Title Page

An anti-inflammatory role for leukemia inhibitory factor receptor signaling in regenerating skeletal muscle

Liam C Hunt ^{1,2}, Aradhana Upadhyay ³, Jalal A Jazayeri ³, Elizabeth M Tudor ¹, Jason D White
*^{1,2}

¹ Faculty of Veterinary Science, University of Melbourne, Flemington road Parkville 3010

² Murdoch Childrens Research Institute, Royal Children's Hospital, Flemington Road Parkville
VIC 3052 Australia

³ Medicinal Chemistry and Drug Action, Monash Institute of Pharmaceutical Sciences, Monash
University (Parkville Campus), 381 Royal Parade, Parkville, Victoria 3052, Melbourne, Australia

*corresponding author: Liam Hunt, Muscular Dystrophy Research Group, Murdoch Childrens
Research Institute; email: liam.hunt@mcri.edu.au

Abstract

Skeletal muscle regeneration in pathology and following injury requires the coordinated actions of inflammatory cells and myogenic cells to remove damaged tissue and rebuild syncytial muscle cells respectively. Following contusion injury to muscle, the cytokine leukemia inhibitor factor (LIF) is up-regulated and knockout of *Lif* negatively impacts on morphometric parameters of muscle regeneration. Although it was speculated that LIF regulates muscle regeneration through direct effects on myogenic cells, the inflammatory effects of LIF have not been examined in regenerating skeletal muscle. Therefore the expression and function of LIF was examined using the antagonist MH35-BD during specific inflammatory and myogenic stages of notexin induced muscle regeneration in mice. LIF protein and mRNA was up-regulated in two distinct phases following intramuscular injection of notexin into tibialis anterior muscles. The first phase of LIF up-regulation coincided with increased expression of pro-inflammatory cytokines; the second phase coincided with myogenic differentiation and formation of new myotubes. Administration of the LIF receptor antagonist MH35-BD during the second phase of LIF up-regulation had no significant effects on transcript expression of genes required for myogenic differentiation or associated with inflammation; there were no significant differences in morphometric parameters of the regenerating muscle. Conversely, when MH35-BD was administered during the acute inflammatory phase, increased gene transcripts for the pro-inflammatory cytokines *Tnf* (Tumor necrosis factor), *Il1b* (Interleukin-1 β) and *Il6* (Interleukin-6) alongside an increase in the number of Ly6G positive neutrophils infiltrating the muscle was observed. This was followed by a reduction in *Myog* (Myogenin) mRNA, which is required for myogenic differentiation, and the subsequent number of myotubes formed was significantly decreased in MH35-BD treated groups compared to sham. Thus antagonism of the LIF receptor during the inflammatory phase of skeletal muscle regeneration appeared to induce an inflammatory response that inhibited subsequent myotube formation. We propose that the predominant role of LIF in skeletal muscle regeneration appears to be in regulating the inflammatory response rather than directly effecting myogenic cells.

Keywords: skeletal muscle, leukemia inhibitory factor, inflammation, myogenic differentiation, regeneration, cytokines

Introduction

Leukemia inhibitory factor (LIF) was first named for its ability to inhibit proliferation of cultured myeloid leukemia cells (Gearing et al., 1987; Hilton et al., 1988; Metcalf et al., 1988). LIF is a cytokine that signals cellular effects by binding to a heterodimer of the gp130 and LIF receptor (LIFR or alternatively gp190) subunits (Gearing et al., 1992). LIF is thus a member of the interleukin-6 (IL-6) group of cytokines that share the common receptor subunit gp130. Cultured myogenic cells are responsive to LIF and thus LIF became a protein of interest in skeletal muscle research.

When recombinant LIF was added to myoblast cultures, the number of cells increased more rapidly over time than compared to controls without LIF (Austin et al., 1992; Austin and Burgess, 1991). Whilst some studies have attributed the increased myoblast cell number induced by LIF to an increased rate of mitosis (Bower et al., 1995; Srikuea et al., 2011; Wang et al., 2008), others including us have reported that LIF does not increase the rate of myoblast mitosis under normal proliferative conditions (Alter et al., 2008; Hunt et al., 2010; White et al., 2001). LIF is capable of promoting survival and inhibiting caspase-dependent apoptosis of myoblasts, which could also be responsible for LIF induced increases in myoblasts cell number (Hunt et al., 2010; White et al., 2001).

In skeletal muscle tissue *Lif* mRNA is up-regulated in response to physical trauma (Barnard et al., 1994; Kurek et al., 1996; Srikuea et al., 2011). LIF knockout mice displayed a decrease in the size of regenerated myofibres following crush injury compared to wild-type; whilst administration of recombinant LIF to the regenerating muscle increased the size of regenerated myofibres in both wild-type and knockout compared to controls without LIF administration (Kurek et al., 1997). Systemic administration of LIF in conjunction with muscle injury however did not improve the functional regeneration of muscle (Gregorevic et al., 2000). Admittedly this study may have been limited by the systemic delivery of LIF, due the very short half-life of LIF of only 6-8 minutes in circulation (Hilton et al., 1991).

Reports on the effect of LIF administration directly to the diaphragm muscles of mdx mice have been mixed. One study observed an overall decrease in the severity of pathology

including an increase in the number of normal fibres, increased size of regenerated fibres, increased functional capacity and decreased fibrotic connective tissue with the LIF treated muscles (Austin et al., 2000). Another found that LIF treated mdx diaphragms showed increased proliferation of myogenic cells but no histological improvement or reduction in myofibre damage (White et al., 2002).

LIF increased myoblast cell number *in vitro* and this was suggested as the mechanism by which LIF improved measures of muscle regeneration following contusion injury or decreased measures of dystrophic pathology including increases in the size of regenerated myofibres. Subsequently it has been reported that LIF inhibits differentiation of myoblasts and subsequent fusion to form myotubes in culture (Alter et al., 2008; Hunt et al., 2011; Jo et al., 2005; Wang et al., 2008). When considering that regenerated myofibres are formed by the differentiation and fusion of myoblasts, the idea that LIF could inhibit differentiation yet promote increases in myofibre size appears somewhat contradictory.

Because LIF is well known as a pleiotropic cytokine, meaning it has different effects on different cell types, it is quite likely that the numerous cell types, other than myoblasts, present in regenerating muscle could be affected by LIF. For example, LIF is shown to promote the maintenance of innervation at neuromuscular junctions in neonatal mice (Kwon et al., 1995) and minimise muscle atrophy and promote re-innervation of rat muscle following transection of peripheral nerves (Tham et al., 1997). LIF is also shown to exert either pro- or anti-inflammatory actions depending on the context. An anti-inflammatory role for LIF has been described wherein knockout of LIF increased the accumulation of neutrophils in inflamed dermis (Banner et al., 1998). Pro-inflammatory roles for LIF have also been reported; inhibition of LIF by antibodies attenuated systemic inflammation induced by endotoxin and LIF may promote inflammation in association with rheumatoid arthritis (Gadient and Patterson, 1999; Jazayeri et al., 2007; Upadhyay et al., 2009).

The inflammation associated with muscle injury is necessary for the removal of damaged tissue and can also promote regeneration by interactions with myoblasts that promote myogenic differentiation (Tidball and Villalta, 2010). Acute muscle injury induced by stimuli such as

myotoxic compounds and contusion is typically associated with a rapid influx of neutrophils into the damaged muscle. Macrophages also respond to the injury and together with neutrophils can promote the lysis and phagocytosis of necrotic muscle cells. Whilst some subsets of macrophages often categorised as M1 macrophages may promote phagocytosis, others categorised as M2 macrophages are important for promoting myogenic differentiation and formation of newly regenerated myofibres.

Inflammatory cells are required for unimpaired regeneration of skeletal muscle. For example knockout of the gene *Ccl2*, which encodes monocyte chemoattractant protein-1 (MCP-1), reduced the number of macrophages in damaged muscle and impaired formation of new syncytial muscle cells following myotoxic injury (Contreras-Shannon et al., 2007; Ochoa et al., 2007; Shireman et al., 2007). However, reduction in the number of macrophages, neutrophils and other leukocytes as well as inhibition of inflammatory cytokines in *mdx* mice can improve the pathology and reduce necrosis of myofibres (Hodgetts et al., 2006; Radley and Grounds, 2006; Spencer et al., 2001; Wehling et al., 2001). Thus while inflammation may be required for regeneration in an acute time frame, the chronic and persistent nature of inflammation in dystrophic muscle can become detrimental.

Although LIF is known to regulate inflammation in numerous contexts, an inflammatory role for LIF in regenerating skeletal muscle has not yet been examined. Therefore this study examined the expression of LIF in conjunction with inflammatory and myogenic markers in regenerating mouse tibialis anterior muscles following injection of the myotoxic compound notexin. Loss of LIF function was examined by use of the synthetic LIF like protein MH35-BD, which antagonises LIFR binding.

MH35 was originally generated in a group of mouse/human LIF chimaeras intended to study the binding of both LIF and LIFR of human and mouse origin (Layton et al., 1994). Although MH35 displayed a lower affinity for the human LIFR than human LIF, this feature was utilised to easily identify potential increases in affinity that could be achieved through mutations in specific regions, two of which were termed B and D corresponding to residues 53-58 and 150-155, and hence was named MH35-BD (Fairlie et al., 2004). These mutations conveyed a higher

degree of affinity for the LIFR than wild-type LIF and two further point mutations at Q29A and G124R not only prevented MH35-BD from inducing proliferation in Ba/F3-LIFR/gp130 cell line, but could competitively inhibit wild-type LIF from inducing proliferation. The term MH35-BD is used to describe this high affinity antagonist that was generated and this protein has been subsequently used to successfully inhibit LIF *in vitro* in several cell types including inhibiting LIF induced expression of matrix proteases in articular chondrocytes (Jazayeri et al., 2007; Upadhyay et al., 2009). MH35-BD administration was targeted to the specific inflammatory and myogenic stages of muscle regeneration; In this way the role of LIF in inflammation and myogenic differentiation during skeletal muscle regeneration was explored.

Methods

Notexin injury

All animal procedures were carried out in accordance with the guidelines of the National Health and Medical Research Council of Australia and with the approval of the University of Melbourne Animal Ethics Committee (AEC). Approval was granted by the AEC under the animal ethics ID 0810901 and all procedures conducted as described within the approved ethics submission. C57BL/6 Mice were housed with 12 hour light/dark cycles and given access to food and water *ad libitum*.

Five male 10 week old C57BL/6 mice per time-point were anaesthetised with 2mg/mL Xylazine and 10mg/mL Ketamine at a volume of 10 μ L per gram of mouse mass. Once the mice were anaesthetised they received a single injection of 50 μ L of 10 μ g/mL notexin (Latoxan, Valence, France), made up in saline, longitudinally into tibialis anterior muscles with a 29G 1mL BD SafetyGlide syringe (BD, Franklin Lakes, USA). The mice were allowed to recover and at 1, 3, 5, 7, 10 and 14 days after notexin injection were asphyxiated by carbon dioxide and the tibialis anterior muscles dissected. Five mice were also sampled without notexin injection to compare to normal uninjured muscle. One tibialis anterior muscle was snap frozen in liquid nitrogen, in order to perform RNA extraction for subsequent qPCR studies, and the contralateral muscle was frozen in liquid nitrogen cooled isopentane for histology.

Real-time quantitative PCR

RNA was extracted from muscle tissue by homogenising the tissue in 1mL of Tri-reagent (Molecular Research Centre, Cincinnati, USA), following the standard protocol to retrieve the RNA containing aqueous layer. The aqueous layer was added in equal volume to a solution comprising one part 90% ethanol and one part SV lysis solution from SV Total RNA Isolation System (Promega, Madison, USA). The resulting solution was then passed through the SV columns and the columns washed with SV wash solution along with the provided DNase treatment. The RNA was eluted from the columns and 1 μ g of RNA per sample was reverse

transcribed with MML-V Reverse Transcriptase (Promega, Madison, USA) to produce cDNA for real-time quantitative PCR (qPCR).

Real-time qPCR reactions were performed in a reaction containing 0.5µL cDNA, 5µL ‘GoTaq qPCR master mix’ (Promega, Madison, USA), 4µL nuclease free water and 0.25µL of both forward and reverse oligonucleotide primers at 20µM. qPCR was performed on a LightCycler 480 (Roche Applied Science, Basel, Switzerland) and thermally regulated with the following parameters:- Pre-incubation at 50°C for five minutes followed by 95°C for five minutes and then 40 cycles of 95°C for 20 seconds for denaturing, 60°C for 30 seconds of annealing and 72°C for 30 seconds of amplicon extension. Oligonucleotide primers sequences are presented in Table 1 along with the PubMed Gene ID number for identification.

Expression data was derived from the cycle threshold (Ct) and normalised to the housekeeper hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), which was the most adequate housekeeper as it possessed the least variability across all samples compared to other housekeepers tested including peptidylprolyl isomerase A (*Ppia*), Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and β-actin (*Actb*) using the “Bestkeeper” excel based tool (Pfaffl et al., 2004). For determining the relative mRNA level by normalised expression the following formula was used:-

$$\text{Normalised expression} = (A^x)/(B^y).$$

Where A is the efficiency of amplification of the housekeeper, B is the efficiency of the gene of interest, x is the housekeeper Ct and y is the gene of interest Ct. Thus all relative mRNA levels presented in histograms are the normalised expression of the gene of interest relative to the housekeeper.

Antibodies

The following primary antibodies were used for immunohistochemical detection: goat anti-LIF clone N-18 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA), mouse monoclonal anti-desmin antibody clone DE-U-10 (Sigma-Aldrich, Castle Hill, Australia), rat

monoclonal anti-F4/80 antibody clone BM8 (Abcam, Cambridge, USA) and purified rat anti-mouse Ly6G antibody clone 1A8 (BD Pharmingen, Franklin Lakes, USA), mouse anti-embryonic heavy chain Myosin antibody (RNMy2/9D2) from Abcam, rat anti-CD206 antibody (MR5D3) and rabbit anti-CD163 (M-96) from Santa Cruz Biotechnology. The secondary antibodies used for immunohistochemical detection were fluorescent AlexaFluor conjugated antibodies (Invitrogen, Carlsbad, USA) targeting the species in which the primary antibody was produced, except for the desmin antibody, where a mouse on mouse fluorescein kit protocol was followed (Vector Laboratories, Burlingame, USA). For dual immunofluorescence if both primary antibodies were raised in the same species, for example with F4/80 and CD206 immunofluorescence, a sequential protocol was followed and the first primary antibody incubated was blocked with biotinylated anti-rat IgG. These sequential immunofluorescent protocols demonstrated no non-specific immunoreactivity when the second primary antibody was omitted; suggesting that blocking with biotinylated anti-rat IgG completely prevents unintended cross reactivity with fluorescent anti-rat secondary antibodies.

Immunohistochemistry

Frozen sections were cut transversely from the midbelly of the tibialis anterior at a thickness of 10µm and mounted on slides, which were then blocked with 5% normal serum (from the species in which the secondary antibodies were raised) and 2% bovine serum albumin in PBS for one hour at room temperature. Sections were then incubated with a 1:200 dilution of the primary antibodies in PBS overnight at 4°C. The sections were then incubated with 1:250 dilutions of the secondary antibodies in the dark for 45 minutes at room temperature. The sections were counterstained with 10µg/mL 4',6-diamidino-2-phenylindole (DAPI) in PBS for 1 minute. Sections were finally mounted with glass coverslips and visualised by fluorescence microscopy.

A negative control for LIF immunostaining was performed to demonstrate the specificity of the antibody by incubating the goat anti-LIF clone N-18 with a five fold molar excess of blocking peptide (LIF N-18 P, Santa Cruz Biotechnology, Santa Cruz, USA) prior to the primary antibody incubation step (see Supplemental Figure S4).

Production of recombinant MH35-BD

The gene encoding the LIF mutant MH35-BD was sub-cloned and expressed as a glutathione-S-transferase (GST) fusion protein in *Escherichia coli*, strain BL21 using the Overnight Express Autoinduction System (Novagen) and purified as described previously (Hunt et al., 2011; Upadhyay et al., 2009).

In vitro testing of MH35-BD LIF antagonist activity

C2C12 cultures were grown in 12-well plates and incubated with either LIF and/or MH35-BD at different concentrations in growth media (10% foetal bovine serum in DMEM) for 24 hours at which point cultures were washed with PBS and incubated with differentiation media (2% horse serum in DMEM) without the exogenous recombinant proteins for 24 hours. Cultures were then lysed in order to measure creatine kinase activity in the differentiated cultures by washing with PBS and lysing with 150µL lysis buffer containing 40mM MES buffer (2-(N-morpholino)ethanesulfonic acid), 50mM Trizma base, 1% (v/v) Triton X-100 and 1 tablet of cOmplete protease inhibitor cocktail per 50mL lysis buffer (Roche Applied Science, Castle Hill, Australia). Insoluble material in the cellular lysate was pelleted by centrifugation at 10,000g for 5 minutes and the resulting supernatant was used for analysis of creatine kinase enzymatic activity. Three technical replicates for each sample were performed by pipetting 10µL of each sample into a 96-well plate followed by 200µL of CK-NAC (Thermo Scientific, Scoresby, Australia). Creatine kinase activity was measured by the change in absorbance at 340nm over 3 minutes (20 second intervals) at 37°C. The change in absorbance was converted to activity in units per litre (U/L) by multiplication with the following factor k.

$$k = (TV \times 1000) / (A \times SV \times P)$$

Where TV is the total reaction volume (0.210mL), A is the millimolar absorption coefficient of NADH at 340nm (6.3), SV is the sample volume (0.010mL) and P is the unique path length of light in the 96-well plate (determined to be 0.533cm by spectrophotometric comparison of absorbances with known path lengths).

Administration of MH35-BD

MH35-BD was administered by intraperitoneal injection with 1mg/kg of mouse mass of the purified MH35-BD diluted in PBS to a concentration of 12.5µg/µL, so an average 25g mouse received 50µL of intraperitoneal MH35-BD. MH35-BD was administered immediately before notexin injection (day 0) or three days after notexin injection for studying the inhibition of LIF on inflammation and myogenesis respectively. As controls, sham treatment was performed with intraperitoneal injection of the MH35-BD vehicle. Mice were sampled at time-points following notexin injection that differed based on the experiment. For the inflammation study where MH35-BD was administered at day 0, mice were sampled at 1, 3, 7 and 14 days relative to notexin injection. For the myogenesis study where MH35-BD was administered at day 3, mice were sampled at 4, 7 and 14 days relative to notexin injection. Tibialis anterior muscles were removed for both RNA extraction and histology. Blood was also sampled by cardiac puncture and the blood was centrifuged at 8,000g for 5 minutes at 4°C and the serum supernatant removed in order to measure MH35-BD levels in serum following administration.

Quantitation of MH35-BD from serum

MH35-BD in serum was quantitated by enzyme linked immunosorbent assay (ELISA) using a mouse LIF immunoassay kit (R&D Systems, Minneapolis, USA). Serum samples were pre-treated to maximize recovery by addition of 50µL 2.5N acetic acid to 50µL of the serum sample and incubated for 10 minutes at room temperature. Samples were then neutralised by adding 30µL of 2.7N sodium hydroxide, 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The recommended protocol for LIF immunoassay was then followed for all samples and LIF standards. After colour generation was complete LIF/MH35-BD in the sample was quantitated from the absorbance at 450nm by interpolation of unknown samples into the LIF standard curve.

Histochemical staining and morphometric analysis

Haematoxylin and eosin staining was performed on the frozen sections with Harris haematoxylin single strength (ProSciTech, Kirwan, Australia) and 1% (w/v) Eosin-Y (ProSciTech, Kirwan, Australia). Sections were dehydrated, cleared in xylene and mounted with Entellan (ProSciTech, Kirwan, Australia) to coverslips. Toluidine blue staining of mast cell granules was performed by immersing sections in a solution containing 1% (w/v) toluidine blue

in 50% (v/v) methanol for 20 seconds. The sections were then dehydrated, cleared in xylene and mounted.

Morphometric assessment of regenerating muscle tissue was performed from haematoxylin and eosin stained sections. Measurements of muscle cell morphology were carried out using ImageJ analysis software (<http://rsb.info.nih.gov/ij/>). The cross-sectional surface area and Feret's minimum diameter (Briguet et al., 2004) of newly regenerated myotubes was calculated from the perimeter of myotubes. The percentage of cross-sectional muscle area occupied by myotubes was measured by summation of all the cross-sectional myotube areas within a field of view and expressing this as a percentage of the field of view total area. The number of myotubes per unit cross-sectional area of muscle tissue was also counted. Treatment groups for morphometric assessment were blinded so as not to bias the outcome.

Quantitation of the number of cells positive for Ly6G immunofluorescence was determined by counting the number of DAPI stained nuclei with positive staining for Ly6G total number of nuclei within several fields of view per biological replicate. The number of total, Ly6G negative and positive nuclei per unit cross sectional area was determined from the area of each field of view and the number of nuclei that were positive for Ly6G expressed as a percentage of total.

Western immunoblotting for mast cell tryptase

Protein lysates from muscle were obtained by disaggregation of muscle tissue into an SDS solution containing 100mM Tris pH 6.8, 2% (w/v) SDS, 5% (v/v) β -mercaptoethanol, 15% (v/v) glycerol and 0.1% (w/v) bromophenol blue. The samples were reduced by incubation at 95°C for 5 minutes and insoluble material pelleted. 20 μ L of protein lysate supernatant was loaded into pre-cast NuPage Novex 4-12% Bis-Tris gels (Invitrogen, Carlsbad, USA) along with the molecular marker SeeBlue Plus2 Pre-stained Standard (Invitrogen, Carlsbad, USA). The proteins were resolved and transferred onto Amersham PVDF-LFP transfer membrane (GE Healthcare Life Science, Rydalmere, Australia) and then blocked with 5% (w/v) BSA in PBS with 0.1% Tween-20 overnight at 4°C. They were then incubated with primary antibodies, rabbit anti-mast cell tryptase antibody (FL-275) sc-32889 (Santa Cruz Biotechnology, Santa Cruz,

USA) and monoclonal anti- β -actin antibody produced in mouse (Sigma-Aldrich, Castle Hill, Australia), as a loading control, overnight at 4°C. AlexaFluor-594 goat anti-rabbit IgG and AlexaFluor-488 goat anti-mouse IgG were used to detect the primary antibodies by incubation for one hour at room temperature. Membranes were then washed several times, dried and then imaged with a Typhoon 9400 (GE Healthcare) to detect AlexaFluor-488 (β -actin) and -594 fluorescence (mast cell tryptase).

Statistical analysis

Where data showed normal distribution, Graphpad Prism (Graphpad Software Inc., La Jolla, USA) was used to perform statistical tests of significance. Student's t-test was used to compare the mean and error of data where only two groups were analysed. For analysis of Ct values obtained from qPCR the pair-wise fixed reallocation randomisation test relative expression software tool (REST) was used to determine significance (Pfaffl, 2001). A 95% confidence interval was accepted where $P \leq 0.05$ was deemed significant and is denoted on histograms with an asterisk (*). All data is graphically represented as the mean $\pm$ the standard error of the mean (SEM) and sample size (n) is reported for all experiments.

Results

LIF expression following notexin injury coincides with inflammation and myogenesis

The expression of *Lif* mRNA was examined by qPCR following notexin injury and compared to expression of genes associated with proliferation, inflammation and myogenesis (Figure 1A). *Lif* mRNA was significantly increased in tibialis anterior muscles 1 day after notexin injection compared to uninjured tibialis anterior muscles (Figure 1B). Following this, transcripts for *Lif* decreased to uninjured levels by day 3, and then began to increase again starting at day 5 and continued to increase up to the final time point of 14 days. The first up-regulation of *Lif* mRNA at day 1 coincided with a sharp increase in the expression of transcripts for tumor necrosis factor- α (*Tnf*) and proliferating cell nuclear antigen (*Pcna*). The second up-regulation of *Lif* mRNA coincided with a peak of myogenin (*Myog*) expression at day 5 and followed a similar trend to myosin heavy chain expression (*Myh1*), which also progressively increased beginning at day 5 up to the final time point of 14 days.

Immunohistochemical detection of LIF protein indicated that whilst LIF immunoreactivity could not be detected in uninjured muscle, mononuclear cells at day 1 after notexin injection displayed LIF immunoreactivity, and at day 7 mononuclear cells as well as newly formed myotubes displayed LIF immunoreactivity (Figure 1C). Pre-incubation of the LIF antibody with the blocking peptide antigen demonstrated that the cellular LIF immunoreactivity was not due to non-specific reactivity of primary or secondary antibodies used (Supplemental Figure S4). The similar relative mRNA expression of LIF (expressed as a ratio of the housekeeper *Hprt*) at day 1 and day 7 corresponded to 15% and 18% of cells (including myotubes) positive for LIF immunoreactivity respectively, suggesting that on a per cell basis LIF expression is maintained at a similar level during these expression peaks but there are clearly more cells overall in the day 7 samples. This agreed with previous reports that LIF is up-regulated following muscle injury and suggested that increased LIF expression occurred in two phases. The first coincided with pro-inflammatory cytokine expression and proliferative markers. The second coincided with myogenic differentiation and the formation of myotubes.

MH35-BD prevents LIF induced inhibition of myogenic differentiation in vitro

Following observations of increased LIF expression in regenerating muscle, the efficacy of the LIF antagonist MH35-BD was tested *in vitro* in order to pursue functional inhibition of LIF during muscle regeneration. Myogenic differentiation of cultured C2C12 myoblasts was inhibited by 24 hour pre-treatment with 10ng/mL LIF prior to induction of differentiation as measured by creatine kinase activity (Figure 2A). When added in conjunction with 10ng/mL LIF, increasing concentrations of MH35-BD progressively increased creatine kinase activity and at a concentration of 10ng/mL of MH35-BD the effect of LIF decreasing creatine kinase activity was abolished. Conversely, when MH35-BD was added at a static concentration of 30ng/mL increasingly higher concentrations of LIF were required to decrease creatine kinase activity (Figure 2B). In the absence of additional exogenous LIF, increasing concentrations of MH35-BD also increased creatine kinase activity above baseline levels, suggesting that there was LIF-like activity naturally in the culture media, which agrees with our previous reports that bovine LIF may be present in the foetal bovine serum containing media (Hunt et al., 2011). The reduction in creatine kinase activity caused by LIF and restoration with the antagonist MH35-BD were paralleled with a visible reduction in the formation of myotubes with LIF and restoration to normal with MH35-BD when viewed by phase contrast microscopy (Figure 2C). Altogether this indicated that MH35-BD was acting as a competitive antagonist of LIF and could minimise LIF inhibiting myogenic differentiation at concentrations as low as one tenth that of LIF and completely abolish the effect of LIF at equal concentrations.

Administration of MH35-BD during different stages of notexin induced regeneration

MH35-BD was administered to block LIF activity during the myogenic and inflammatory phases of regeneration. We hypothesised that because MH35-BD promoted myogenic differentiation *in vitro* by antagonising LIF, that MH35-BD would promote myogenic differentiation if administered when myogenic differentiation was occurring. *Myod1* and *Myog* expression peaked following notexin injury at days 3 and 5 respectively suggesting that the peak of myogenic differentiation occurs around this time. Therefore MH35-BD was administered 3 days after intramuscular notexin injection by intraperitoneal injection of 1mg MH35-BD per

kilogram of mouse mass and sham intraperitoneal injections as controls. A schematic representation of the administration and sampling protocol is presented in Figure 3A. After MH35-BD administration, high levels of MH35-BD could be detected in serum by LIF ELISA, which was greatly elevated above endogenous LIF levels in sham controls until at least day 7 (4 days after administration; Figure 3B).

MH35-BD was also administered prior to notexin injection, so that high levels of MH35-BD could be achieved during the early inflammatory response. In this study MH35-BD was administered in the same fashion (1mg/kg intraperitoneal) except immediately prior to intramuscular notexin injection (day 0; Figure 3C). High levels of MH35-BD were present in serum following this administration until at least day 3 (Figure 3D).

Effect of MH35-BD administration on myogenic markers and inflammatory cytokines

The effects of the different MH35-BD administrations on transcripts relating to myogenesis and inflammation were examined by qPCR. Administration of MH35-BD at day 3 did not significantly alter gene transcripts for any genes that were examined associated with myogenic differentiation or genes for inflammatory cytokines compared to sham (Figure 4A). In contrast, administration of MH35-BD at day 0 significantly reduced mRNA for *Myog* at day 3 and increased transcripts for the cytokines *Tnf*, *Il1b* and *Il6* compared to sham at day 1 (Figure 4B). Although *Myod1* transcripts appeared somewhat decreased at day 3 with MH35-BD compared to control, which followed a similar pattern to *Myog*, this was not significantly different ($0.05 < P < 0.10$). Altogether this suggested that whilst administration of MH35-BD at day 3 appeared to have no significant effect on inflammation and myogenesis, administration of MH35-BD at day 0 may have induced an increased acute inflammatory response and subsequently inhibited myogenic differentiation.

Effects of MH35-BD administration on morphometric parameters of regenerating muscles

The effects of the different MH35-BD administrations on the histology of the regenerating muscle were examined. Representative images of haematoxylin and eosin stained muscle sections from mice with sham and MH35-BD administration at day 3 and day 0 are presented in Figure 5A and 5B respectively. Qualitative differences between MH35-BD treatments and shams appeared minimal; though the frequency of small centrally nucleated myotubes present at day 7 following notexin injections appeared decreased with administration of MH35-BD at day 0 compared to sham.

The quantity, diameter and surface area of centrally nucleated myotubes present in regenerating muscles at day 7 was measured to determine if these administrations had any effect on myogenesis or myotube formation. Following the observation of no changes in cytokine and myogenic gene expression with administration of MH35-BD at day 3, there were also no differences between MH35-BD and sham for all morphological parameters measured (Figure 5C). This further suggested that the administration of MH35-BD at day 3 did not affect myogenic differentiation and myotube formation.

Administration of MH35-BD at day 0 decreased the percentage of muscle cross-sectional area that was occupied by myotubes compared to sham (Figure 5D). This suggested that myogenic differentiation and fusion was inhibited such that a reduction in the size or number of regenerating myotubes was caused by MH35-BD treatment. The size of regenerating myotubes was not altered with MH35-BD treatment; mean myotube cross-sectional areas and mean diameters of myotubes were not significantly different compared to sham. However there was a reduction in the number myotubes per unit area of the whole muscle with this MH35-BD treatment.

To confirm changes in myotube appearance with this treatment, desmin and embryonic myosin heavy chain (eMHC) immunoreactivity was used. Both desmin and eMHC immunoreactivity is present in newly regenerated myotubes and can detect myogenic cells that appear differentiated yet mononucleated as well as very small myotubes that may not be detected by haematoxylin and eosin histochemistry (Figure 6A). Neither desmin nor eMHC immunoreactivity was present in the timepoints collected prior to day 7; therefore desmin

immunoreactivity was used to compare differences in smaller myotubes than possible with haemotoxylin and eosin at day 7. A reduction in the percentage of the regenerating muscles at day 7 occupied by newly regenerated myotubes with MH35-BD administration at day 0 was observed by quantitating desmin immunofluorescence. The mean percentage of whole tibialis anterior cross sections that were positive for desmin immunoreactivity and thus occupied by regenerated myotubes was significantly reduced with MH35-BD treatment compared to sham (Figure 6B). The percentage of whole muscle occupied by desmin was concordant with the percentage of muscle occupied by myotubes with haemotoxylin and eosin validating both measures indicative of reduced myogenic fusion. Although the distribution of cross-sectional areas of myotubes determined by haemotoxylin and eosin appeared shifted slightly toward the left, suggesting increased number of smaller myotubes with MH35-BD, the smaller myogenic cells that could be detected by desmin showed no difference in distribution (Figure 6C&D). Together this confirmed at least a reduction in the quantity of myotubes formed and a possible reduction in the size of myotubes formed in the MH35-BD treated group suggesting that myoblast fusion was impaired.

Effect of MH35-BD administration on macrophage markers

Specific macrophage markers were analysed to determine if the inflammatory changes induced by administration of MH35-BD at day 0 were associated with changes in the number of macrophages present in the regenerating muscle. The mean percentage of whole tibialis anterior cross sections that were positive for the macrophage marker F4/80 were not significantly different between MH35-BD and sham groups at both days 3 and 7 (Figure 7A&B), suggesting that macrophages may not have been affected by this MH35-BD administration.

The effect of this MH35-BD administration at day 0 was examined further by qPCR for transcripts relating to macrophages and the different macrophage phenotypes (Figure 8). Transcripts for *Emr1* and *Ccl2*, which encode F4/80 and the monocyte chemoattractant protein-1 (MCP-1) respectively, were not significantly different between MH35-BD and sham groups; agreeing with earlier results suggesting there were no changes in overall macrophage numbers. *Cd86* and *Cd163* transcripts, which are antigens present on classically activated M1 and

alternatively activated M2 phenotype macrophages respectively, were also not significantly altered by MH35-BD administration.

Transforming growth factor- β (TGF- β) and interleukin-10 (IL-10) are factors that have been associated with M2 phenotype of macrophages and also associated with myogenic differentiation. TGF- β can be produced by macrophages and can prevent mitogen induced inhibition myogenic differentiation (Lefaucheur and Seville, 1995; Zentella and Massague, 1992). Gene transcripts for *Tgfb1* were not significantly different with MH35-BD treatment compared to sham, suggesting this was not associated with inhibition of myogenic differentiation caused by MH35-BD. IL-10 is known to cause deactivation of classically activated macrophages (polarisation towards M2 phenotype) by reducing cytolytic activities and inflammatory cytokine production (Gordon, 2003). Coincident with the increased expression of inflammatory cytokines *Tnf*, *Il1b* and *Il6*, there was also a significant increase in gene transcripts for the anti-inflammatory cytokine *Il10* at day 1 with MH35-BD treatment compared to sham. This altered cytokine expression profile caused by MH35-BD might be expected to alter polarisation of macrophages towards the specific macrophage phenotypes.

Coincident with no changes in transcriptional markers specific to the macrophage phenotypes there was also no alteration in either the absolute number of macrophages per cross sectional area or the proportional number of macrophages positive for phenotype specific antigens. Dual immunofluorescence for F4/80 and CD206; F4/80 and CD163; and CD206 and CD163 were performed. Whilst F4/80 is present on all monocytes/macrophages, CD206 and CD163 are unique to M2 macrophages and CD163 in some instances is only found on the IL-10 polarised M2c macrophages (Villalta et al., 2011). The number of macrophages were counted with F4/80⁺/CD206⁻, F4/80⁺/CD206⁺, F4/80⁺/CD163⁺ cells indicating M1, M2 and M2c macrophages respectively with CD206⁺/CD163⁻ and CD206⁺/CD163⁺ reflecting the proportion of M2a to M2c macrophages. No significant differences could be detected with this MH35-BD treatment at either day 3 (Figure 8A) or day 7 (Figure 8B) after notexin injection. Thus there appeared to be no difference in macrophage numbers in general or the number of macrophages with polarisation specific phenotypic markers following this MH35-BD administration.

Effect of MH35-BD administration on mast cell markers

Mast cells are also associated with pro-inflammatory cytokine production and skeletal muscle regeneration (Helliwell et al., 1990). CD117 or the stem cell factor receptor is a marker commonly used for detection of mast cells. Gene transcripts for *Kit*, which encodes CD117, were significantly increased at day 1 with MH35-BD administration at day 0, suggesting a possible increase in the number of mast cells (Figure 9A). Histochemical toluidine blue staining to detect metachromatically purple stained mast cell granules indicated that a small number of mast cells with cytoplasmic granules were present in the perimysium in uninjured muscle (Figure 9B). However at 1 and 3 days following notexin injection, no metachromatic purple staining could be observed, suggesting a loss of cytoplasmic mast cell granules, possibly via degranulation. Thus toluidine blue staining could not be used to assess any differences between MH35-BD and sham treatment groups with notexin injury. Mast cell tryptase (MCT) is a protease produced and secreted by mast cells (Kovanen et al., 1995) and was examined by western immunoblotting of protein lysates from uninjured and MH35-BD and sham treated mouse muscles. MCT expression increased at days 1 and 3 following notexin injection compared to uninjured, suggesting that mast cell numbers may have increased in response to injury, but there was no difference in MCT expression between MH35-BD and sham treatments (figure 9C). This suggested that changes in *Kit* expression may not have been particularly indicative of mast cell numbers and that mast cell numbers were not likely altered with MH35-BD administration.

Effect of MH35-BD administration on neutrophil markers

The effect of MH35-BD administration at day 0 on neutrophils was examined by qPCR for neutrophil associated genes and Ly6G immunofluorescence. The gene *Ly6g* encodes the Ly6G antigen, which is present on predominantly neutrophils and granulocytes. Transcripts for *Ly6g* were significantly increased 1 and 3 days after notexin injection with MH35-BD treatment compared to sham. In addition *Cxcl2* transcripts, which encode macrophage inflammatory protein-2 (MIP-2) a protein produced by macrophages and a chemoattractant for neutrophils, were significantly increased with MH35-BD treatment (Figure 10A). Together this suggested that neutrophil numbers may be increased with MH35-BD administration through increased production of neutrophil chemoattractants.

Immunofluorescent staining of neutrophils in the MH35-BD and sham regenerating muscles with an Ly6G antibody revealed a clear qualitative increase in the number of neutrophils present at day 1 after notexin with MH35-BD compared to sham (Figure 10B). Quantitatively, there was a significant increase the number of nuclei positive for Ly6G per unit area of the muscle with MH35-BD treatment (Figure 10C). This corresponded to an increase from 22% to 43% of total nuclei within the muscle at day 1, which were Ly6G positive neutrophils, with MH35-BD treatment. However whilst there was an absolute increase in Ly6G positive nuclei per unit area in MH35-BD treated muscles, the number of total nuclei per unit area of muscle did not increase. Associated with the increase in Ly6G positive nuclei there was a decrease in the number of nuclei per unit area that were Ly6G negative with MH35-BD treatment. Therefore, there may have been a decrease in the number of an unidentified cell type with MH35-BD treatment.

Although all mice received an equal dose of 1mg/kg of MH35-BD, there were variations in serum MH35-BD concentrations at day 1 following administration at day 0, likely due to the pharmacokinetics for each individual mouse. This allowed for comparisons between the MH35-BD present in serum and number of neutrophils present in the muscle at day 1. There was a significant positive linear correlation ($r^2=0.89$, $P=0.0168$) between MH35-BD concentration in serum and the absolute number of neutrophils present per unit area in the muscle. This further suggested that MH35-BD was responsible for increased neutrophils in the muscle.

Discussion

Results obtained from these studies provide important insights into the expression and function of LIF during skeletal muscle regeneration. Studies into *Lif* mRNA and protein expression following notexin induced injury demonstrated that LIF was up-regulated in two distinct phases during the period of regeneration. The first up-regulation of LIF was at day 1 after notexin injection and coincided with an acute inflammatory response phase, during which increased pro-inflammatory cytokine expression was observed. The second up-regulation of LIF was from 5-14 days after notexin injection and coincided with the myogenic phase, during which increased *Myog* expression and formation of myotubes was observed.

Administration of a competitive antagonist of LIF, MH35-BD, to mice at day 3 achieved high levels of MH35-BD in circulation through days 4-7 after notexin injection. This administration had no major effect on transcriptional and histological measurements of inflammation or myogenesis. Administration of MH35-BD at day 0 achieved high levels of MH35-BD in circulation through days 1-3 after notexin injection. In contrast to the administration at day 3, administration of MH35-BD at day 0 did have a significant effect on measurements of inflammation and myogenesis.

Expression of LIF during notexin induced regeneration is associated with inflammation

Previous studies in regenerating crush injured muscle found that *Lif* mRNA was up-regulated within as little as 3 hours after injury and persisted for up to 4 days (Barnard et al., 1994; Kurek et al., 1996). Similarly in a more recent study, *Lif* mRNA was shown to be up-regulated rapidly and transiently, from 3-12 hours post injury, in rat gastrocnemius muscles following contusion injury (Srikuea et al., 2011). In the present study, *Lif* mRNA appeared to be up-regulated in two separate phases following notexin injury. The first phase was closely associated with increased pro-inflammatory cytokine expression and the initial influx of inflammatory cells. The second appeared to coincide with myogenic differentiation (up-regulation of *Myog*) and the formation of myotubes and expression of *Myh1*. This was novel in that other studies examining *Lif* mRNA expression following muscle injury typically only observed an up-regulation during the early

stages of regeneration coinciding with inflammation. This expression profile was also observed when examining *Lif* mRNA expression from notexin injured muscle collected from the MH35-BD experiments (Supplemental Figure S5). Whether or not the two phases of *Lif* mRNA up-regulation observed herein is unique to the notexin model of muscle injury utilised herein however is difficult to know. LIF protein immunoreactivity was detected within the mononucleated cells present during the inflammatory phase and also in mononucleated cells and myotubes present during the myogenic phase of regeneration, indicating the increased mRNA levels at these times compared to uninjured, coincided with a visible increase in protein immunoreactivity.

In both inhibitor studies MH35-BD could be detected in serum greatly in excess of endogenous LIF with some serum concentrations as high as 2ng/mL. However, endogenous LIF in serum did increase following notexin injury. Whilst an average of 14pg/mL could be detected in serum from uninjured mice, a significantly increased average of 40pg/mL could be detected 1 day after notexin injections, which subsequently decreased to uninjured levels as the muscle regenerated (see Supplemental Figure S1). This might suggest that LIF is produced systemically in response to muscle injury or that locally produced LIF in injured muscle may enter the circulation.

It is plausible that this systemic increase in LIF could play a role in the muscle inflammation following notexin injury. LIF is known to mediate proopiomelanocortin mRNA expression and adrenocorticotrophic hormone (ACTH) secretion on the hypothalamic pituitary axis (Akita et al., 1996; Ray et al., 1996). Through release of hormones such as ACTH, LIF could potentially increase systemic cortisol release (Bamberger et al., 2000; Woods et al., 2008), which via glucocorticoid receptors could inhibit the inflammation locally within injured muscle. Thus LIF could be acting systemically to regulate and minimise an inflammatory response to muscle injury.

Given that administration of the LIF antagonist MH35-BD during the first inflammatory phase of LIF up-regulation produced a pro-inflammatory effect, it would suggest that this up-regulation of LIF relates directly to an anti-inflammatory role for LIF in this context.

Administration of MH35-BD during the second or myogenic phase of LIF up-regulation had no effect on inflammatory, myogenic or morphometric parameters measured, which suggests that the up-regulation of LIF at this time does not relate to inflammation or myogenesis.

Administration of MH35-BD during stages of myogenic differentiation did not affect muscle regeneration

Administration of MH35-BD during the myogenic differentiation phase of regeneration did not promote myogenic differentiation as was hypothesised. Administration of MH35-BD at this time yielded approximately half the concentration of MH35-BD in serum compared to when administered at day 0, which was likely due to pharmacokinetic variations between individual mice limiting the distribution of MH35-BD. However, it is unlikely that this difference in serum MH35-BD accounts for a lack of effect for the following reasons. On average, the serum levels of MH35-BD with administration during the myogenic phase reached half that of administration during the inflammatory phase, which did cause an affect. When testing MH35-BD activity *in vitro*, the same concentration of MH35-BD as LIF (10ng/mL of both) could completely block the effect of LIF, meaning doses of MH35-BD would not have to be particularly high in comparison to endogenous LIF in order to inhibit LIF. The levels of MH35-BD that were achieved in serum were on average 15 fold in excess of endogenous LIF. Assuming similar distribution of the proteins in the tibialis anterior muscle tissue, it seems highly likely that sufficient MH35-BD was present to inhibit LIF activity in the muscle, but that this simply had no effect on muscle regeneration

Indeed this MH35-BD administration was capable of inducing an effect though not on muscle regeneration, suggesting that the administration was efficacious but simply did not alter muscle regeneration. Gene expression of lymphocyte markers was investigated to examine a potential role for lymphocytes in acute muscle injury, inflammation and regeneration and the effects of MH35-BD on lymphocyte markers. Transcript expression of the T cell markers *Cd4* and *Cd8a* was particularly low in uninjured muscle and during early regeneration, but increased during the later stages of regeneration around 7-14 days after notexin injury (see Supplemental Figure S2). Similarly, increased expression of interferon- γ , *Ifng*, and a B cell marker *Cd19* occurred around this time. Thus lymphocyte markers did not appear to correspond to an acute

inflammatory response but rather to the later stages of regeneration. Administration of MH35-BD at day 0 had no significant effects on transcript expression of the lymphocyte markers, however administration of MH35-BD at day 3 did. *Cd8a* expression was significantly decreased with this MH35-BD treatment at days 4 and 7 after notexin injection, providing evidence that this administration of MH35-BD did allow distribution to the injured tibialis anterior muscle and was efficacious. It also suggested that the MH35-BD treatment may have modulated the number of CD8⁺ cytotoxic T cells in the regenerating muscle at this time. Thus the study of lymphocyte markers provided evidence that the MH35-BD administration at day 3, for which no other effects were observed, did affect the muscle but not in the ways hypothesised. It also suggests lymphocytes may be involved in later stages of skeletal muscle injury and regeneration and LIF may play a role modulating populations of cytotoxic T cells, however this does not appear to affect the formation and regeneration of syncytial muscle cells.

Administration of MH35-BD during inflammatory stages affected inflammation and subsequent muscle regeneration

Administration of MH35-BD during the early inflammatory phase of regeneration had a multitude of effects that culminated in decreased myogenic differentiation and syncytial muscle cell formation. At the earliest stages 1 day after notexin injection, MH35-BD treatment increased expression of the pro-inflammatory cytokines *Tnf*, *Il1b* and *Il6*, which coincided with increased expression of markers for neutrophils and increased numbers of neutrophils. This was followed by decreased *Myog* expression 3 days after notexin injection and fewer syncytial muscle cells present 7 days after notexin injection.

This temporal order of events suggests that the primary effect of MH35-BD administration is disturbance of the inflammatory balance, which leads to a delay in myogenic differentiation. LIF has been shown to inhibit production of the pro-inflammatory cytokine TNF- α by isolated peritoneal macrophages *in vitro* (Hendriks et al., 2008). Because antagonism of LIF with MH35-BD increased *Tnf* expression, this suggested that LIF may minimise TNF- α production by macrophages in regenerating muscle. It is also possible that increased expression of *Tnf* and the other pro-inflammatory cytokines may be associated with an increase in neutrophils present in the muscle at this same time.

Neutrophils present in muscle following damage are known sources of the pro-inflammatory cytokines, including IL-1 β (Fielding et al., 1993). The cause of increased neutrophils with MH35-BD treatment may be due to increased expression of *Cxcl2*, which encodes MIP-2 a protein produced by macrophages that is a chemoattractant for neutrophils (Iida and Grotendorst, 1990; Pelus and Fukuda, 2006; Wolpe et al., 1989). Increased *Tnf* expression with MH35-BD treatment may also be responsible for increased neutrophil numbers, as direct administration of recombinant TNF- α into muscle can cause accumulation of neutrophils (Peterson et al., 2006). A decrease in *Pcna* expression was also observed coinciding with increased neutrophil numbers at day 1, and might represent an overall increase in the proportion of non-proliferative cells, such as neutrophils, in the muscle (see Supplemental Figure S3). It has been reported that knock-out of the *Lif* gene leads to increased neutrophil numbers present in inflamed dermis (Banner et al., 1998), supporting the possibility of LIF modulating neutrophil numbers. During early pregnancy in mice, loss of *Lif* is associated with an inhibition of Ly6G⁺ neutrophils decreasing over time, and it was suggested that this could be due to LIF promoting neutrophil apoptosis (Schofield and Kimber, 2005). Because of the pleiotropic nature of LIF, it can potentially display both anti-inflammatory and pro-inflammatory properties, depending on the context such as tissue or cell system being studied. Whether LIF affects skeletal muscle inflammation in any way has not been studied until now. These studies suggest that LIF may be crucial as a negative feedback mechanism preventing an overwhelming inflammatory response, which was produced here by inhibiting LIFR signaling with MH35-BD. Therefore in this context LIF may be anti-inflammatory.

Because there were changes with MH35-BD administration in expression of cytokines known to affect polarisation of macrophages and due to past reports that LIF itself can skew macrophages towards an M2 like phenotype (Duluc et al., 2007), alterations in macrophage phenotypes were expected. However no such changes could be detected as there was no significant change in the number of macrophages expressing markers of alternative activation. This suggests that although LIF may be able to alter the expression levels of cytokines potentially expressed by macrophages, the MH35-BD administration did not alter macrophage polarisation in this context.

Mast cells present in inflamed muscle may also be a source of pro-inflammatory cytokines (Gorospe et al., 1994; Helliwell et al., 1990). Although changes in *Kit* gene expression, which is associated with mast cell function (Galli et al., 1993; Moritz et al., 1998), suggested possible changes in mast cell populations with MH35-BD treatment, deeper examination by detection of mast cell tryptase (MCT) suggested that mast cell populations were unaltered. Mast cells store the protease MCT in cytoplasmic granules and release this extracellularly upon stimulation via exocytotic degranulation. MCT expression was examined in the present study from whole muscle extracts and therefore could potentially correspond to MCT contained within mast cells and extracellularly and thus was not limited to detection of only mast cells that had not degranulated, as toluidine blue staining appeared to be. The expression of MCT in muscles increased following notexin injection, suggesting increased mast cell numbers present in the inflamed muscle. However there was no significant difference in MCT expression with MH35-BD compared to sham treated muscles, suggesting there was no change in mast cell populations with MH35-BD treatment. Therefore changes in *Kit* gene expression were likely not representative of changes in mast cell populations. Altogether this suggests that altered cytokine expression in MH35-BD treated muscle and subsequent reductions in myotube formation was not associated with a change in mast cell populations.

Altered cytokine expression is the most likely explanation for decreased myogenin expression and subsequent myotube formation. *Tnf*, *Il1b* and *Il6* are all potentially mitogenic for myoblasts as well as inhibitors of myogenic differentiation (Alter et al., 2008; Alvarez et al., 2002; Broussard et al., 2004; Li, 2003). Addition of these recombinant cytokines can decrease expression of *Myog*/myogenin by myoblasts in culture and inhibit differentiation. Therefore the inhibition of myogenic differentiation in regenerating muscle observed with MH35-BD treatment is unlikely to be caused through a direct effect of MH35-BD on myoblasts but rather may be caused indirectly by increased expression of *Tnf*, *Il1b* and *Il6*. A direct effect of MH35-BD on myoblasts leading to decreased differentiation was unlikely considering MH35-BD treatment promoted myogenic differentiation of myoblasts in culture conditions. Expression of *Myog* was significantly decreased at day 3 by MH35-BD treatment. Whilst MH35-BD appeared to inhibit myotube formation at day 7, MH35-BD treatment had no effect on *Myog* expression at day 7, a time at which *Myog* levels were greatest. Because a direct effect of MH35-BD on myogenic

differentiation is unlikely, the rapid and transient increase in expression of inflammatory cytokines with MH35-BD treatment during the early stages of regeneration most likely only affected *Myog* expression at day 3 but not day 7.

An indirect effect of MH35-BD on myoblasts differentiation, through increased cytokine expression, could lead to a decreased number of differentiated myoblasts in the muscle capable of fusing into myotubes and the decrease in myotube numbers that was observed in the muscle 7 days after notexin injection. This observation was consistent with previous reports suggesting that knock-out of *Lif* leads to decreased parameters of muscle regeneration (Kurek et al., 1997). Evidence provided in the present study however clarify and further develop mechanisms for these findings by suggesting that the negative effects on muscle regeneration caused by loss of LIF function are due to an altered inflammatory response and not a direct effect on myogenic cells.

Because an increase in LIF was observed systemically following notexin injury and LIF could potentially influence glucocorticoid production through the hypothalamic pituitary axis, it remains possible that the effect of MH35-BD administration may not have been only inhibiting LIFR signaling locally within the injured muscle, but also systemically. Thus we cannot exclude the possibility that the systemic administration of MH35-BD prevented a systemic anti-inflammatory response from LIF, thus producing a local pro-inflammatory response in the muscle compared to sham. Nevertheless, this still highlights an important role for LIF as an anti-inflammatory factor within muscle following injury.

It is important to note that although MH35-BD was used in this study as a LIF antagonist, MH35-BD may also block other cytokines known to bind the LIFR such as cardiotrophin-1 (CT-1), oncostatin-M (OSM) and ciliary neurotrophic factor (CNTF). For example, OSM can potentially compete for LIFR binding and be blocked by MH35-BD (Upadhyay et al., 2009). CT-1 is suggested to be present during myotoxin induced muscle regeneration (Nishikawa et al., 2005) and also inhibits myogenic differentiation *in vitro* through the same signaling mediators as LIF (Miyake et al., 2009). Cytokines signaling through the LIFR elicit similar effects due to use of the shared receptor and common signaling mediators. *Lif* knock-out however has been shown

to have a greater effect on maintenance of axons and motor endplates than knock-out of the genes for CT-1 or CNTF and therefore could be the predominant ligand for the LIFR in certain tissues and contexts (Holtmann et al., 2005). Despite the possibility of LIF being the predominant ligand for LIFR, the effects observed with MH35-BD treatment cannot be conclusively and exclusively linked to an effect of LIF alone. Therefore it would be of interest to know whether the observed effects of MH35-BD could be attributed to the combined inhibition of LIF and other LIFR utilising cytokines or inhibition of LIF by itself.

Conclusions

These studies provide compelling evidence for the role of LIFR signaling mediating the inflammatory response in regenerating muscle; in particular modulating the inflammatory balance of cytokine expression and neutrophil cell populations which leads to altered myogenic differentiation. These findings however do not support an important role for LIFR signaling directly affecting cellular responses of myoblasts that lead to changes in myogenic differentiation.

Acknowledgements

The authors acknowledge the contributions of Kitipong Uaesoontrachoon and Charles Pagel for providing oligonucleotide primer sequences for the genes *Myod1* and *Myog*. Su Toulson should also be acknowledged for her contributions toward the sampling of mice and subsequent processing of those samples.

Funding information

This work was funded by the Muscular Dystrophy Association of Australia (MDA) and supported by the Victorian Government's Operational Infrastructure Support Program.

Competing interests

The authors declare that no competing interests exist.

References

- Akita S, Malkin J, Melmed S (1996) Disrupted murine leukemia inhibitory factor (LIF) gene attenuates adrenocorticotrophic hormone (ACTH) secretion. *Endocrinology* 137:3140
- Alter J, Rozentzweig D, Bengal E (2008) Inhibition of Myoblast Differentiation by Tumor Necrosis Factor Is Mediated by c-Jun N-terminal Kinase 1 and Leukemia Inhibitory Factor. *Journal of Biological Chemistry* 283:23224
- Alvarez B, Quinn LBS, Busquets S, Quiles MT, López-Soriano FJ, Argilés JM (2002) Tumor necrosis factor- α exerts interleukin-6-dependent and-independent effects on cultured skeletal muscle cells. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1542:66-72
- Austin L, Bower J, Bennett T, Lynch G, Kapsa R, White J, Barnard W, Gregorevic P, Byrne E (2000) Leukemia inhibitory factor ameliorates muscle fiber degeneration in the mdx mouse. *Muscle & nerve* 23:1700-1705
- Austin L, Bower J, Kurek J, Vakakis N (1992) Effects of leukaemia inhibitory factor and other cytokines on murine and human myoblast proliferation. *Journal of the neurological sciences* 112:185-191
- Austin L, Burgess A (1991) Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. *Journal of the neurological sciences* 101:193-197
- Bamberger AM, Schulte HM, Wullbrand A, Jung R, Beil FU, Bamberger CM (2000) Expression of leukemia inhibitory factor (LIF) and LIF receptor (LIF-R) in the human adrenal cortex: implications for steroidogenesis. *Molecular and cellular endocrinology* 162:145-149
- Banner LR, Patterson PH, Allchorne A, Poole S, Woolf CJ (1998) Leukemia inhibitory factor is an anti-inflammatory and analgesic cytokine. *The Journal of neuroscience* 18:5456
- Barnard W, Bower J, Brown MA, Murphy M, Austin L (1994) Leukemia inhibitory factor (LIF) infusion stimulates skeletal muscle regeneration after injury: Injured muscle expresses lif mRNA. *Journal of the neurological sciences* 123:108-113
- Bower J, Vakakis N, Nicola N, Austin L (1995) Specific binding of leukemia inhibitory factor to murine myoblasts in culture. *Journal of cellular physiology* 164:93-98
- Briguet A, Courdier-Fruh I, Foster M, Meier T, Magyar JP (2004) Histological parameters for the quantitative assessment of muscular dystrophy in the mdx-mouse. *Neuromuscular Disorders* 14:675-682
- Broussard SR, McCusker RH, Novakofski JE, Strle K, Shen WH, Johnson RW, Dantzer R, Kelley KW (2004) IL-1 impairs insulin-like growth factor I-induced differentiation and downstream activation signals of the insulin-like growth factor I receptor in myoblasts. *The Journal of Immunology* 172:7713
- Contreras-Shannon V, Ochoa O, Reyes-Reyna SM, Sun D, Michalek JE, Kuziel WA, McManus LM, Shireman PK (2007) Fat accumulation with altered inflammation and regeneration in skeletal muscle of CCR2 $^{-/-}$ mice following ischemic injury. *American Journal of Physiology-Cell Physiology* 292:C953
- Duluc D, Delneste Y, Tan F, Moles MP, Grimaud L, Lenoir J, Preisser L, Anegon I, Catala L, Ifrah N (2007) Tumor-associated leukemia inhibitory factor and IL-6 skew monocyte differentiation into tumor-associated macrophage-like cells. *Blood* 110:4319-4330
- Fairlie WD, Uboldi AD, McCoubrie JE, Wang CC, Lee EF, Yao S, De Souza DP, Mifsud S, Metcalf D, Nicola NA (2004) Affinity maturation of leukemia inhibitory factor and conversion to potent antagonists of signaling. *Journal of Biological Chemistry* 279:2125

Fielding R, Manfredi T, Ding W, Fiatarone M, Evans W, Cannon J (1993) Acute phase response in exercise. III. Neutrophil and IL-1 beta accumulation in skeletal muscle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 265:R166

Gadient RA, Patterson PH (1999) Leukemia Inhibitory Factor, Interleukin 6, and Other Cytokines Using the GP130 Transducing Receptor: Roles in Inflammation and Injury. *Stem Cells* 17:127-137

Galli SJ, Zsebo KM, Geissler EN (1993) The kit ligand, stem cell factor. *Advances in immunology* 55:1-96

Gearing DP, Comeau MR, Friend DJ, Gimpel SD, Thut CJ, McGourty J, Brasher KK, King JA, Gillis S, Mosley B (1992) The IL-6 signal transducer, gp130: an oncostatin M receptor and affinity converter for the LIF receptor. *Science* 255:1434

Gearing DP, Gough N, King J, Hilton D, Nicola N, Simpson R, Nice E, Kelso A, Metcalf D (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). *The EMBO journal* 6:3995

Gordon S (2003) Alternative activation of macrophages. *Nature Reviews Immunology* 3:23-35

Gorospe JRM, Tharp M, Demitsu T, Hoffman EP (1994) Dystrophin-deficient myofibers are vulnerable to mast cell granule-induced necrosis. *Neuromuscular Disorders* 4:325-333

Gregorevic P, Hayes A, Lynch GS, Williams DA (2000) Functional properties of regenerating skeletal muscle following LIF administration. *Muscle & nerve* 23:1586-1588

Helliwell T, Gunhan O, Edwards R (1990) Mast cells in neuromuscular diseases. *Journal of the neurological sciences* 98:267-276

Hendriks JJA, Slaets H, Carmans S, de Vries HE, Dijkstra CD, Stinissen P, Hellings N (2008) Leukemia inhibitory factor modulates production of inflammatory mediators and myelin phagocytosis by macrophages. *Journal of neuroimmunology* 204:52-57

Hilton D, Nicola N, Metcalf D (1988) Specific binding of murine leukemia inhibitory factor to normal and leukemic monocytic cells. *Proceedings of the National Academy of Sciences* 85:5971

Hilton DJ, Nicola NA, Waring PM, Metcalf D (1991) Clearance and fate of leukemia inhibitory factor (LIF) after injection into mice. *Journal of cellular physiology* 148:430-439

Hodgetts S, Radley H, Davies M, Grounds MD (2006) Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNF α function with Etanercept in mdx mice. *Neuromuscular disorders : NMD* 16:591-602

Holtmann B, Wiese S, Samsam M, Grohmann K, Pennica D, Martini R, Sendtner M (2005) Triple knock-out of CNTF, LIF, and CT-1 defines cooperative and distinct roles of these neurotrophic factors for motoneuron maintenance and function. *The Journal of neuroscience* 25:1778

Hunt L, Tudor E, White J (2010) Leukemia inhibitory factor-dependent increase in myoblast cell number is associated with phosphatidylinositol 3-kinase-mediated inhibition of apoptosis and not mitosis. *Experimental Cell Research* 316:1002-1009

Hunt LC, Upadhyay A, Jazayeri JA, Tudor EM, White JD (2011) Caspase-3, myogenic transcription factors and cell cycle inhibitors are regulated by leukemia inhibitory factor to mediate inhibition of myogenic differentiation. *Skeletal muscle* 1:17

Iida N, Grotendorst GR (1990) Cloning and sequencing of a new gro transcript from activated human monocytes: expression in leukocytes and wound tissue. *Molecular and cellular biology* 10:5596

Jazayeri JA, De Weerd N, Raye W, Kivivuori S, Zabihi E, Carroll GJ (2007) In vitro evaluation of leukemia inhibitory factor receptor antagonists as candidate therapeutics for inflammatory arthritis. *Journal of Interferon & Cytokine Research* 27:281-290

Jo C, Kim H, Jo I, Choi I, Jung SC, Kim J, Kim SS, Jo SA (2005) Leukemia inhibitory factor blocks early differentiation of skeletal muscle cells by activating ERK. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1743:187-197

Kovanen PT, Kaartinen M, Paavonen T (1995) Infiltrates of activated mast cells at the site of coronary atheromatous erosion or rupture in myocardial infarction. *Circulation* 92:1084-1088

Kurek JB, Bower JJ, Romanella M, Koentgen F, Murphy M, Austin L (1997) The role of leukemia inhibitory factor in skeletal muscle regeneration. *Muscle & nerve* 20:815-822

Kurek JB, Nouri S, Kannourakis G, Murphy M, Austin L (1996) Leukemia inhibitory factor and interleukin-6 are produced by diseased and regenerating skeletal muscle. *Muscle & nerve* 19:1291-1301

Kwon YW, Abbondanzo SJ, Stewart CL, Gurney ME (1995) Leukemia inhibitory factor influences the timing of programmed synapse withdrawal from neonatal muscles. *Journal of neurobiology* 28:35-50

Layton MJ, Owczarek CM, Metcalf D, Clark RL, Smith DK, Treutlein HR, Nicola NA (1994) Conversion of the biological specificity of murine to human leukemia inhibitory factor by replacing 6 amino acid residues. *Journal of Biological Chemistry* 269:29891-29896

Lefaucheur JP, Sebille A (1995) Muscle regeneration following injury can be modified in vivo by immune neutralization of basic fibroblast growth factor, transforming growth factor [beta] 1 or insulin-like growth factor I. *Journal of neuroimmunology* 57:85-91

Li YP (2003) TNF- is a mitogen in skeletal muscle. *American Journal of Physiology-Cell Physiology* 285:C370

Metcalf D, Hilton D, Nicola N (1988) Clonal analysis of the actions of the murine leukemia inhibitory factor on leukemic and normal murine hemopoietic cells. *Leukemia: official journal of the Leukemia Society of America, Leukemia Research Fund, UK* 2:216

Miyake T, Alli NS, Aziz A, Knudson J, Fernando P, Megeney LA, McDermott JC (2009) Cardiotrophin-1 maintains the undifferentiated state in skeletal myoblasts. *Journal of Biological Chemistry* 284:19679

Moritz DR, Rodewald H-R, Gheyselinck J, Klemenz R (1998) The IL-1 Receptor-Related T1 Antigen Is Expressed on Immature and Mature Mast Cells and on Fetal Blood Mast Cell Progenitors. *The Journal of Immunology* 161:4866-4874

Nishikawa J, Sakuma K, Sorimachi Y, Yoshimoto K, Yasuhara M (2005) Increase of Cardiotrophin 1 immunoreactivity in regenerating and overloaded but not denervated muscles of rats. *Neuropathology* 25:54-65

Ochoa O, Sun D, Reyes-Reyna SM, Waite LL, Michalek JE, McManus LM, Shireman PK (2007) Delayed angiogenesis and VEGF production in CCR2^{-/-} mice during impaired skeletal muscle regeneration. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 293:R651

Pelus LM, Fukuda S (2006) Peripheral blood stem cell mobilization: the CXCR2 ligand GRO [beta] rapidly mobilizes hematopoietic stem cells with enhanced engraftment properties. *Experimental hematology* 34:1010-1020

Peterson JM, Feedback KD, Baas JH, Pizza FX (2006) Tumor necrosis factor- promotes the accumulation of neutrophils and macrophages in skeletal muscle. *Journal of Applied Physiology* 101:1394

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

Pfaffl MW, Tichopad A, Prgomet C, Neuvians TP (2004) Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. *Biotechnology letters* 26:509-515

Radley HG, Grounds MD (2006) Cromolyn administration (to block mast cell degranulation) reduces necrosis of dystrophic muscle in mdx mice. *Neurobiology of disease* 23:387-397

Ray DW, Ren SG, Melmed S (1996) Leukemia inhibitory factor (LIF) stimulates proopiomelanocortin (POMC) expression in a corticotroph cell line. Role of STAT pathway. *Journal of Clinical Investigation* 97:1852

Schofield G, Kimber SJ (2005) Leukocyte subpopulations in the uteri of leukemia inhibitory factor knockout mice during early pregnancy. *Biology of reproduction* 72:872-878

Shireman PK, Contreras-Shannon V, Ochoa O, Karia BP, Michalek JE, McManus LM (2007) MCP-1 deficiency causes altered inflammation with impaired skeletal muscle regeneration. *Journal of leukocyte biology* 81:775

Spencer MJ, Montecino-Rodriguez E, Dorshkind K, Tidball JG (2001) Helper (CD4+) and Cytotoxic (CD8+) T Cells Promote the Pathology of Dystrophin-Deficient Muscle. *Clinical Immunology* 98:235-243

Srikuea R, Esser KA, Pholpramool C (2011) Leukaemia inhibitory factor is expressed in rat gastrocnemius muscle after contusion and increases proliferation of rat L6 myoblasts via c-Myc signalling. *Clinical and Experimental Pharmacology and Physiology* 38:501-509

Tham S, Dowsing B, Finkelstein D, Donato R, Cheema SS, Bartlett PF, Morrison WA (1997) Leukemia inhibitory factor enhances the regeneration of transected rat sciatic nerve and the function of reinnervated muscle. *Journal of neuroscience research* 47:208-215

Tidball JG, Villalta SA (2010) Regulatory interactions between muscle and the immune system during muscle regeneration. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology* 298:R1173

Upadhyay A, Sharma G, Kivivuori S, Raye WS, Zabihi E, Carroll GJ, Jazayeri JA (2009) Role of a LIF antagonist in LIF and OSM induced MMP-1, MMP-3, and TIMP-1 expression by primary articular chondrocytes. *Cytokine* 46:332-338

Villalta SA, Rinaldi C, Deng B, Liu G, Fedor B, Tidball JG (2011) Interleukin-10 reduces the pathology of mdx muscular dystrophy by deactivating M1 macrophages and modulating macrophage phenotype. *Human molecular genetics* 20:790-805

Wang X, Wu H, Zhang Z, Liu S, Yang J, Chen X, Fan M (2008) Effects of interleukin-6, leukemia inhibitory factor, and ciliary neurotrophic factor on the proliferation and differentiation of adult human myoblasts. *Cellular and Molecular Neurobiology* 28:113-124

Wehling M, Spencer MJ, Tidball JG (2001) A nitric oxide synthase transgene ameliorates muscular dystrophy in mdx mice. *The Journal of cell biology* 155:123-132

White JD, Davies M, Grounds MD (2001) Leukaemia inhibitory factor increases myoblast replication and survival and affects extracellular matrix production: combined in vivo and in vitro studies in post-natal skeletal muscle. *Cell and Tissue Research* 306:129-141

White JD, Davies M, McGeachie J, Grounds MD (2002) An evaluation of leukaemia inhibitory factor as a potential therapeutic agent in the treatment of muscle disease. *Neuromuscular Disorders* 12:909-916

Wolpe SD, Sherry B, Juers D, Davatellis G, Yurt RW, Cerami A (1989) Identification and characterization of macrophage inflammatory protein 2. *Proceedings of the National Academy of Sciences* 86:612

Woods AM, McIlmoil CJ, Rankin EN, Packer AA, Stevens JC, Macievic JA, Brown AB, Porter JP, Judd AM (2008) Leukemia inhibitory factor protein and receptors are expressed in the bovine adrenal cortex and increase cortisol and decrease adrenal androgen release. *Domestic Animal Endocrinology* 35:217-230

Zentella A, Massague J (1992) Transforming growth factor beta induces myoblast differentiation in the presence of mitogens. *Proceedings of the National Academy of Sciences* 89:5176

Gene name	Protein name	ID	Forward (5'-3')	Reverse (5'-3')
<i>Hprt</i>	HPRT	15452	GATTAGCGATGATGAACCAGGTT	TCCAAATCCTCGGCATAATGAT
<i>Kit</i>	CD117	16590	AGCGTCTTCCGGCACAACGG	TGTGGGCCTGGATTTGCTCTTTGT
<i>Cd163</i>	CD163	93671	TGCGCCGACGTGTTCCGAAG	GCTGGCCACTTGCTATGCAGGG
<i>Cd19</i>	CD19	12478	AGCTGGCCCAACCAGTTGGC	AGGACTGGGACCCAAGCGAGG
<i>Cd4</i>	CD4	12504	CGTGCTGGGTGGCTCCTTCG	CTTCTGCATCCGGTGGGGGC
<i>Cd8a</i>	CD8	12525	GGCTCAGTGAAGGGGACCGGA	AGCGGCCTGGGACATTTGCAAA
<i>Cd86</i>	CD86	12524	TCAGCCTAGCAGGCCAGCA	GGCTCTCACTGCCTTCACTCTGC
<i>Emr1</i>	F4/80	13733	ACAGCCACGGGGCTATGGGA	GCACCCAGGAGCAGCCCCAG
<i>Il6st</i>	gp130	16195	CATAGTCGTGCCTGTGTGCT	GTGACCACTGGGCAATATGA
<i>Ifng</i>	IFN- γ	15978	AGTTTGAGGTCAACAACCCACAGGT	CCACCCCGAATCAGCAGCGA
<i>Il10</i>	IL-10	16153	TGCGGCTGAGGCGCTGTCATC	ACTCTTCACCTGCTCCACTGCCTT
<i>Il1b</i>	IL-1 β	16176	CTCGGCCAAGACAGGTCGCTC	CCCCCACACGTTGACAGCTAGG
<i>Il6</i>	IL-6	16193	TCTCTGCAAGAGACTTCCATCCAGT	AGTAGGGAAGGCCGTGGTTGTCA
<i>Lif</i>	LIF	16878	CAAGAATCAACTGGCACAGC	AGTGGGGTTCAGGACCTTCT
<i>Lifr</i>	LIFR	16880	AGAAGAACTGGCTCCCATTTG	GGATGTCGTCCCATTTCCT
<i>Ly6g</i>	Ly6G	546644	TGCCTTCCCCTCTGCCCACT	AAGCACCCCTGCCATGGTCC
<i>Ccl2</i>	MCP-1	20296	GCTCAGCCAGATGCAGTTAACGC	TGGGGTCAGCACAGACCTCTCT
<i>Cxcl2</i>	MIP2	20310	GCTACAGGGGCTGTTGTGGCC	CAGGCTCCTCCTTTCCAGGTCA
<i>Myh1</i>	MYHC	17879	GCTCAAAGCCCTGTGTTACC	CATAGACGGCTTTGGCTAGG
<i>Myod1</i>	myoD	17927	TACAGTGGCGACTCAGATGC	TAGTAGGCGGTGTCGTAGCC
<i>Myog</i>	myogenin	17928	AGTGAATGCAACTCCCACAG	ACGATGGACGTAAGGGAGTG
<i>Pcna</i>	PCNA	18538	GGGCGTGAACCTCACCAGCA	CGTGCAAATTACCCGACGGC
<i>Tgfb1</i>	TGF- β	21803	CGGCTGCTGACCCCCACTGA	ACGTTTGGGGCTGATCCCGTT
<i>Tnf</i>	TNF- α	21926	CAAATGGCCTCCCTCTCAT	TGGGCTACAGGCTTGTCACT

Table 1 Oligonucleotide primer sequences used for qPCR

A)

C)

B)

D)

A)

B)

C)