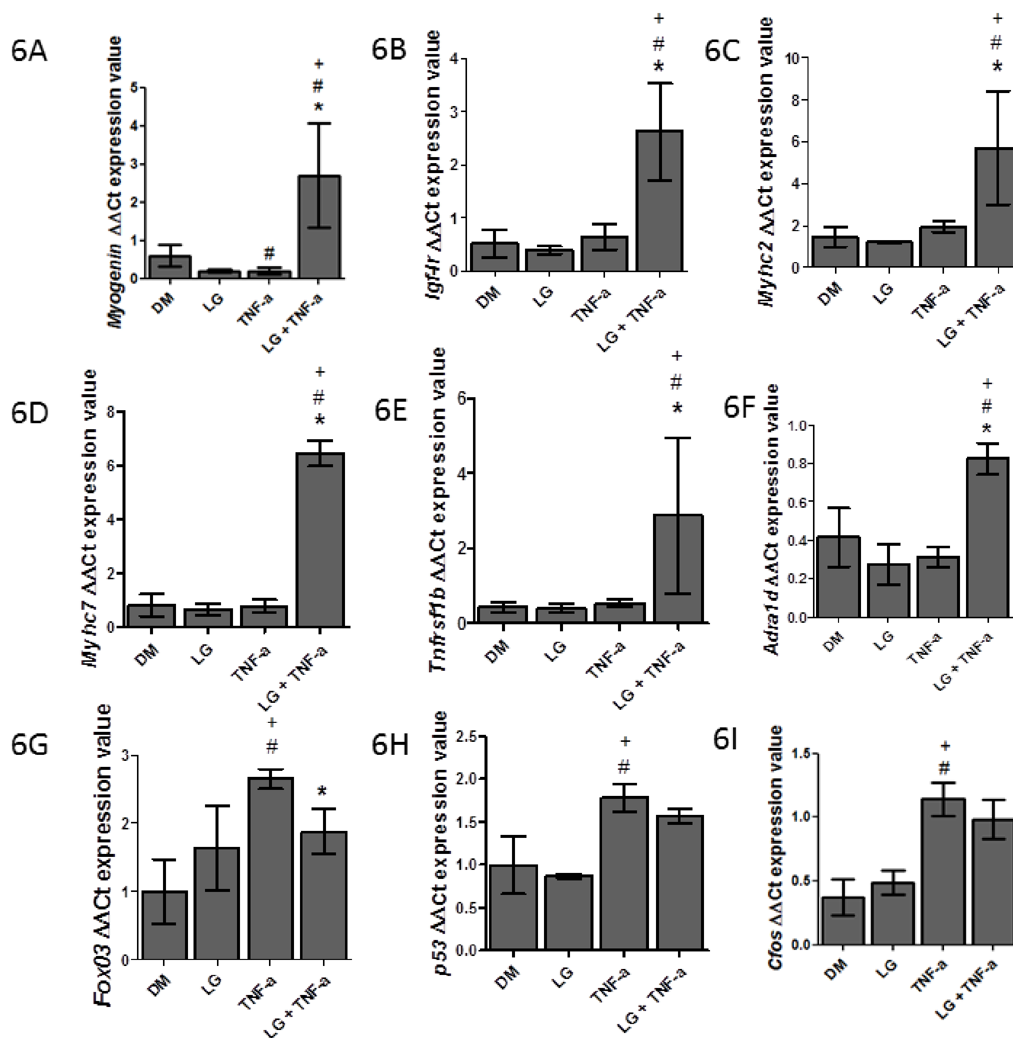

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969 **Figure 6:** The signaling events (figure 4) and morphological adaptations (figure 5) in response to
970 L-glutamine on a background of elevated TNF-α were driven by extensive upregulation of genes
971 associated with growth and survival including; *Myogenin* (**6A**), *Igf-Ir* (**6B**), *Myhc2 (**6C**) & 7* (**6D**),
972 *Tnfsfr1b* (**6E**) *Adra1d* (**6F**) and transcriptionally restoring some of the important genes associated
973 with cell death and myotube atrophy, particularly *Foxo3* back to baseline *(**6G**)* as well as average
974 reductions (yet non-significant) for *p53* (**6H**) and *Cfos* (**6I**), that were otherwise increased post
975 TNF-α administration. All experiments were conducted on 3 separate occasions (*n* = 3), RT-PCR
976 assays were performed in duplicate. *Significantly different vs. TNF-α. # Significantly different vs.
977 DM. + Significantly different vs. LG.
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