**Differential effects of TNFα on satellite cell differentiation.**

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I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it any university for a degree.

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Date: …….

Tumour necrosis factor alpha (TNFα) is a pleiotropic cytokine and has a wide variety of dose dependent cellular effects ranging from cell growth and differentiation, to inducing apoptosis. It has long been implicated in muscle and non-muscle inflammatory disorders, such as muscle wasting in chronic disease states, and rheumatoid arthritis. However, a physiological role for TNFα in muscle regeneration has been proposed as elevated levels of the cytokine are present when muscle regeneration processes are initiated: TNFα is secreted by infiltrating inflammatory cells, and by injured muscle fibres. Adult skeletal muscle contains a population of resident stem cell-like cells called satellite cells, which become activated, proliferate and differentiate following muscle injury to bring about repair of damaged muscle. Much research on the effects of TNFα on satellite cell differentiation has been conducted in recent years. It is however difficult to get a complete characterisation of the cytokine's action as all models used slightly differ. We aimed therefore at providing comprehensive assessment of the effects of increasing doses of chronically supplemented TNFα on differentiating C2C12 cells. Cells were allowed to differentiate with or without TNFα supplementation for 7 days. Differentiation was induced at day 0. The effect on differentiation was assessed at days 1, 3, 5, and 7 by western blot analysis, and supplementary immunohistochemical analysis at days 1, 4, and 7 of markers of differentiation - muscle regulatory factors: MyoD and myogenin, markers of the cell cycle p21, PCNA, and the integral signalling molecule, p38MAPK. TNFα supplementation at day 1 tended to positively regulate early markers of differentiation. With continued supplementation however, markers of differentiation decreased dose dependently in treated cultures as the initial effect appeared to be reversed: A trend towards a dose dependent decrease in MyoD, myogenin and p21 protein existed in

treated cultures at days 3, 5, and 7. These findings were significant at day 5 (p21, $p<0.05$), and day 7 (myogenin, $p<0.05$). A significant dose dependent decrease in p38 phosphorylation was evident at day 3 ($p<0.05$), while phospho-p38 was dose dependently increased at day 7 ($p<0.05$). Taken together, these data show that TNFα supplementation for 24 hours following the induction of differentiation *in vitro*, tends to increase levels of early markers of differentiation, and with continued TNFα supplementation decrease markers of differentiation in a dose dependent fashion. This study provides a comprehensive characterisation of the dose and time dependent effects of TNFα on satellite cell differentiaton *in vitro*. The model system used in the current study, allows us to make conclusions on more chronic disease states.

Tumor nekrose faktor alfa (TNFα) is ‘n pleiotropiese sitokien wat ‘n wye verskeidenheid, dosis afhanklike, sellulêre effekte te weeg bring. Hierdie sellulêre effekte sluit sel groei en differensiasie tot sel dood in. TNFα is by beide spier en nie-spier inflammatoriese stoornisse soos spier tering in kroniese siektetoestande, en rumatiese artritis betrek. ‘n Fisiologiese rol vir TNFα is egter voorgestel aangesien verhoogde vlakke van die sitokien tydens inisiasie van spier herstel meganismes teenwoordig is: TNFα word deur infiltrerende inflammatoriese selle, asook deur beseerde spier vesels afgeskei. Volwasse skeletspier bevat ‘n populasie stamselagtige selle, sogenoemde satelliet selle. Laasgenoemde word geaktiveer, prolifereer en differensieër volgende spierbesering, om sodoende herstel van beskadigde spier te weeg te bring. Baie navorsing op die effekte van TNFα op satelliet sel differensiasie is onlangs uitgevoer. Dit is egter aansienlik moeilik om volgens hierdie navorsing‘n algehele beeld van TNFα se aksies te vorm aangesien alle modelle wat gebruik word verskil. Ons doel was daarom om ‘n omvangryke assessering van toenemende konsentrasies kronies gesupplementeerde TNFα op differensieërende C2C12 selle op ‘n enkele model uit te voer. Selle was vir 7 dae met of sonder TNFα supplementasie gedifferentieër. Differensiasie was by Dag 0 geïnduseer. TNFα se effek op differensiasie is op dae 1, 3, 5, en 7 deur middel van western blot analise geassesseer. Aanvullende immunohistochemiese bepalings op dae 1, 4, en 7 is verder deurgevoer. Merkers vir differensiasie het die spier regulatoriese faktore MyoD en miogenien, sel siklus merkers p21 en PCNA, asook die integrale sein transduksie molekule p38MAPK ingesluit. TNFα supplementasie by dag 1 het geneig om vroeë merkers van differensiasie positief te reguleer. Met voortdurende supplementasie is die vroeë positiewe effekte (op ‘n dosis afhanklike manier) egter omgekeer: ‘n neiging teenoor

(‘n dosis afhanklike) vermindering in MyoD, miogenien en p21 proteïen het in behandelde kulture op dae 3, 5, en 7 bestaan. Hierdie bevindinge was beduidend by dag 5 (p21, $p<0.05$), en dag 7 (miogenien, $p<0.05$). A beduidende dosis afhanklike afname in p38 fosforilasie was duidelik by dag 3 ($p<0.05$), terwyl fosfo-p38 by dag 7 verhoog het met verhoogde konsentrasie TNFα ($p<0.05$). Bogenoemde saamgevat, dui aan dat TNFα supplementasie 24h volgende die induksie van differensiasie *in vitro*, verhoogde vlakke van vroeë differnsiasie merkers te weeg bring. Met voortdurende TNFα supplementasie, word differensiasie merkers egter met toenemende dosis verminder. Hierdie studie voorsien ‘n omvattende karakterisering van die dosis- en tyd afhanklike effekte van TNFα op satelliet sel differesiasie *in vitro*. Die model sisteem in hierdie studie gebruik, maak afleidings oor meer kroniese siektetoestande moontlik.

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**INDEX**

**List of tables**



**List of Figure titles**

**Figure 3.3** Protein expression profile of p21 (A), PCNA (B) in differentiating C2C12 cells. Error bars indicate SEM for 3 independent experiments

**Figure 3.4** Ratio of phosphorylated- to total p38α/β in differentiating C2C12 cells. Error bars indicate SEM for 3 independent experiments.

**Figure 3.5** Schematic representation of MyoD protein expression relative to harvest day control levels (Mean ± SEM, n=3) (A). Representative western blot (B).

**Figure 3.6** Schematic representation of myogenin expression relative to harvest day control levels (Mean ± SEM, day 3, 5 n=7; day 7 n=4) (A). Statistical analysis: Mann Whitney U test control vs high dose, # $p < 0.017$. Representative western blot (B).

**Figure 3.7** Schematic representation of p21 expression relative to harvest day control levels (Mean ± SEM, day 1, 3, 5 n=3; day 7 n=5) (A). Statistical analysis: Kruskal Wallis ANOVA, * $p < 0.05$, # $p < 0.05$ (Dunn post hoc test). Representative western blot (B).

**Figure 3.8** Schematic representation of PCNA expression relative to harvest day control levels (Mean ± SEM, n=3) (A). Representative western blot (B).

**Figure 3.9** Schematic representation of the ratio of phospho-p38α/β relative to harvest day control levels (Mean ± SEM, day 1, n=3 day 7, n=4) (A). Statistical analysis: Kruskal Wallis ANOVA *$p<0.05$, # $p<0.05$ (Dunn post hoc test). Representative western blot (B). Tp38 = representative total p38 blot.

**Figure 3.10** MyoD staining. Immunohistochemistry of day 1 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left hand panel (A-D) shows cells stained for MyoD (FITC,

green). The right hand panel (E-H) shows merged signals, MyoD (FITC), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

**Figure 3.11** MyoD staining. Immunohistochemistry of day 4 control- (A & C) and high dose (B & D) TNFα treated C2C12 cultures. The left panel (A & B) shows cells stained for MyoD (Texas red). The right panel (C & D) shows merged signals, MyoD (Texas red), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

**Figure 3.12** Myogenin staining. Immunohistochemistry of day 4 control- (A & C) and high dose (B & D) TNFα treated C2C12 cultures. The left panel (A & B) shows cells stained for myogenin (FITC, green). The right panel (C & D) shows merged signals, myogenin (FITC), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

**Figure 3.13** Immunohistochemistry of day 4 control (A) and high dose (B) TNFα treated C2C12 cultures. Merged signals of MyoD (Figure 3.14), myogenin (Figure 3.15) and Hoechst (stains all nuclei blue) are shown. Images are at a 60X magnification.

**Figure 3.14** Myogenin staining. Immunohistochemistry of day 7 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left panel (A-D) shows cells stained for myogenin (Texas red). The right panel (E-H) shows merged signals, myogenin (Texas Red), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

**Figure 3.15** phopsho-p38α/β staining. Immunohistochemistry of day 7 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left panel (A-D) shows cells stained for phospho-p38α/β (FITC, green). The right panel (E-H) shows merged signals, phospho-p38α/β (FITC), and Hoechst (stains all nuclei blue). Images are at a 60X magnification.

**Figure 3.16** Immunohistochemistry of day 7 control, low, medium and high dose (A-D) TNFα treated C2C12 cultures. Merged signals of myogenin (Figure 3.17), phospho-p38α/β (Figure 3.18) and Hoechst (stains all nuclei blue) are shown. Images are at a 60X magnification.

List of abbreviations

| | |
|---|---|
| Ab | Antibody |
| AIDS | Acquired Immunodeficiency Syndrome |
| ANOVA | Analysis of Variance |
| AP-1 | Activated Protein-1 |
| bHLH | basic Helix Loop Helix |
| cdk | cyclin dependent kinases |
| COPD | Chronic Obstructive Pulmonary Disease |
| DD | Death Domain |
| DED | Death Effector Domain |
| DMEM | Dulbecco's Modified Eagle's Medium |
| ECL | Enhanced Chemiluminescence |
| ERK | Extracellular Regulated Kinase |
| ES | Embryonic Stem cell |
| FADD | Fas-Associated Death Domain |
| FAN | Neutral SMase Activation Factor |
| FGF | Fibroblast Growth Factor |
| FGFR | Fibroblast Growth Factor Receptor |
| FITC | Fluorescein Streptavidin |
| HGF | Hepatocyte Growth Factor |
| IκBα | NFκB Inhibitory subunit |
| IGF | Insulin Growth Factor |

| | |
|---|---|
| IL | Interleukin |
| JNK | c-jun $NH_3$-terminal Kinases |
| LIF | Leukaemia Inhibitory Factor |
| M | Mitosis stage |
| MAPK | Mitogen Activated Protein Kinase |
| MAPKAPK-2 | MAPK Activated Protein Kinase |
| MAPKK/MKK | MAPK Kinases |
| MAPKKK | MAPK Kinase Kinases |
| MEF2 | Myocyte Enhancer Binding Factor 2 |
| MEK1 | First isoform of MAPKK |
| MEK2 | Second isoform of MAPKK |
| MHC | Myosin Heavy Chain |
| mpc | myogenic precursor cells |
| MRF | Myogenic Regulatory Factor |
| NFκB | Nuclear Factor Kappa B |
| NIK | NFκB- Inducing Kinase |
| PBS | Phosphate Buffered Solution |
| PCNA | Proliferating Cellular Nuclear Antigen |
| PI3K | Phosphatidylinositol 3-Kinase |
| PVDF | Polyvinylidine Fluoride |
| Rb | Retinoblastoma protein |
| RIP | Receptor Interacting Protein |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| RT | Room temperature |
| S | Synthesis phase |
| SAPK | Stress Activated Protein Kinase |
| SDS-PAGE | Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis |
| SEM | Standard Error of the Mean |
| SM | Skeletal Muscle |
| TBS-T | Tris Buffered Saline-Tween20 |
| TGFβ | Transforming Growth Factor beta |
| TNFα | Tumour Necrosis Factor alpha |
| TNFR | Tumour Necrosis Factor Receptors |
| TRADD | TNF Receptor-Associated Death Domain |
| TRAF2 | TNF Receptor-Associating Factor 2 |
| VCAM | Vascular cell adhesion molecule |

# CHAPTER 1: INTRODUCTION

## 1.1 Skeletal muscle

### 1.1.1 Skeletal muscle morphological structure (general overview)

Adult skeletal muscle is composed of postmitotic, multinucleated (Morgan *et al.*, 2003) elongated cells known as muscle fibres (myofibres) (Charge *et al.*, 2004; Vander *et al.*, 2001). Muscle fibres are grouped and arranged into bundles known as fasciculi, clearly visible at cross section through skeletal muscle (Figure 1.1 A). An extensive connective tissue network is found within skeletal muscle: Each muscle fibre is individually surrounded by connective tissue known as the endomysium, each myofibre bundle by the perimysium, while the epimysium surrounds an entire muscle (McComas, 1996). Skeletal muscle is richly supplied of essential nutrients by a network of blood vessels dispersed throughout the perimysium (Charge *et al.*, 2004). At the end of each muscle collagen fibres (tendons) connect the muscle to bone, allowing whole muscle shortening and synchronised contraction and force generation (McComas, 1996). Effective force generation and muscle contraction is the product of each of the muscle components. This is adequately illustrated in individuals where a single defective or missing muscle component (such as the affected dystrophin gene in muscular dystrophy sufferers) severely affects muscle function.

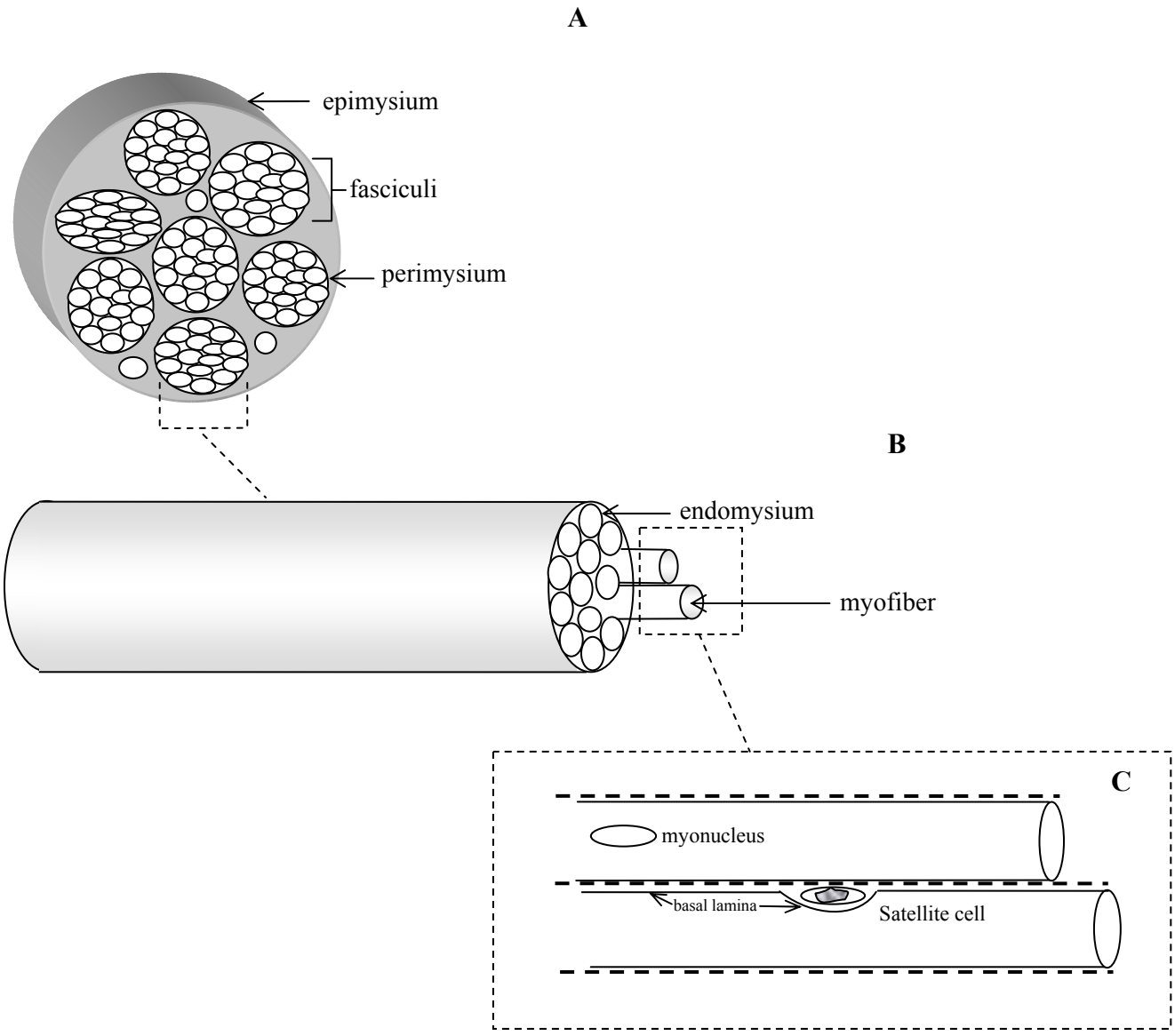



**Figure 1.1** Basic organisation of skeletal muscle. A cross section of skeletal muscle, showing muscle fiber bundles (fasciculi) (A). A single muscle fiber bundle consisting of myofibers (B). Satellite cell position within muscle (C). Figure adapted and modified from Hawke & Garry (2001).

### 1.1.2 Contractile apparatus and force generation of skeletal muscle

Myofibres contain the basic contractile units of skeletal muscle, the sarcomere. A significant portion of cytoplasm is filled with so called thick and thin filaments neatly arranged into cylindrical bundles referred to as myofibrils (Figure 1.1 B) and are concerned with the contractile function of the muscle cell (McComas, 1996). Muscle contraction, or shortening, is brought about by a sliding mechanism whereby the myosin rich thick filaments and actin rich thin filaments slide over each other and shorten the muscle upon stimulation (Charge *et al.*, 2004).

## 1.2. Skeletal muscle function and integrity

Skeletal muscle (SM) is chiefly involved in the facilitation of locomotor activity, breathing, and coordinated and directed movements (Charge *et al.*, 2004; Song *et al.*, 1998). By virtue of its function, SM is rather susceptible to injury, however, despite the challenges SM faces during the daily cycle of use, it remains a relatively stable tissue, with little turnover of nuclei (Charge *et al.*, 2004). Adult SM has a remarkable capability to self-repair in response to the physiological demands of growth, training and injury (Hawke *et al.*, 2001). It has been said that maintaining an efficiently working skeletal musculature is granted by its notable ability to regenerate (Charge *et al.*, 2004).

As skeletal myofibers are highly specialised and terminally differentiated, their regeneration requires some source of renewable cells capable of myogenic differentiation (Zammit *et al.*, 2001). The identification of so-called muscle satellite cells by Mauro in 1961 (Mauro, 1961) led to great advances in our understanding of

muscle regeneration as it became clear that satellite cells are indeed that renewable source of cells. This is responsible for all postnatal muscle growth, hypertrophy and differentiation are accomplished by satellite cells (Blau *et al.*, 2001; Hawke *et al.*, 2001; Seale *et al.*, 2000a).

### 1.3. Satellite cells (stem cell like progenitors of skeletal muscle)

Satellite cells were initially described and named according to their location relative to the mature myofibre. Residing between the basal lamina and the sarcolemma, satellite cells occupy indentations on the periphery of myofibres (Figure 1.1 C) (Charge *et al.*, 2004; Hawke *et al.*, 2001; Mauro, 1961; Morgan *et al.*, 2003; Muir *et al.*, 1965; Seale *et al.*, 2000a). They exist as a pool of mononucleated, undifferentiated, stem cell-like cells (Hawke *et al.*, 2001). Stem cells are referred to as non-specialized cells capable of self-renewal and of differentiation along multiple lineages. Cells from the first two divisions of the zygote are totipotent, which means that any cell type from the embryo as well as cells from the placenta can be formed from them (Alison *et al.*, 2002). A second type of stem cell, the embryonic stem cell (ES), has the potential to form any cell type from the three germ layers: meso-, endo-, and ectoderm. ES are however not capable of forming the placenta, and can therefore not form a new embryo (Alison *et al.*, 2002). The third type of stem cell, the so called adult stem cell, is resident in most organs of mature animals. By acting as a source of new cells, adult stem cells are responsible for postnatal growth and repair (Blau *et al.*, 2001). Satellite cells are referred to as stem cell-like cells as they too, are undifferentiated and self-renewing. Furthermore, they have been shown to be able to differentiate into adipogenic cells as well (Asakura *et al.*, 2001).

In their un-activated state, satellite cells are said to be quiescent and divide infrequently. As stem cells undergo a limited number of cell divisions, chances of accumulating mutations during DNA replication are reduced (Alison *et al.*, 2002). Such an inactive state does not however imply a totally inactive basal state, but rather a state in which only limited maintenance genes are expressed. The difficulty of studying satellite cell populations *in vivo* lies with their small population size in muscle, and is further hindered by the fact that a mere isolation process is stimulus enough for their activation and subsequent proliferation (Charge *et al.*, 2004).

Quiescent satellite cells express a distinct set of markers (See Appendix A for table of satellite cell markers), and are distinguished from adult myoblasts by various morphological characteristics, including their reduced organelle number, and increased nuclear-to-cytoplasmic ratio. Relative to the neighbouring myonucleui, quiescent satellite cells nuclei are small with increased amounts of heterochromatin (highly condensed regions on chromosomes physically obstructing the binding of transcription and other factors). Such characteristics are indicative of the fact that satellite cells (when unstimulated), are mitotically quiescent and metabolically- and transcriptionally less active (Charge *et al.*, 2004; Hawke *et al.*, 2001).

### 1.3.1 Studying skeletal muscle differentiation *in vitro*

Following the appropriate cues (see further sections), satellite cells become activated, and initiate multiple rounds of proliferation, and subsequently differentiate into mature myofibers (Charge *et al.*, 2004; Hawke *et al.*, 2001). The major muscle differentiation steps can be reproduced *in vitro* with the aid of muscle satellite cell lines (Charge *et al.*,

2004; Hawke *et al.*, 2001). Differentiation is induced by a process of serum removal, a process whereby the serum concentration of culture medium is dropped considerably. The C2C12 murine skeletal muscle cell line (amongst other) is commonly used to study myogenesis *in vitro*. Primary tissue cultures are also often employed by researchers.

### 1.3.2 Cell cycle

#### 1.3.2.1 Overview of the cell cycle

Cellular events that take place from the completion of one division until the start of the following division constitute the cell cycle (Klug *et al.*, 2000). Cells spend most of their time in interphase, the initial stage of the cell cycle. Interphase is further subdivided into 3 phases namely the G1, S, and G2 phases. At an advanced point in G1 phase cells continue on one of two paths: either proceeding to S phase and DNA replication, or entering what is known as G0 phase. Despite not proliferating, cells in G0 remain metabolically active (Schafer, 1998) and are referred to as quiescent. Tremendous metabolic activity and cell growth take place throughout interphase (Vander *et al.*, 2001). Following the S phase, physical cell division (both nuclear and cytoplasmic) occurs during the M, or mitosis stage of the cell cycle. It is therefore only in the mitosis stage of the cell cycle that any visible signs of division become evident. Proliferating cellular nuclear antigen (PCNA) is an accessory factor to DNA polymerase and is, as such, used as a marker of cell proliferation (Layne *et al.*, 1999; Schafer, 1998; Seale *et al.*, 2000a).

#### 1.3.2.2 Regulation of the cell cycle

The cell cycle is under strict regulation. Several so called checkpoints have been identified within the cell cycle. Depending on the condition of the cell at these checkpoints, a decision to proceed with or halt the cycle beyond the checkpoint is effected. Cyclin dependent kinases (cdk) are the major promoters of cell cycle progression. Their action (moving cells through the various phases) is dependent on the interaction with different subsets of subunits of a group of proteins known as cyclins of which cyclin D1 is a well studied example (Guo *et al.*, 1995; Hawke *et al.*, 2001; Schafer, 1998). Cdk inhibitors negatively regulate cell progression by adhering to cdk/cyclin complexes, thereby blocking their action. Five cdk inhibitors have been identified to date: p15, p16, p18, p21, and p27 (Peter *et al.*, 1994).

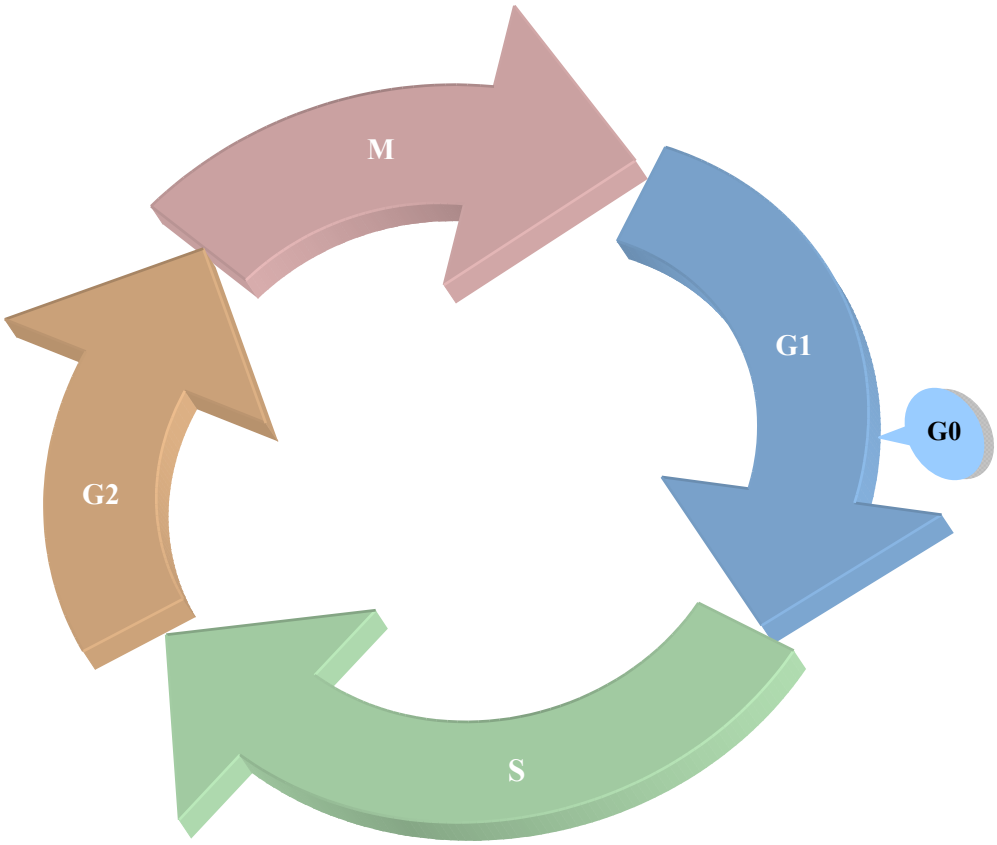



**Figure 1.2** The cell cycle. See text for details.

### 1.3.3 The activation of satellite cells

In response to a wide variety of stimuli including denervation, stretching, over exertion, agents released from damaged tissue and the inflammatory response resultant of muscle damage (Grounds, 1998) satellite cells exit their normal quiescent state, enter the cell cycle, (i.e. move from G0 to G1 phase of cell cycle) and initiate multiple rounds of proliferation. Once proliferating, satellite cells are referred to as adult myoblasts or myogenic precursor cells (mpc) (Charge *et al.*, 2004). Following multiple rounds of proliferation, most of the myoblasts migrate to the injured fibre where they either fuse to the existing myofiber (thereby progressing muscle repair), or together to form a new myofiber. Recent reports have, however, indicated that certain satellite cells already fuse after only 1 or 2 cell divisions (Schultz, 1996). The remainder of the activated satellite cell population once again become quiescent, thereby completing the process of self-renewal. Such a process is vital in preventing depletion of the satellite cell pool (Charge *et al.*, 2004).

### 1.3.4 Activated satellite cells at the molecular level

At a molecular level, the activation of satellite cells is accompanied by a rapid up-regulation of two groups of myogenic transcription factors that are important for myogenesis: the myogenic regulatory factor (MRF) family and the myocyte enhancer binding factor 2 (MEF2) family of proteins (Charge *et al.*, 2004; Wu *et al.*, 2000).

### 1.3.5 The myogenic regulatory factor (MRF) family of transcription factors

The MRFs, a family of basic helix loop helix (bHLH) transcription factors consists of the Myf5, MyoD, myogenin and MRF4 proteins (Charge *et al.*, 2004; Sabourin *et al.*,

2000; Seale *et al.*, 2000a; Song *et al.*, 1998; Zhou *et al.*, 1994) that are all found specifically in skeletal muscle (Wu *et al.*, 2000). Quiescent satellite cells have no detectable expression of the MRFs, but activated satellite cells are characterised by their rapid up-regulation (Charge *et al.*, 2004; Sabourin *et al.*, 1999; Seale *et al.*, 2000a). The MRFs drive the myogenic differentiation program at molecular level to bring about satellite cell differentiation into mature myotubes. The temporal MRF expression patterns during satellite cell activation, proliferation and differentiation during muscle regeneration are comparable to those during embryogenesis (Seale *et al.*, 2000a). As transcription factors, the MRF mode of action involves the induction of muscle-specific gene transcription. The bHLH transcription factors heterodimerise with other bHLH proteins before binding to a site (referred to as an E-box) in the promoter region of muscle specific genes (Sabourin *et al.*, 2000; Wu *et al.*, 2000).

The MyoD and Myf5 transcription factors are considered the primary MRFs, responsible for the commitment of satellite cells to myogenic differentation. The secondary MRFs, myogenin and MRF4 are responsible for the regulation of terminal differentiation (Sabourin *et al.*, 2000; Seale *et al.*, 2000a).

#### 1.3.5.1 Primary myogenic regulatory factors: Myf 5 and MyoD

Activated satellite cells express either Myf5 or MyoD initially, before coexpressing both MyoD and Myf5 (Cornelison *et al.*, 1997; Sabourin *et al.*, 2000; Seale *et al.*, 2000a). Interestingly, mice with a homozygous deletion of MyoD develop normally, however mice lacking both MyoD and Myf5 do not form skeletal muscle (Rudnicki *et al.*, 1992).

However, in the absence of MyoD, less activated myogenic progenitors progress through the differentiation program (Seale *et al.*, 2000a). Myf5 on the other hand plays an important role as a molecular determinant of satellite cell self-renewal (Charge *et al.*, 2004; Sabourin *et al.*, 2000; Seale *et al.*, 2000b).

MyoD is a key factor to the commitment of myoblasts to the differentiation program, and is greatly expressed in activated, proliferating myoblasts transitioning to differentiation (Charge *et al.*, 2004; Sabourin *et al.*, 2000; Seale *et al.*, 2000a). Besides activating muscle specific genes during myogenesis, MyoD expression also leads to cell cycle arrest (Song *et al.*, 1998); in a mechanism thought to involve transcriptional activation of the gene coding for the cell cycle inhibitor p21 (Langen *et al.*, 2004; Parker *et al.*, 1995; Sabourin *et al.*, 2000). This draws attention to an important consideration: Upon stimulation, the myogenic differentiation program is initiated in myoblasts, whereby expansion of the myogenic pool takes place, thus ensuring the provision of many new myonuclei for fusion and new myotube formation. Although myoblasts do undergo multiple rounds of proliferation upon activation, cell cycle exit is however required for myogenic differentiation, as proliferation and differentiation are two mutually exclusive events (i.e. mitogenic vs. myogenic stimuli) (Andres *et al.*, 1996).

#### 1.3.5.2 Secondary myogenic regulatory factors

The secondary MRFs are required downstream of MyoD and Myf 5 to further the differentiation of myoblasts into mature myofibers. Sequential up-regulation of

myogenin and MRF4 therefore takes place in myoblasts entering their terminal differentiation phase (Charge *et al.*, 2004; Hawke *et al.*, 2001; Sabourin *et al.*, 2000). Cell cycle arrest proteins (such as p21) are irreversibly activated allowing myoblasts to permanently exit the cell cycle (Charge *et al.*, 2004; Sabourin *et al.*, 2000; Song *et al.*, 1998).

While myogenin promotes the transcription of muscle-specific genes, the proposed role for MRF4 in the myogenic program appears to be extended beyond that of myogenin. MRF4 expression is found in myonuclei of newly formed myotubes and regenerated myofibres, at a time after fusion. It is therefore suspected to play a role in myofibre maturation (Charge *et al.*, 2004; Zhou *et al.*, 2001).

### 1.3.6 The MEF2 family of proteins

The MEF2 family of transcription factors binds directly to the promoters of most muscle-specific genes at A/T (Adenine/Thymine) rich DNA sequences. The MEF2 proteins act in conjunction with the MyoD transcription factors specifically, to initiate the skeletal muscle differentiation program (McKinsey *et al.*, 2002).

## 1.4 Response of satellite cells to muscle injury: role of growth factors

Skeletal muscle damage (myotrauma) ranges from macromolecule damage to tears in the sarcolemma, basal lamina and the supportive connective tissue, or even the contractile proteins of the myofiber (Vierck *et al.*, 2000). Direct physical trauma can be brought about by extensive physical exercise like resistance training, or lengthening contractions that lead to functional and histological signs of injury (Charge *et al.*, 2004;

Toumi *et al.*, 2003; Tsivitse *et al.*, 2005; Vierck *et al.*, 2000). Severe muscle injuries can be induced by either extreme cold; crushing, or toxins (Warren *et al.*, 2002). Indirect causes of muscle injury include inherent genetic defects (muscular dystrophies for example), (Charge *et al.*, 2004).

Damage to the myofiber leads to the release of a milieu of growth factors, cytokines and other agents and mediators from both the injured tissue as well as from the inflammatory response following injury (Shephard *et al.*, 1998; Tidball, 2002; Toumi *et al.*, 2003; Vierck *et al.*, 2000).

### 1.4.1 Growth factors/agents released from injured muscle following myotrauma

The growth factors referred to include hepatocyte growth factor (HGF); fibroblast growth factors (FGFs); insulin-like growth factors (IGFs); transforming growth factor beta TGFβ, and Interleukin (IL)-6 family of cytokines, including leukaemia inhibitory factor (LIF). Many of the agents released from the damaged area act as messengers and are chemo-attractants to inflammatory- and satellite cells (Bischoff, 1997; Vierck *et al.*, 2000). Platelet activating factor (Shephard *et al.*, 1998), myostatin, neural derived factors, nitric oxide, testosterone as well as ATP are other factors seem to play a role in the emerging picture of muscle regeneration (Charge *et al.*, 2004; Hawke *et al.*, 2001; Seale *et al.*, 2000a; Vierck *et al.*, 2000).

#### 1.4.1.1 Hepatocyte Growth Factor (HGF)

HGF, first identified as being a mitogen for mature hepatocytes (Hawke *et al.*, 2001), is now considered imperative to regulating satellite cells following muscle damage. It is

particularly important in the early phases of regeneration where it serves as a chemo-attractant to satellite cells (Bischoff, 1997; Hawke *et al.*, 2001), and stimulates the quiescent satellite cell to enter the cell cycle and commence proliferation (Charge *et al.*, 2004; Hawke *et al.*, 2001). HGF is released from the damaged myofibre during the first three days following injury (Jennische *et al.*, 1993) at levels directly proportional to the degree of injury (Hawke *et al.*, 2001; Tatsumi *et al.*, 2001; Vierck *et al.*, 2000). Its receptor, c-met, is expressed on quiescent satellite cells (Cornelison *et al.*, 1997; Hawke *et al.*, 2001), myoblasts and adjacent myofibres (Hawke *et al.*, 2001).

Although the HGF/c-met pathway is important for expanding the mpc population during the early stage of regeneration, down regulation of this pathway (and mostly all mitogenic stimuli) is necessary for differentiation to commence (Coolican *et al.*, 1997; Wu *et al.*, 2000).

#### 1.4.1.2 Fibroblast Growth Factors (FGFs)

Nine different isoforms of FGF have been identified, (Hawke *et al.*, 2001) of which FGF-6 is muscle specific (Floss *et al.*, 1997), and up-regulated in response to muscle damage (Floss *et al.*, 1997). Although FGF's precise role in muscle regeneration remains unclear (Charge *et al.*, 2004), its regulation of the satellite cell population is analogous to that of HGF in that it promotes proliferation of the mpc population without increasing their subsequent differentiation (Charge *et al.*, 2004; Hawke *et al.*, 2001).

Both FGF and HGF receptors (FGFR and c-met respectively) are transmembrane receptor tyrosine kinases (Charge *et al.*, 2004) that dimerise upon ligand binding, become auto-phosphorylated and subsequently activate downstream signalling events.

#### 1.4.1.3 Insulin-like Growth Factors (IGFs)

IGF-I and –II play an important role in the growth and development of various tissues, and are up-regulated during muscle regeneration. IGF-I and –II promote both expansion of the myogenic pool as well as differentiation of myoblasts *in vitro*. IGF-I may promote muscle regeneration by promoting satellite cell differentiation and cell survival, chiefly through a phosphatidylinositol 3-kinase (PI3K) pathway (Charge *et al.*, 2004; Coolican *et al.*, 1997; Hawke *et al.*, 2001).

#### 1.4.1.4 IL-6 family of cytokines

IL-6 and LIF are members of an IL-6 family of cytokines expressed both by myoblasts and macrophages (part of the immune system – see section below). While LIF promotes proliferation of satellite cells, IL-6 function involves (amongst others) degradation of necrotic tissue, and synchronising the cell cycle in satellite cells (Hawke *et al.*, 2001).

All the abovementioned growth factors (together with the agents released as part of the inflammatory reaction – discussed below) are necessary for the activation, chemotaxis and proliferation of satellite cells in a temporal and concentration-dependent manner. The concentrations of these factors are only transiently increased following acute injury

as differentiation can only occur once these growth factors have been withdrawn (ie mitogenic vs myogenic stimuli).

### 1.5 Inflammation is necessary for muscle regeneration

The immune response to infection is analogous to the immune response elicited by muscle damage (Vierck *et al.*, 2000). Depending on the extent of damage, and the ensuing inflammatory response, inflammation can be considered a functionally beneficial response. Growth factors and cytokines released from injured muscle fibres (described above) are responsible for activating local inflammatory cells residing in the muscle, that in turn, chemotactically attract circulating inflammatory cells (Rappolee *et al.*, 1992; Tidball, 1995) and satellite cells (Vierck *et al.*, 2000) to the site of injury.

#### 1.5.1 Neutrophils and macrophages govern an immune response to myotrauma

The early phase of muscle injury is characterized by the activation and rapid invasion of the injured muscle by inflammatory cells (Charge *et al.*, 2004; Hawke *et al.*, 2001; Seale *et al.*, 2000a; Shephard *et al.*, 1998; Tidball, 2002; Vierck *et al.*, 2000).

Neutrophil numbers are particularly increased at the site of damage within an hour of the injury (Anastasi *et al.*, 1997; Toumi *et al.*, 2003). First to arrive at the site of injury, neutrophils have a phagocytic function (Lowe *et al.*, 1995; Shephard *et al.*, 1998), and release proteases and reactive oxygen species (ROS) that serve to degrade the cellular debris resultant of muscle damage. Although removal of cellular debris is an important contribution to muscle regeneration, neutrophils can be harmful to muscle as

neutrophil-derived ROS and proteases molecules damage healthy tissues as well (Tidball, 2002; Toumi *et al.*, 2003).

Following neutrophil infiltration, macrophages become the dominant inflammatory cell peaking around 48h post-injury (Charge *et al.*, 2004; Hawke *et al.*, 2001; Orimo *et al.*, 1991; Tidball, 1995). Similar to neutrophils, macrophages phagocytose cellular debris (Charge *et al.*, 2004; Seale *et al.*, 2000a; Shephard *et al.*, 1998; Vierck *et al.*, 2000) and necrotic myofibers as well (Hawke *et al.*, 2001). Macrophages play a central role in the immune response to myotrauma, and are a rich source of leukotrienes, prostaglandins, various growth factors and cytokines such as IL-1, ,IL-6, IL-8, IL-10, IL-12, LIF, interferon-β, and importantly, TNFα (Hawke *et al.*, 2001; Shephard *et al.*, 1998; Tidball, 2005; Vierck *et al.*, 2000). The proinflammatory cytokine tumour necrosis factor alpha (TNFα) is produced in large amounts by activated macrophages (Saghizadeh *et al.*, 1996), and has been recognised as a mitogenic (Li, 2003) factor for myoblasts. TNFα and its dose dependent effects on satellite cell differentiation is the central focus of this thesis.

### 1.6 TNFα as a relevant factor in muscle adaptation and regeneration

TNFα is barely detectable in the serum of healthy individuals at picogram-per-milliliter levels (Li *et al.*, 2000; Parissis *et al.*, 1999). Myocytes are a source of TNFα as they constitutively synthesize the cytokine, as do healthy human and rat skeletal muscle tissue (Kuru *et al.*, 2003; Li, 2003; Saghizadeh *et al.*, 1996). Following muscle injury, TNFα expression largely increases (Broussard *et al.*, 2003; Collins *et al.*, 2001; Warren *et al.*, 2002); from two sources (Li, 2003): Macrophages infiltrate the damaged area and

also release TNFα as part of the inflammatory response (discussed previously). TNFα mRNA levels are up regulated for 24 – 48 hours before returning to basal levels in C2C12 cells given a differentiation stimulus (i.e. serum withdrawal) (Li *et al.*, 2001b). Furthermore, circulating TNFα levels are found to increase markedly following strenuous exercise in healthy individuals (Camus *et al.*, 1998; Ostrowski *et al.*, 1999). At the same time, TNFα expression is increased in injured muscle fibers (De Bleecker *et al.*, 1999). The up-regulation of TNFα expression by injured muscle is not merely a response to inflammation as it is not proportionately associated with the grade of inflammation (Li, 2003). Since elevated levels of TNFα are present at the time when muscle regeneration processes are initiated, the cytokine can be considered a relevant factor in skeletal muscle regeneration and adaptation.

TNFα is expressed as a 26kDa integral transmembrane precursor from which a soluble 17kDa subunit is proteolytically cleaved and released (Natoli *et al.*, 1998; Warren *et al.*, 2002). As key mediator of the inflammatory response, TNFα is known to play important roles in (amongst others) the chemotaxis of leukocytes, the expression of adhesion molecules on neutrophils and endothelial cells, and the regulation of production of other pro-inflammatory cytokines (Collins *et al.*, 2001). When blocking TNFα, the production of other pro-inflammatory cytokines such as IL-1;-6, and-8 is inhibited (Butler *et al.*, 1995; Haworth *et al.*, 1991). TNFα could therefore be pictured at the very apex of a cytokine cascade such that many major pro-inflammatory cytokines are linked in cascade to it (Andreakos *et al.*, 2002). As chronically elevated levels of TNFα are associated with chronic inflammatory diseases such as rheumatoid

arthritis, many treatment strategies are aimed against the cytokine due to the very fact that it is at an apex of a pro-inflammatory cytokine cascade (Andreakos *et al.*, 2002).

In addition to being a prime mediator of the immune system, TNFα is also implicated in growth and differentiation of many cell types (Li, 2003; Li *et al.*, 2001b; Tracey *et al.*, 1993). It has for example been shown that TNFα elicits a hypertrophic growth response in cardiac myocytes, a cell type comparible to skeletal myocytes (Yokoyama *et al.*, 1997).

### 1.7 TNF Receptors

The diverse range of effects of TNFα are mediated by two distinct transmembrane cell surface receptors, TNFR-1 (also known as p55TNFR, p60, CD120a) and TNFR-2 (also known as p75TNFR, p80, CD120b) (Li *et al.*, 2001a; MacEwan, 2002; Natoli *et al.*, 1998; Sack, 2002). Both receptors are expressed simultaneously on virtually all cell types (Natoli *et al.*, 1998). TNFR are transmembrane receptors (MacEwan, 2002) that signal as homotrimers upon ligand (TNFα) binding (Baker *et al.*, 1996; Balkwill, 2000). TNFα ligand is itself presented in a trimerised form (Natoli *et al.*, 1998). Signal transduction following ligand binding occurs mainly via TNFR1, where signalling through TNFR2 remains less well characterized (Natoli *et al.*, 1998; Sack, 2002).

#### 1.7.1 TNF signalling activates both pro-survival and pro-apoptotic pathways

The cytoplasmic domain of TNFR1 contains a so-called death domain (DD) to which a number of associating transducer molecules, each with their respective DDs, bind and thereby signal cell death (MacEwan, 2002). TNF receptor–associated death domain

(TRADD) is a vital adaptor to TNFR1 as it recruits further downstream transducers: Fas-associated death domain (FADD), TNF receptor-associating factor 2 (TRAF2) and receptor interacting protein (RIP) (Gaur *et al.*, 2003; MacEwan, 2002; Natoli *et al.*, 1998) (Figure 1.3).

FADD carries out the cell death action associated with TNFR1 by interacting with the death circuitry, including caspase-8, through its own death effector domain (DED). Caspases are cysteine-aspartate-directed proteases responsible for apoptotic cell death via destruction of "the cell's own repair mechanisms". Caspases-2, -8, -9, and -10 are so-called apoptotic initiator caspases, whereas caspases -3, -6, and -7 are executioner caspases and responsible for carrying out cell death mechanisms (MacEwan, 2002).

TRAF2 and RIP, on the other hand, are not involved in the induction of cell death. TRAF2 also interacts directly with TNFR2, thereby forming a link between TNFR1, and -2 and explains the somewhat overlapping responses seen between the two receptors (Natoli *et al.*, 1998).

TRAF2 is required for the activation of two further downstream signalling molecules (MacEwan, 2002; Natoli *et al.*, 1998). Interaction of the protein kinase nuclear factor kappa B (NFκB)-inducing kinase (NIK) with TRAF2 leads to the phosphorylation and subsequent inactivation of the NFκB inhibitory subunit (IκBα). This sets into motion the activation of the transcription factor NFκB and its subsequent translocation to the nucleus where it regulates transcription of genes involved in (amongst others) immune responses, anti-apoptosis responses, as well as cell growth and proliferation (Gaur *et*

*al.*, 2003; Hsu *et al.*, 1996; Li *et al.*, 2001a; MacEwan, 2002). RIP does contain a kinase sequence, but its role as kinase has not yet been ascertained (MacEwan, 2002). RIP has however been linked to NFκB activation (Devin *et al.*, 2003; MacEwan, 2002).

The second pathway activated by TRAF2 involves the activation of the stress activated protein kinases (SAPK), p38 MAPK and JNK (MacEwan, 2002; Natoli *et al.*, 1998). Activated SAPK phosphorylate a number of transcription factors including c-Jun, activating transcription factor-2 (ATF2), and AP-1 (Natoli *et al.*, 1998), and MEF2 (Ono *et al.*, 2000).

TNFR1 stimulation therefore leads to antagonistic signals in the target cell. Whilst cell death is signalled for through FADD, gene transcription is activated through TRAF2-dependent pathways (Figure 1.3). How the fate of a cell (i.e. cell death vs. gene transcription) is ultimately determined remains to be elucidated. Although NFκB is activated within minutes of TNF receptor activation, the transcription and translation of cytoprotective genes would take far longer than it would the cell death circuitry to become effective. Yet, most cells are resistant to apoptosis except when TNF is co-supplemented with protein/RNA synthesis inhibitors such as cycloheximide. It follows that cytoprotective messages independent of NFκB exist in the cell. Signals inhibiting the cell death machinery are another possibility considered for explaining cytoprotective signals dominating those of cell death (Natoli *et al.*, 1998).

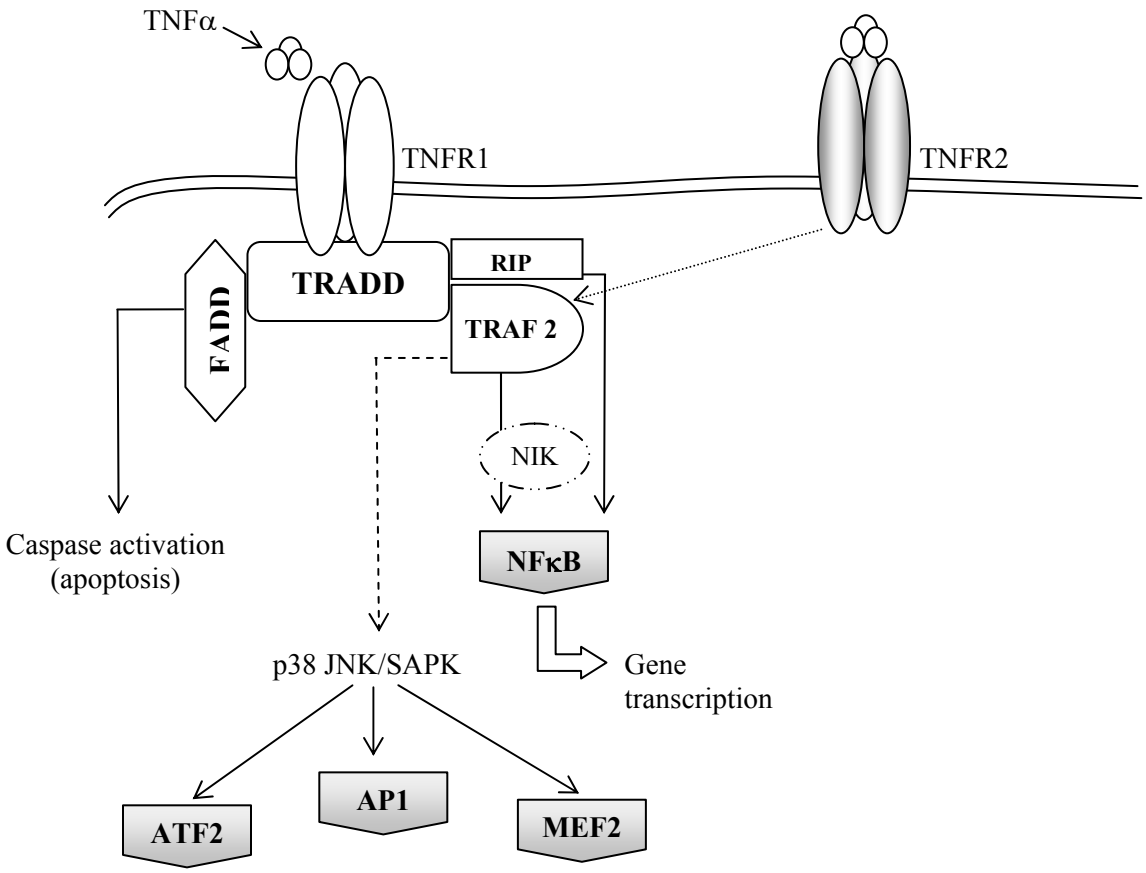



**Figure 1.3** TNFα signalling induces both pro-apoptotic and pro-survival pathways.

### 1.7.2 Other signalling effects in response to TNFR stimulation

TNFRs interact with an adaptor protein called neutral SMase activation factor (FAN), which is responsible for the degradation of sphingolipids into ceramide-containing signalling intermediates (Kolesnick *et al.*, 1998). A signalling intermediate further downstream of TNFR is protein kinase C (PKC). PKC reportedly phosphorylates (and so inactivates) the secondary MRF myogenin (Sabourin *et al.*, 2000; Zhou *et al.*, 1994).

As TNFα is implicated in a wide array of cellular processes of both beneficial and detrimental consequence to the cell, it could be well understood that TNFα signalling is indeed extremely complex, and well beyond the scope of this thesis. A more comprehensive description of TNFα signalling was excellently reviewed by MacEwan (MacEwan, 2002).

## 1.8 Molecular mechanisms governing myogenesis

Myogenesis is a multi-step process that involves the activation of satellite cells, expansion of the myogenic pool capable of donating myonuclei, the expression of muscle specific markers such as the MRFs, exit from the cell cycle, differentiation into myocytes and subsequent fusion into myotubes (Cabane *et al.*, 2004; Cabane *et al.*, 2003; Langen *et al.*, 2002; Layne *et al.*, 1999; Lee *et al.*, 2002). The myogenic transcription factors of the MRF and MEF2 families of transcription factors (described previously) regulate the expression of vital muscle-specific genes such as myosin heavy and light chains and muscle creatine kinase (Cabane *et al.*, 2003). Extracellular signals largely contribute to the decision of the cell to differentiate or not. It has been noted that when extracellular signals favour myogenesis instead of mitogenesis,

myoblasts undergo cell cycle arrest at the early stage of differentiation. Furthermore, induction of cell cycle inhibitors such as p21 and p57, together with myogenin, prevents cells from re-entering the cell division cycle (Lee *et al.*, 2002). The intracellular pathways regulating the initiation of myogenesis and the muscle specific transcription factors have not been fully identified. The extracellular regulated kinase (ERK) and p38 mitogen activated protein kinase (p38 MAPK) from the mitogen activated protein kinase (MAPK) family (Lee *et al.*, 2002); together with the IGF/phosphatidylinositol-3-kinase (PI3K) pathway have all been implicated in myogenesis (Cabane *et al.*, 2004; Wu *et al.*, 2000). The MAPKs are indeed important components of intracellular pathways relaying extracellular signals to transcription factors in the nucleus (Lavoie *et al.*, 1996; Ono *et al.*, 2000; Wu *et al.*, 2000). The MAPK pathway is constituted of a series of kinase activation events, initiated at the plasma membrane and culminates in the nucleus with the phosphorylation of nuclear transcription factors.

At least three MAPK families have been identified namely the extracellular signal-regulated kinases (ERKs), the c-jun NH3-terminal kinases (JNK)/stress activated protein kinase (SAPK), and the p38 MAPK family (Clark *et al.*, 2003; Jones *et al.*, 2005; Wu *et al.*, 2000). Each of the MAPK pathways are ordered as parallel signalling cascades (Figure 1.4) in which MAPK kinase kinases (MAPKKK) phosphorylate dual specificity MAPK kinase (MAPKK or MKK), which in turn phosphorylate both threonine and tyrosine residues and finally activating the MAPK of the pathway (Clark *et al.*, 2003; Ono *et al.*, 2000). ERKs are activated by MEK1 and -2, JNK by MKK 4 and -7, and p38 by MKK 3 and -6 (Clark *et al.*, 2003; Ip *et al.*, 1998; Robinson *et al.*,

1997). Whilst ERK is activated by mitogenic signals, p38 and JNK are activated by proinflammatory and stressful stimuli (Clark *et al.*, 2003).

As mentioned previously, ERK and p38 MAPK, have been implicated in the regulation of the myogenic program (Wu *et al.*, 2000).

### 1.8.1 ERK involvement in myogenesis

The ubiquitously expressed ERKs are strongly activated by mitogenic signals (Cabane *et al.*, 2003; Lavoie *et al.*, 1996). Despite being implicated in myogenesis, their exact involvement is controversial, with some reports deeming the ERK pathway necessary for muscle differentiation whilst others hold that an inhibition of ERK activity promotes myogenesis (Lee *et al.*, 2002; Wu *et al.*, 2000). ERK appears to play a biphasic role in myogenesis where its inhibition during the early phases of myoblast differentiation, and a subsequent increase in activity at the late stages of differentiation is required for normal myogenesis (Wu *et al.*, 2000).

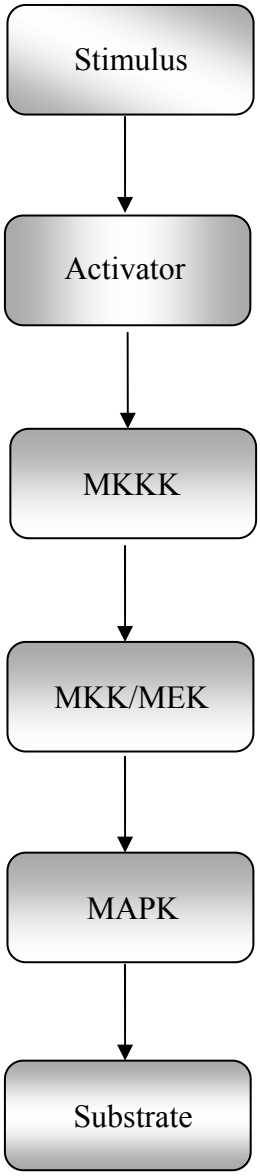



**Figure 1.4** Schematic illustration of the parallel signaling cascade leading to MAPK activation.

### 1.8.2 p38 MAPK as a modulator of myogenesis

Four isoforms of the p38 MAPK family have been identified: p38 MAPKα, -β, -γ, and -δ. The p38α and p38β isoforms are ubiquitously expressed, whilst p38γ is predominantly expressed in skeletal muscle (Enslen *et al.*, 2000; Ono *et al.*, 2000). Induction of differentiation by serum removal is a common method for studying myogenesis *in vitro* (Wu *et al.*, 2000) (see section on "studying skeletal muscle differentiation *in vitro*"). In a study conducted by Wu (Wu *et al.*, 2000), serum removal leads to the induction of p38 activity in C2C12 myoblasts within 1 day, and continues to increase for three days, correlating with the time around which myotubes become visible. Sustained p38 activity therefore appeared to be part of the muscle differentiation pathway, distinct from the pathways stimulated by cytokines, and stress (Wu *et al.*, 2000). p38 was found to be functionally linked to muscle differentiation as its activation preceded accumulation of myogenin and MyoD (Wu *et al.*, 2000).

Recent studies with kinase inhibitors such as SB-203580 (inhibits p38α, and -β isoforms), recent studies confirm that p38 MAPK plays an important role in muscle differentiation (Cabane *et al.*, 2003). Interestingly, such inhibition led to proliferation, as opposed to differentiation of H9c2 cardiac myoblasts in differentiation medium (Lee *et al.*, 2002). In a study conducted by Cabane et al, (Cabane *et al.*, 2003) for example, it was shown that inhibition of p38 blocks the expression of both early (MyoD, p21, and p27) and late (MHC, and myosin light chain isoform 3F) markers of differentiation. Furthermore, an inability to form myotubes was evidenced in cultures treated with the p38 inhibitor SB-203580. It was concluded from the aforementioned study that a

functional p38 MAPK pathway is required for both the induction and completion of differentiation (Cabane *et al.*, 2003).

Interestingly, the role of p38α/β *in vivo* appears to be the opposite of that found in cell lines. A study by Weston (Weston *et al.*, 2003) indicated that in the context of the developing limb, myogenesis was significantly enhanced following p38α/β inhibition. The study further highlighted the importance of assessing myogenesis in the presence of non-myogenic factors as well (Weston *et al.*, 2003). Further research is required to elucidate the current disparities in the literature.

Many effects of p38 are mediated by its substrate MAPK activated protein kinase (MAPKAPK-2) (Clark *et al.*, 2003; Ono *et al.*, 2000). Activated MAPKAPK-2 phosphorylates further downstream substrates such as heat shock protein 27 (HSP27) (Stokoe *et al.*, 1992), serum response factor (SRF) (Heidenreich *et al.*, 1999), and various transcription factors including ATF-2, p53, MEF2A, and MEF2C (Ono *et al.*, 2000).

A major function of p38MAPK pathway is the regulation of pro inflammatory gene expression in cells of the immune system. p38MAPK is, as such, identified as a potential therapeutic target in inflammatory disease (Ono *et al.*, 2000).

### 1.8.3 The phosphoinositide-3-kinase (PI3-kinase) pathway

The PI3-kinase pathway is activated greatly by IGF, a factor released following muscle damage (Cabane *et al.*, 2004; Wu *et al.*, 2000). The IGF-PI3-kinase pathway appears to

act in parallel with the p38 MAPK pathway, both these pathways being vital for myogenesis (Cabane *et al.*, 2004; Wu *et al.*, 2000). Interestingly, Akt (also known as protein kinase B/PKB), a key downstream substrate to PI3K, was found to be a further downstream effector of p38 (Cabane *et al.*, 2004). An interaction between the IGF-PI3-kinase- and p38 MAPKs pathways was therefore suggested (Cabane *et al.*, 2004).

### 1.9 Differential effects of TNFα on muscle

A wide variety of studies on the effects of TNFα on the skeletal muscle differentiation program are found in the literature. The emerging picture is that TNFα has an effect on all aspects of myogenesis from activation and proliferation of satellite cells to cell cycle exit, expression of markers of differentiation such as myogenin and MHC, to myotube formation (Guttridge *et al.*, 2000; Langen *et al.*, 2001; Langen *et al.*, 2004; Layne *et al.*, 1999; Li, 2003; Li *et al.*, 2000; Li *et al.*, 2001b; Li *et al.*, 1998).

Most reports in the literature classify TNFα as an agent that negatively regulates myogenesis. However, an interesting and relevant finding by Li & Schwartz (Li *et al.*, 2001b) indicates that TNFα mRNA level and activity is up regulated for up to 48 hours in C2C12 myoblasts responding to serum restriction. What is more, the endogenous TNFα stimulated "muscle gene expression" as measured by MHC expression. The aforementioned effect was abolished by the introduction of TNFα-neutralizing antibodies (Li *et al.*, 2001b). It was concluded from the aforementioned study that TNFα is required for the normal differentiation program in muscle (Li *et al.*, 2001b). The findings from the aforementioned study further substantiate the consideration that TNFα is a relevant factor in muscle regeneration.

Studies of cultures treated with exogenous TNFα have mostly contrasting outcomes on differentiation.

### 1.9.1 Anabolic effects of TNFα

In a study by Li (Li, 2003), TNFα was shown to exert a mitogenic effect on satellite cells. Proliferating primary myoblasts incubated with recombinant TNFα at concentrations ranging from 2 to 6ng/ml for 24h, showed a significantly progressive increase in DNA content (indicating enhanced proliferation). Moreover, TNFα (6ng/ml, for a 16h incubation period) increased the number of proliferating myoblasts incorporating BrdU, suggesting increased DNA synthesis as a result of faster cell cycle progression from G1 to S phase (Li, 2003). The study further illustrated that TNFα is capable of activating quiescent satellite cells to enter the cell cycle. Li therefore demonstrated that TNFα not only enhanced satellite cell proliferation once initiated (so called progression factor) (Li, 2003), but that it also stimulated normally quiescent satellite cells to proliferate (so called competence factor) (Li, 2003).

### 1.9.2 Catabolic effects of TNFα

TNFα's involvement in myogenesis has only recently been recognized. The cytokine was first identified as a factor reducing tumour size whilst causing muscle wasting in tumour-bearing rats (Warren *et al.*, 2002) and has long been associated with cachectic muscle wasting (Li *et al.*, 2001b), a chronic wasting disease characterised by disproportionate skeletal muscle loss seen in many chronic disease states (Langen *et al.*, 2002; Langen *et al.*, 2004). TNFα was in fact once referred to as cachectin in acknowledgment of its catabolic actions (Li *et al.*, 2000; Li *et al.*, 2001b). Muscle wasting is commonly found in individuals suffering chronic conditions such as cancer, acquired immunodeficiency syndrome (AIDS), chronic obstructive pulmonary disease (COPD), and chronic heart failure (Anker *et al.*, 1997; Guttridge *et al.*, 2000; Schols *et*

*al.*, 1998; Weinroth *et al.*, 1995). The ensuing loss of muscle mass contributes to weakness and fatigue in affected individuals (Li *et al.*, 1998). Muscle wasting in the aforementioned diseases contributes significantly to morbidity and mortality in affected individuals (Langen *et al.*, 2004; Li *et al.*, 2000; Li *et al.*, 1998). Elucidating the relevant factors and mechanisms involved in such muscle wasting is therefore of clinical relevance.

It is significant that muscle wasting seen in chronic wasting conditions is associated with an elevation in circulating inflammatory cytokine levels, particularly TNFα. TNFα is, as such, proposed to be a major trigger of the series of catabolic events that constitute muscle wasting (Langen *et al.*, 2001; Langen *et al.*, 2004; Li *et al.*, 1998). Support for this is evidenced by the diminished muscle mass found in animals treated with exogenous TNFα (Buck *et al.*, 1996), or in experimental conditions mimicking disease states exhibiting presenting elevated endogenous TNFα levels (Li *et al.*, 2001a), such as tumour implantation (Tessitore *et al.*, 1993). Muscle wasting is manifested as a loss of muscle protein such as myosin heavy chain (MHC), (a major myofibrillar protein characteristic of later stages- and terminal differentiation) although the mechanisms underlying such catabolism remain unclear. TNFα induces loss of muscle specific proteins (like MHC) (Langen *et al.*, 2001; Li *et al.*, 1998) and as such it is clearly implicated as a mediator of muscle wasting.

Muscle wasting could be considered as an anabolism-catabolism imbalance: where muscle degradation exceeds that of muscle protein synthesis (Langen *et al.*, 2001). Such an imbalance was indeed found in the case of experimental models of cancer

cachexia (Buck *et al.*, 1996) and sepsis (Vary *et al.*, 1996) where inflammatory mediators were proposed to trigger the wasting events. Little is known about the proposed direct effects of TNFα on muscle (Li *et al.*, 1998). Earlier studies (Goodman, 1991) explored the catabolic actions of TNFα *in vivo*, subjecting rats to supra-physiologic concentrations of the cytokine (Garcia-Martinez *et al.*, 1993). Contrary to expectations, no significant change in protein breakdown was found in these studies, leading researchers to believe that a direct role for TNFα in muscle breakdown/proteolysis does not exist (Garcia-Martinez *et al.*, 1993; Goodman, 1991). Investigators have since also exposed excised skeletal muscle to supra-physiologic concentrations of TNFα for periods as short as 3h, finding no significant alterations in protein breakdown (or amino acid release) (Goodman, 1991; Rofe *et al.*, 1987). Findings from such studies lead researchers to conclude that TNFα's role in muscle breakdown is not a direct one. Cell culture protocols have become a widespread means of studying inflammatory cytokine effects on skeletal muscle. However, studying skeletal muscle *in vitro*/ in culture does have its shortcomings. An important consideration is that the environment the cells are grown in are devoid of many of the anabolic (and other) factors found in a whole body system that could affect the outcome of the body's response to the factor being investigated. Cell culture models do however remain a valuable tool for studying satellite cell differentiation *in vitro*.

### 1.9.3 Effect of TNFα on C2C12 cells

Various researchers have assessed the direct effects of inflammatory cytokines including TNFα on differentiated C2C12 myotubes. It was found in one such study that there was a concentration dependent decrease in MHC protein in myotubes treated with

TNFα (1-10ng/ml) every 24h (Li *et al.*, 1998). The same research group confirmed their cell culture based findings, published in 2001, when they again found a significant concentration dependent decrease in MHC protein in TNFα treated C2C12 myotubes (Li *et al.*, 2001b). However, this in contrast with the studies investigating the direct effects of TNFα on muscle described above.

An experiment by Langen and his group (Langen *et al.*, 2001) compared the effects of pro-inflammatory cytokines (on C2C12 cells), both during myogenic differentiation and on already differentiated myotubes. After treating differentiated myotubes with TNFα at doses ranging from 1-50 ng/ml for 72 hours, the overall effects of TNFα on the myotubes were insignificant and occurred only at the very highest dose (50ng/ml) supplemented (Langen *et al.*, 2001). A dose dependent increase in total protein content was in actual fact found, thereby excluding the possibility that the cytokine exerted any direct catabolic effects on the myotubes, - a finding contradictory to that of Li *et al* 1998, 2001 (discussed above). Allowing myoblasts to differentiate in the presence of TNFα on the other hand, did have a significant outcome on their subsequent differentiation (Langen *et al.*, 2001). C2C12 myoblasts treated with varying doses of TNFα (0.1-10 ng/ml) for 72h, showed a dose dependent suppression of differentiation markers myogenin and MHC (Langen *et al.*, 2001). Moreover, addition of the cytokine (particularly the 1ng/ml and 10ng/ml doses) prevented the formation of myotubes.

In a further study by Langen *et al* (Langen *et al.*, 2004) C2C12 cells were again allowed to differentiate in the presence of TNFα (10ng/ml) for either 24 or 48 hours. The treated cells exhibited attenuated muscle specific gene mRNA levels (Langen *et*

*al.*, 2004). The up regulation of muscle specific gene transcripts during normal differentiation was markedly attenuated in the cultures differentiated in the presence of TNFα (Langen *et al.*, 2004). Both MyoD gene transcripts and protein levels were significantly decreased by 72h post serum-restriction in the TNFα treated cultures (Langen *et al.*, 2004). The outcome of a further experiment in the same study indicated that TNFα prevented cell cycle exit of myoblasts through sustained expression of the cyclin D1 protein. TNFα's apparent effect on the differentiation of myoblasts would explain part of its involvement in muscle pathology.

The muscle differentiation program of C2C12 cells allowed to differentiate in the presence of 20ng/ml of TNFα for 72 hours was inhibited, as evidenced by reduced expression of the myogenin and MyoD proteins (Guttridge *et al.*, 2000). TNFα is known to be an effective activator of NFκB (Guttridge *et al.*, 2000; Li *et al.*, 2001a). NFκB positively regulates cell proliferation by regulating the expression of the cell cycle progression factor cyclin D1 (Guttridge *et al.*, 2000). This presents one possible mechanism by which TNFα hinders the differentiation program of myoblasts, again referring to the mitogenic properties TNFα has in skeletal muscle (Li *et al.*, 2003).

The findings from the above and similar studies draws attention to the consideration that inflammatory cytokine involvement in muscle wasting of chronic wasting syndromes such as cancer, AIDS, COPD etc may indeed be indirect (as previously suggested). By inhibiting the formation of myotubes from satellite cells TNFα reduces the regenerative capacity of muscle.

### 1.9.4 TNFα in cytokine-hormone interactions

An indirect way of promoting protein loss would be to negatively regulate the hormones/anabolic growth factors regulating muscle growth such as IGF-I (Broussard *et al.*, 2003), insulin, glucagon, thyroid hormone and catecholamines (Layne *et al.*, 1999; Tessitore *et al.*, 1993) for example. IGF-I is an anabolic factor known to stimulate protein synthesis, myofiber hypertrophy and stimulate myogenesis (Broussard *et al.*, 2003; Layne *et al.*, 1999; Strle *et al.*, 2004). TNFα obstructs IGF-I mediated muscle growth by inhibiting functionally important phosphorylation of major docking proteins of the IGF-I receptor, thereby inducing a state of IGF-I receptor resistance (Broussard *et al.*, 2003). Low doses of TNFα (0.01 – 1ng/ml) dose-dependently attenuated IGF-I stimulated protein synthesis (including that of myogenin) in C2C12 myoblasts (Broussard *et al.*, 2003). It was shown that ceramide, a sphingosine-based lipid second messenger (Strle *et al.*, 2004) is required for proinflammatory cytokine attenuation of IGF action (Strle *et al.*, 2004).

Reactive oxygen species (ROS) form part of proinflammatory cytokine signaling (Garg *et al.*, 2002). Skeletal myocytes treated with proinflammatory cytokines (including TNFα) resulted in the production of ROS and reactive nitrogen species (Williams *et al.*, 1994). Reactive oxygen intermediates (ROI) are implicated in both pro-survival and pro-apoptotic signaling (Garg *et al.*, 2002) and are required for the activation of several signalling molecules (of the TNF-stimulated signaling pathway) including NFkB, activated protein-1 (AP-1), JNK, MAPK, as well as the induction of apoptosis (Garg *et al.*, 2002; Langen *et al.*, 2002).

Oxidative stress has, however, also been implicated in muscle wasting (Langen *et al.*, 2002). Chronically elevated proinflammatory cytokine levels would significantly alter the redox state of the cell (through TNFα-induced ROS). ROS may influence several signalling pathways (including ERK, JNK, and NFκB) (Langen *et al.*, 2002) and mitochondrial respiration. It could further be directly linked to muscle protein breakdown as oxidation of sulfhydryl groups on contractile proteins could lead to degradation of contractile proteins, particularly in chronic disease states (Supinski, 1998). TNFα's dose- and time dependent effects could perhaps, in part, be ascribed to elevated levels of ROS accompanying (chronically) elevated levels of TNFα.

The findings from studies such as the ones described herein, further brings to attention the fact that the effects of TNFα are dose- and time dependent. However, an in depth characterisation of the dose dependent effects of TNFα on satellite cell differentiation is required, to obtain more insight into its mechanisms of action.

## AIMS OF THE STUDY

The aims of the current thesis were to:

1) Characterise the temporal patterns of expression of the following proteins during differentiation of C2C12 cells
    - MyoD,
    - Myogenin,
    - p21,
    - PCNA, and
    - p38 MAPK for the purposes of subsequently
2) Investigating the differential effects of chronically supplemented low, medium and high doses of TNFα on differentiating satellite cells on the expression of these proteins.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 *In vitro* model: C2C12 cell cultures

### 2.1.1 Culture conditions

The C2C12 murine skeletal muscle satellite cell line was obtained from the European Collection of Cell Cultures (ECACC). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Highveld Biological (PTY) Ltd, RSA) supplemented with 10% fetal bovine serum (Highveld Biological (PTY) Ltd, RSA), 2mM L-glutamine (Sigma Aldrich), and antibiotics (1% Penicillin/Streptomycin (PenStrep), Highveld Biological (PTY) Ltd, RSA) (here on referred to as culture medium). Cells were cultured in T75 flasks (75cm$^2$ flasks, Greiner Bio One) until 80% confluent (i.e covering 80% of the culture flask surface), at which time cells were split and seeded into T25 (25cm$^2$) flasks (at a seeding density of 300 000 cells per T25) in culture medium for a further 24 hours. Following the 24 hours, culture medium was replaced by differentiation medium: DMEM supplemented with 2% horse serum (Sigma Aldrich), 2mM L-glutamine, and antibiotics (as in culture medium above) to initiate differentiation. Cultures treated with TNFα received differentiation medium supplemented with TNFα (recombinant murine TNFα, Cytolab Ltd, Israel) at either low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses (Table 2.1). Fresh differentiation medium (vehicle, and in the aforementioned doses) was added every 24 hours for 7 days. All cells were cultured in an environment of 95% humidity, 20% $O_2$, 5% $CO_2$ at 37°C, and were handled in sterile laminar flow hoods.

**Table 2.1.** Varying doses of TNFα supplemented to differentiation medium of C2C12 cell cultures

| Dose description | [Stock] mg/ml | Final dose ng/ml |
|---|---|---|
| Control | ~ | ~ |
| Low dose TNFα | 1 | 0.5 |
| Medium dose TNFα | 10 | 5 |
| High dose TNFα | 20 | 10 |

## 2.2 Harvesting and preparing cells for analysis

Cells were harvested at specific points along the course of their differentiation: at days 0, 1, 3, 5, 7 (Figure 2.1). Cultures to be harvested were washed with cold Phosphate Buffered Saline (PBS) prior to the addition of lysis buffer composed of 2.5 mMTris/HCl, 1mM EDTA, 1mM EGTA, 250mM sucrose, 50mM NaPPi, 1mM DTT, 0.1mM PMSF, 1% (v/v) Nonidet P-40, 0.1% (m/v) SDS, 0.5% (v/v) Na Deoxycholate, and a cocktail of protease inhibitors. Flasks were incubated on ice for 2-5 minutes before adherent cells were scraped from the culture flask, and the whole cell lysates were further sonicated before being stored at -80°C for protein determination and further analysis Sample protein concentration was determined by the Bradford protein determination method (Bradford, 1976). Aliquots of each sample (containing either 20 or 30μg protein depending on gel lane volume restrictions) were made and stored at -80°C for analysis by western blotting.

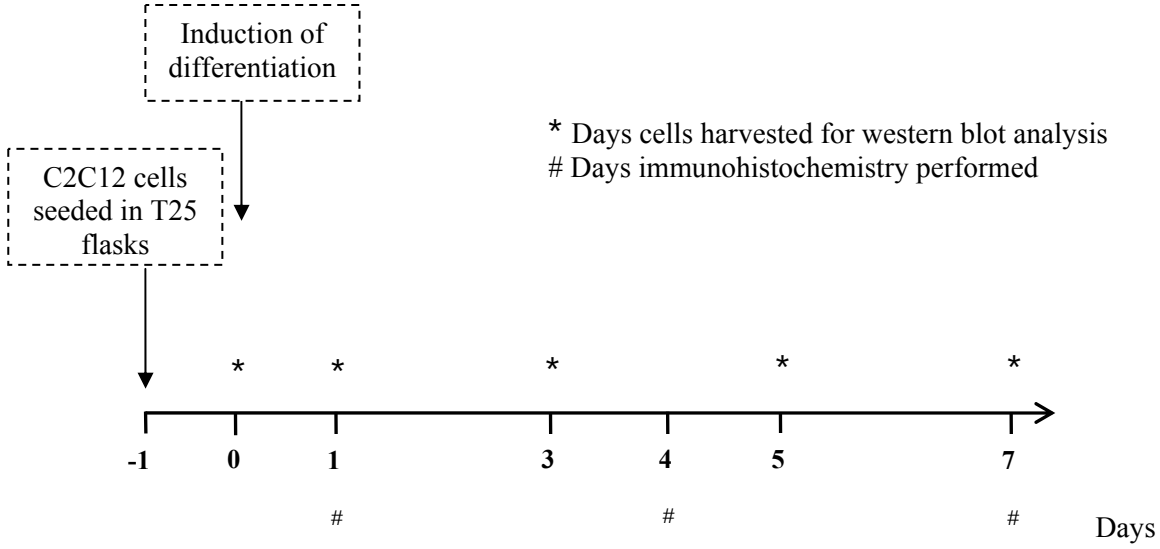



**Figure 2.1** Schematic representation of the study design. C2C12 cells were treated every 24 hours with fresh differentiation medium, or medium supplemented with increasing doses of cytokine (see text). * Time points at which cells were harvested for western blot analysis. # Time points at which immunohistochemistry was performed.

### 2.3 Western blot analysis

Whole cell lysates were separated on 10% polyacrylamide gels by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). A prestained protein marker (peqGOLD) was loaded on each gel to verify molecular weight of specific bands. 20 or 30μg of protein (sample description above) was loaded per lane. Gels were run for 75 minutes (or until the front moved off the gel), at 130V (constant) and 400mA (Mini Protean System, Bio-Rad, Hercules). Following SDS-PAGE, proteins were transferred to polyvinylidine fluoride (PVDF) membranes (Immobilon, Millipore, USA) via wet transfer (100V (constant), 350mA, 65 minutes). To prevent non-specific binding, membranes were blocked in Blotto (5% (w/v) non-fat dried milk powder in Tris Buffered Saline-Tween20 (TBS-T, 0.05%)) for 1-2 hours at room temperature. Membranes were incubated with primary antibodies (diluted in either Blotto (for Santa-Cruz antibodies) or TBS-T (for Cell signalling antibodies)) against the desired proteins (Table 2.2) at 4°C for (minimum) 24 hours. Following 2 wash steps (TBS-T) of 7.5 minutes each, membranes were incubated in either anti-rabbit (Amersham Biosciences, UK limited, and Dako Cytomation, Denmark) or anti-mouse (Dako Cytomation, Denmark) horseradish peroxidase-conjugated secondary antibody for 1-1.5 hours at room temperature. Following the incubation period, membranes were washed for a further three times, 5 minutes in TBS-T, followed by a final 15 minute wash step in TBS. Antibodies were detected with enhanced chemiluminescence (ECL) kit (Amersham Biosciences, UK limited), and bands were visualised on x-ray film (Hyperfilm, Amersham Biosciences, UK limited). Bands visualised were quantified by densitometry with the UN-SCAN-IT© program (Silk Scientific Corporation, Utah, USA).

**Table 2.2.** Primary antibodies used in western blot analysis.

| | Molecular weight (kDa) | Species raised in | Manufacturer | Dilution |
|---|---|---|---|---|
| MyoD | 38 | rabbit | Santa Cruz* | 1/100 |
| Myogenin | 43 | mouse | Santa Cruz* | 1/200 |
| p21 | 21 | mouse | Santa Cruz* | 1/100 |
| PCNA | 45 | mouse | Santa Cruz* | 1/200 |
| phospho-p38α/β MAPK | 43 | rabbit | Cell Signaling# | 1/500 |
| Total p38α/β MAPK | 43 | rabbit | Cell Signaling# | 1/500 |

*Santa Cruz, Santa Cruz, CA
# Cell Signaling, MA, USA

### 2.3.1 Statistical analysis

Data were expressed as mean ± SEM. Due to the small sample size (n=3-7), non parametric tests were conducted. A statistician advised the employment of the Kruskal Wallis ANOVA for comparisons between the 4 groups (control and 3 TNFα treated groups) at a specific time point. Dunn post hoc analysis was performed if a significant difference ($p<0.05$) was found. When wanting to compare control cultures to only one of the treated groups (control vs. high dose TNFα treated cultures for example), a Mann Whitney U test was conducted. A Bonferroni type correction was then subsequently made whereby only a P-value of $p<0.017$ was considered significant. All statistical analyses were conducted with the aid of *Statistica* version 7 software.

### 2.4 Immunohistochemistry

C2C12 cells for staining were cultured in 8 chamber plates (Nunc, NY, USA), and induced to differentiate as described previously. Cells at day 1, 4, and 7 of their differentiation program were manipulated for immunostaining (See Appendix A for schematic representation of immunohistochemistry procedure). Half of the wells served as PBS controls to the remainder of the treated wells. Wells were washed once with sterile PBS (0.1M, for staining) before fixing with 1:1 methanol-acetone for 10 minutes at 4°C, thereafter allowed to air dry for 20 minutes, and subsequently rinsed with PBS. Non-specific binding was blocked by incubating in 5% donkey serum (in PBS) for 20 minutes at room temperature. The following steps were conducted in a dark and moist environment. Primary antibodies (in PBS, see Table 2.3 for dilutions and descriptions) were added to wells of the 8 chamber plate (excluding PBS control wells) for 90 minutes at room temperature.

**Table 2.3.** Primary antibodies used for immunostaining of C2C12 cells.

| | **Species raised in** | **Manufacturer** | **Dilution** |
|---|---|---|---|
| MyoD | rabbit | Santa Cruz | 1/50 |
| Myogenin | mouse | Santa Cruz | 1/50 |
| phospho-p38α/β MAPK | rabbit | Cell Signaling | 1/50 |

Wells were rinsed with PBS following the incubation period, where after secondary antibodies were added to all wells for a further 30 min (see table 2.4 for dilutions). The nuclear marker Hoechst (1/200) was then added to all wells for a 10 minute incubation period. Wells were again washed thoroughly with PBS, and a drop of mounting fluid

added to each well. Images of stained cells were captured immediately or within two weeks of staining. Images were captured on an Olympus IX81 microscope fitted with CellR® software.

**Table 2.4**. Secondary antibodies used in immunostaining of C2C12 cells.

| Secondary antibody | Fluorescent marker conjugated to antibody | Manufacturer | Dilution |
|---|---|---|---|
| Donkey anti-mouse | Fluorescein Streptavidin (FITC) | Invitrogen, CA, USA | 1/200 |
| Donkey anti-rabbit | Fluorescein Streptavidin (FITC) | Invitrogen, CA, USA | 1/200 |
| Horse anti-mouse | Texas Red Streptavidin | Jackson ImmunoResearch | 1/200 |

# CHAPTER 3: RESULTS

## 3.1 Protein expression profiles in a differentiation model

Satellite cell differentiation over time was visualised by phase contrast microscopy (Figure 3.1). Following the induction of differentiation, myoblast cytoplasm becomes more profuse. Cell extensions become evident by day 3, and myotube formation is prominent by day 5. By day 7 the majority of cells have differentiated and some degree of myotube fusion has taken place.

The temporal cellular protein expression profiles of various markers in differentiating C2C12 cells were first investigated in our model (Figures 3.2 – 3.5).

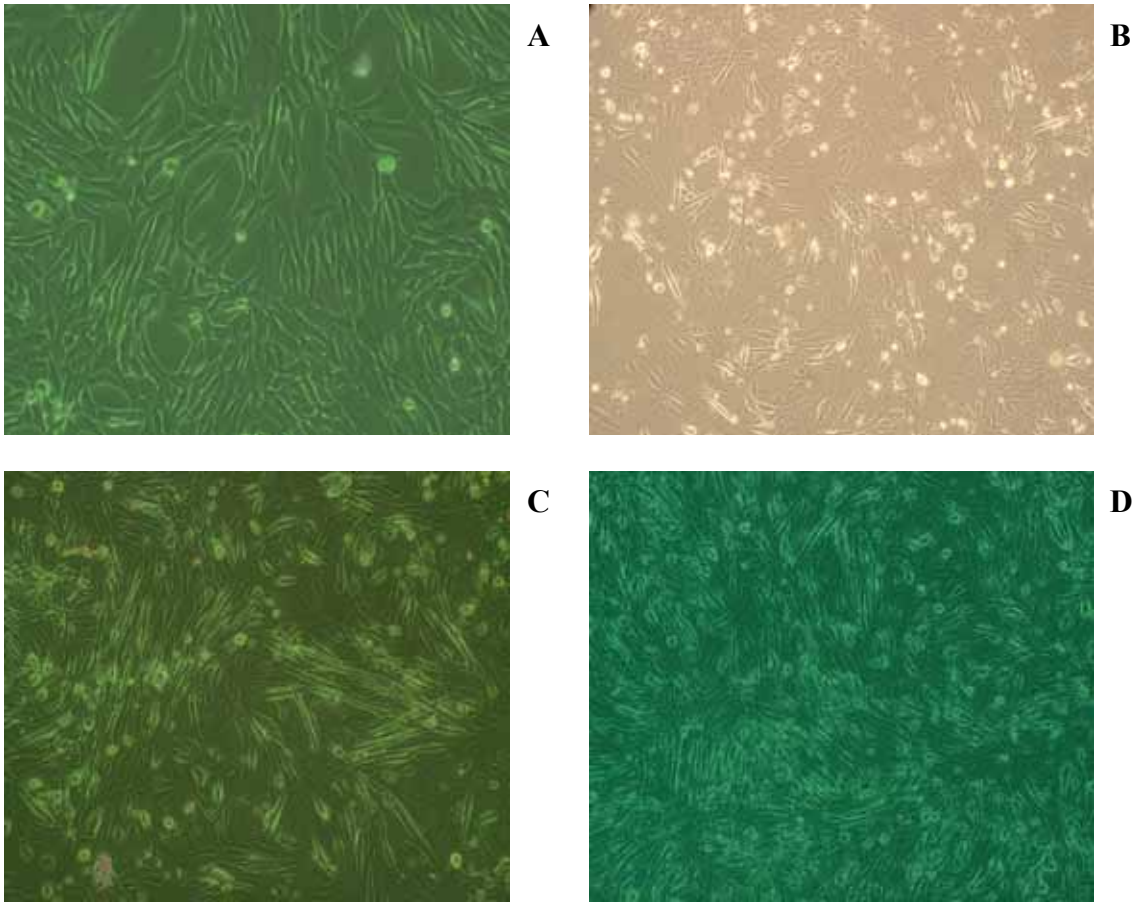



**Figure 3.1** Differentiation of C2C12 cells: Day 1 (A), Day 3 (B), Day 5 (C), Day 7 (D). Phase contrast images were taken with (A, C and D), or without (B) a green filter. Photographs were taken using a 20X objective.

### 3.1.1 MyoD and Myogenin

The MRFs MyoD and Myogenin are widely used as markers of differentiation. Their protein expression patterns during satellite cell differentiation were, as such, assessed as part of our study.

The primary MRF, MyoD (Figure 3.2A) is required for the commitment of myoblasts to differentiation. As expected, a dramatic increase in MyoD protein was evidenced between baseline (day 0) and day 1 (i.e. over the first 24 hours following the differentiation stimulus). MyoD protein expression was reduced between days 1 and 3, and sustained between days 3 and 5. Interestingly, MyoD protein displayed a biphasic mode of expression, as an elevation in MyoD protein was again seen between days 5 and 7 of the differentiation program in our model.

Myogenin, as a secondary MRF, is characterised as a marker of middle to terminally differentiated myotubes. As such, no detectable levels of myogenin expression were found at baseline (Figure 3.2B). Myogenin protein expression increased steadily up to day 5 in our model, prior to a drop in myogenin between days 5 and 7.

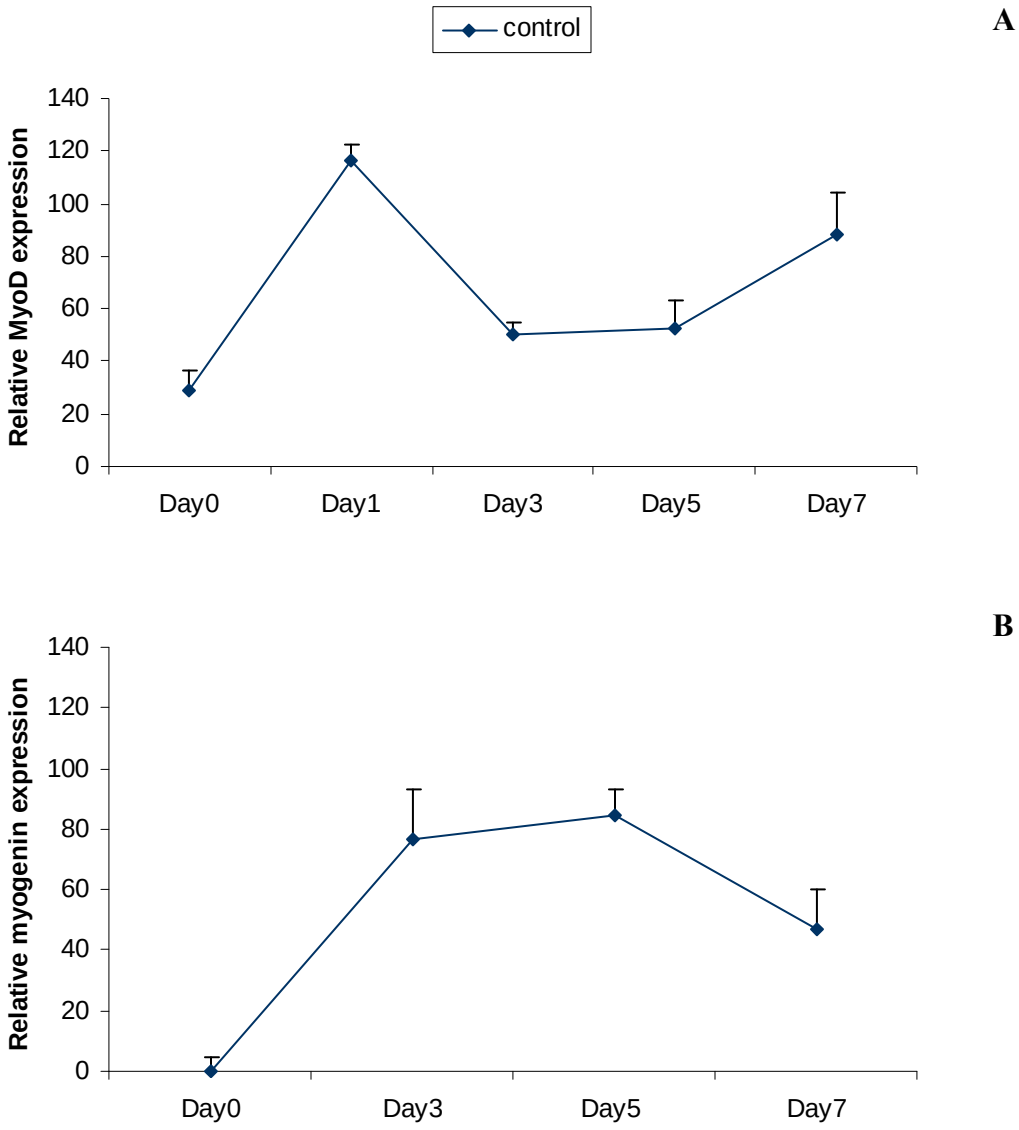


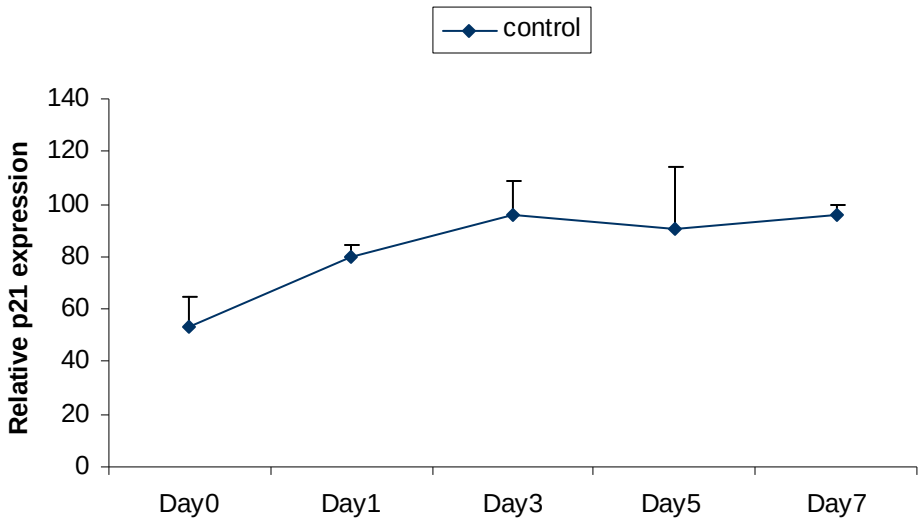

**Figure 3.2** MyoD (A) and myogenin (B) protein expression profile in differentiating C2C12 cells. Error bars indicate SEM for 3 independent experiments.

### 3.1.2 p21 and PCNA, markers of the cell cycle.

As the cell cycle (and its subsequent inhibition) is such an important component of the differentiation pathway, we further went on to assess the protein expression of p21 and PCNA. This allowed us to provide a more complete characterisation of the C2C12 cell differentiation process and the subsequent effects of TNFα thereon (see 3.2 below).

p21 expression in our model (Figure 3.3A) showed a steady rise for three days following the induction of differentiation at day 0. There after, p21 protein leveled off and remained elevated up to day 7, the last time point for measurement in our study.

PCNA was assessed as a marker of proliferation in the present study. No marked changes in the protein expression profile of PCNA were detected for the first 5 days following the induction of differentiation (Figure 3.3B). A slight elevation in PCNA expression was however maintained for three days following the differentiation stimulus, followed by a return to (approximately) basal level at day 5. A considerable increase in PCNA expression between days 5 and 7 was detected in our model.



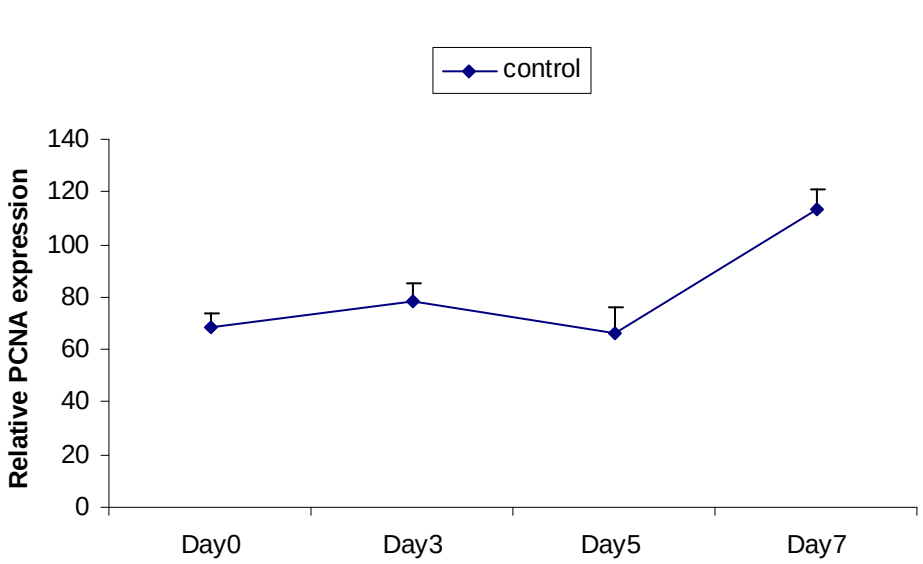




**Figure 3.3** Protein expression profile of p21 (A), PCNA (B) in differentiating C2C12 cells. Error bars indicate SEM for 3 independent experiments.

### 3.1.3 p38 MAPK

p38 MAPK as integral signaling molecule in myogenesis was assessed in this study. Total p38 remained relatively constant over time. A marked increase in p38α/β phosphorylation was detected over the first 24 hours following the differentiation stimulus (Figure 3.4). A drop in such phosphorylation followed between days 1 and 5. p38 MAPK in our model exhibited a biphasic pattern of phosphorylation as a rise in phospho-p38 again occurred between days 5 and 7 of the differentiation program. The phospho-p38 pattern of expression described here is analogous to that of MyoD.

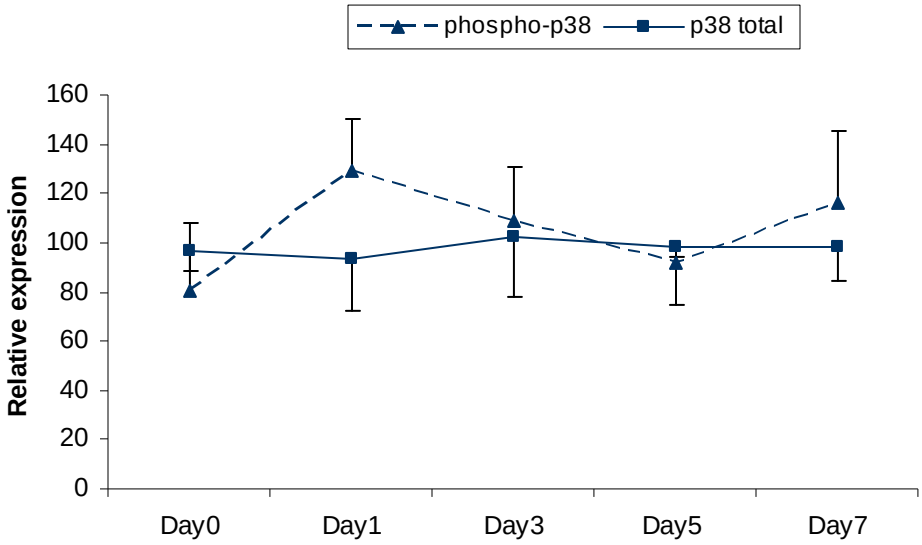



**Figure 3.4** Ratio of phosphorylated- to total p38α/β in differentiating C2C12 cells. Error bars indicate SEM for 3 independent experiments.

### 3.2 Dose-dependent effects of TNFα on differentiating satellite cells

The graphs presented from hereon forth illustrate the dose-dependent effects of chronically supplemented TNFα on differentiating satellite cells. Protein expression levels (as determined by western blot analysis) are expressed relative to each harvest day control, such that control values equal 1 (or otherwise stated 100%) and the dose-dependent effects are thereby visualized relative to the control value.

#### 3.2.1 MyoD expression: cells commited to myogenic differentiation

Compared to controls, all TNFα treated cultures showed a trend towards elevated MyoD protein at day 1 (Figure 3.5). Low and medium dose cytokine treated cultures reached near significance compared to controls (Mann Whitney U test control vs. low $p=0.036$; control vs. medium $p=0.036$). With continued TNFα supplementation however, MyoD protein seemed to decrease in cytokine treated cells relative to controls at days 3 and 5. A slight (though statistically insignificant) elevation in MyoD protein in low dose (0.5ng/ml) TNFα treated cultures compared to controls seems possible for day 7, whereas medium- (5ng/ml), and high dose (10ng/ml) treated cultures showed a trend towards decreased MyoD protein, similar to that seen at days 3 and 5.

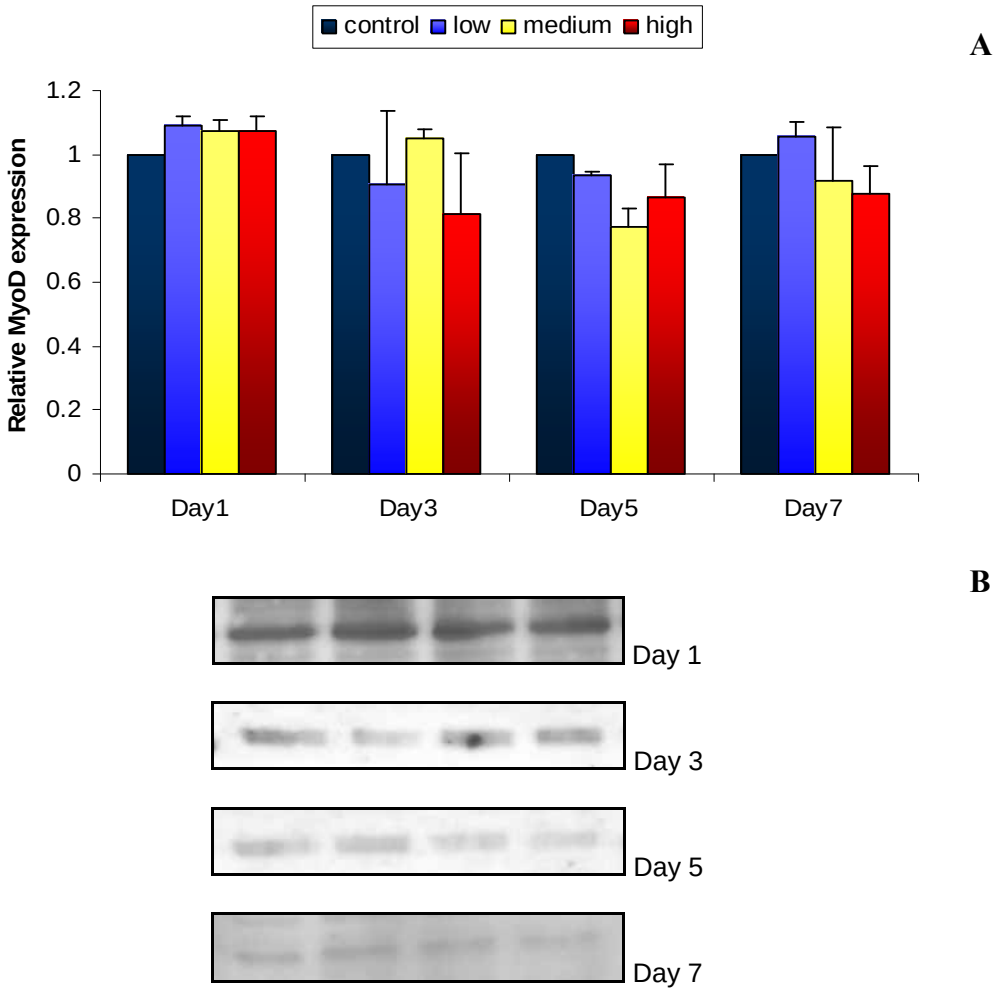



**Figure 3.5** Schematic representation of MyoD protein expression relative to harvest day control levels (Mean ± SEM, n=3) (A). Representative western blot (B).

Myoblasts were allowed to differentiate in the presence or absence of TNFα at low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses for 7 days. Cultures were treated every 24 hours where fresh differentiation medium (controls) or medium supplemented with TNFα was administered. Whole cell lysates were analysed by western blotting techniques.

### 3.2.2 Myogenin as a marker of middle to terminally differentiated myotubes

As a marker of middle to terminally differentiated myotubes, myogenin was assessed from day 3 onwards (Figure 3.6). In the present study, supplementation with a high dose of TNFα negatively regulated myogenin expression more so than supplementation with any of the other doses. Although statistically insignificant at days 3 and 5, a trend towards decreased myogenin expression in high dose treated cultures (relative to controls) was evident at the aforementioned timepoints. A dose dependent decrease in myogenin protein in all cytokine treated cultures became evident at day 7, where high dose TNFα treated cultures exhibited significantly less myogenin protein compared to controls (Mann Whitney U test, control vs. high $p<0.017$).

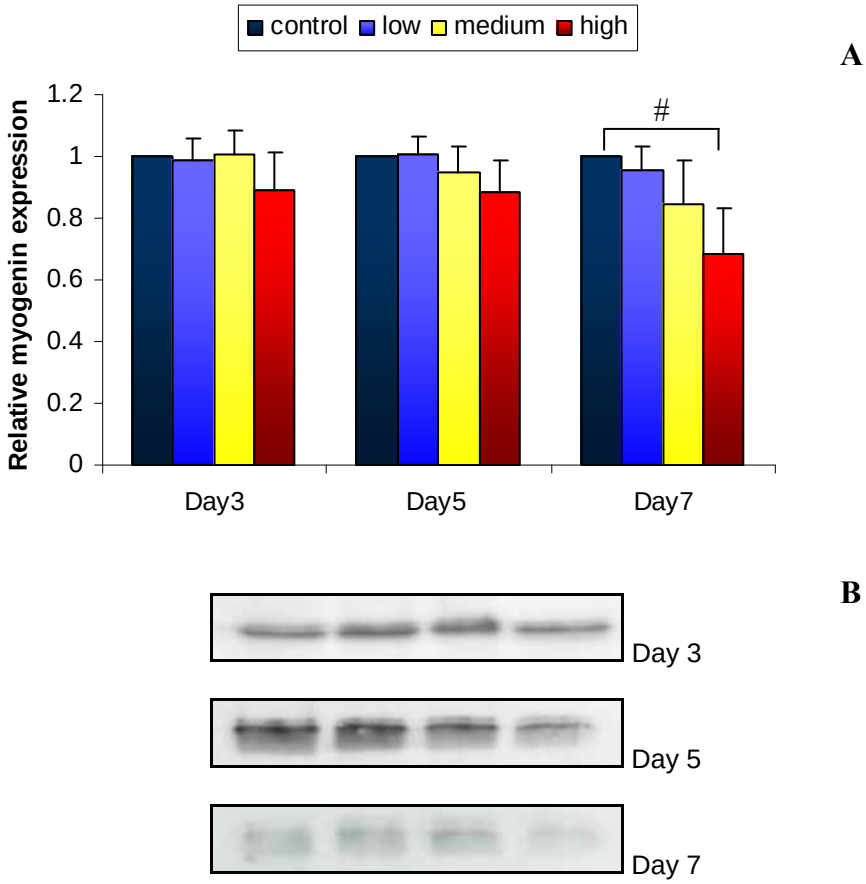



**Figure 3.6** Schematic representation of myogenin expression relative to harvest day control levels (Mean ± SEM, day 3, 5 n=7; day 7 n=4) (A). Statistical analysis: Mann Whitney U test control vs. high dose, # $p < 0.017$. Representative western blot (B).

Myoblasts were allowed to differentiate in the presence or absence of TNFα at low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses for 7 days. Cultures were treated every 24 hours where fresh differentiation medium (controls) or medium supplemented with TNFα was administered. Whole cell lysates were analysed by standard western blotting techniques.

### 3.2.3 Cell cycle inhibition: promotion of differentiation by p21

The pattern of p21 protein expression in treated cells was similar to that of MyoD: At day 1, TNFα treated cultures displayed elevated levels of p21 protein compared to controls (Figure 3.7). Low and medium dose cytokine treated cultures reached near significance when compared to controls (Mann Whitney U test control vs. low $p=0.036$; control vs. medium $p=0.036$). With continued TNFα supplementation, this seemingly positive effect on differentiating myoblasts was reversed. At day 3, a trend existed for TNFα treated cultures to show a slight decrease in p21 protein compared to controls. A dose dependent decrease in p21 was observed at day 5 in TNFα treated cultures ($p<0.05$, Kruskal Wallis ANOVA). Post hoc analysis indicated that high dose treated cultures showed significantly less p21, and therefore cell cycle exit, compared to control cultures (Dunn post hoc analysis). Similar to the trend of MyoD expression in treated cells at day 7: p21 expression was slightly elevated in low dose cytokine treated cultures, but reduced in medium- and high dose treated cultures relative to controls.

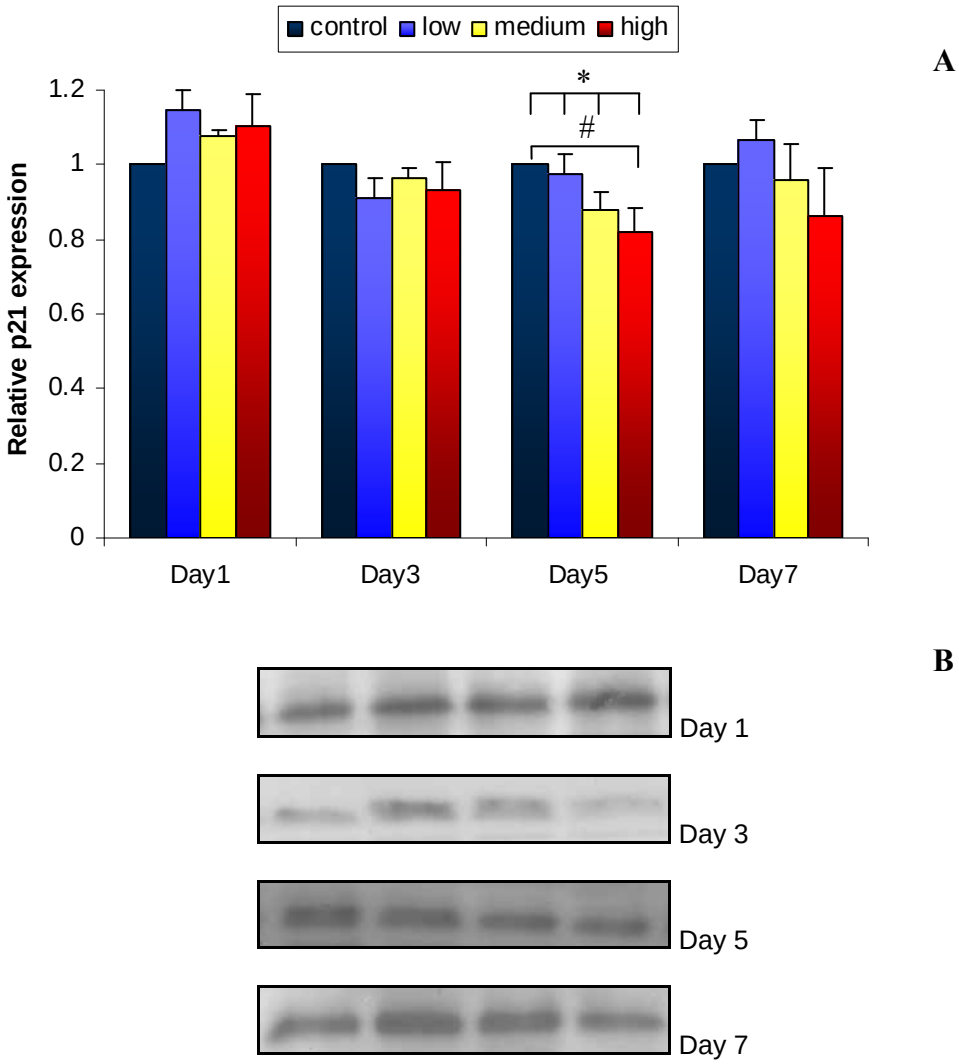



**Figure 3.7** Schematic representation of p21 expression relative to harvest day control levels (Mean ± SEM, day 1, 3, 5 n=3; day7 n=5) (A). Statistical analysis: Kruskal Wallis ANOVA, *$p<0.05$, # $p<0.05$ (Dunn post hoc test). Representative western blot (B).

Myoblasts were allowed to differentiate in the presence or absence of TNFα at low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses for 7 days. Cultures were treated every 24 hours where fresh differentiation medium (controls) or medium supplemented with TNFα was administered. Whole cell lysates were analysed by standard western blotting techniques.

### 3.2.4 PCNA

Proliferation of C2C12 cells in our model was assessed by PCNA expression in differentiating satellite cells (Figure 3.8)

No significant difference in PCNA protein existed between control and TNFα treated cultures (Figure 3.8). A slight (statistically insignificant) trend for increased PCNA protein with increasing doses of cytokine does however, seem possible at day 5. PCNA was not assessed at day 1.

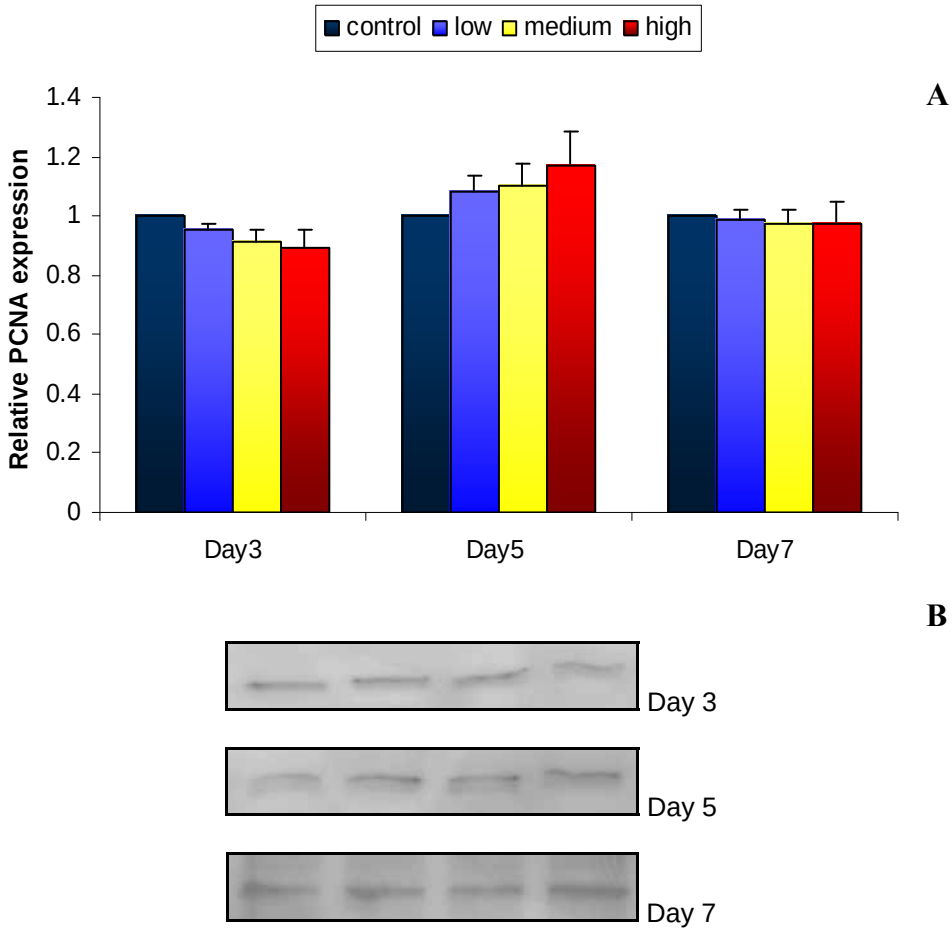



**Figure 3.8** Schematic representation of PCNA expression relative to harvest day control levels (Mean ± SEM, n=3) (A). Representative western blot (B).

Myoblasts were allowed to differentiate in the presence or absence of TNFα at low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses for 7 days. Cultures were treated every 24 hours where fresh differentiation medium (controls) or medium supplemented with TNFα was administered. Whole cell lysates were analysed by standard western blotting techniques.

### 3.2.5 p38 MAPK.

The differential effects of TNFα on an integral pro-differentiation signalling pathway were assessed at the hand of p38α/β MAPK (Figure 3.9). The addition of TNFα did not significantly alter the phosphorylation of p38 at day 1 (except for a slight statistically insignificant elevation with high dose TNFα supplementation). With continued TNFα supplementation however, a dose dependent decrease in phospho-p38 followed at day 3 of the differentiation program was observed (Kruskal Wallis ANOVA $p<0.05$). Although less phospho-p38 was found in all cytokine treated cultures at day 5, this finding was not significant. A reversal in the pattern seen at days 3 and 5 was evident at day 7, as there was a dose dependent increase in phospho-p38 at this time point (Kruskal Wallis ANOVA $p<0.05$). Post hoc analysis indicated that high dose treated cultures had significantly higher p38 phosphorylation compared to controls.

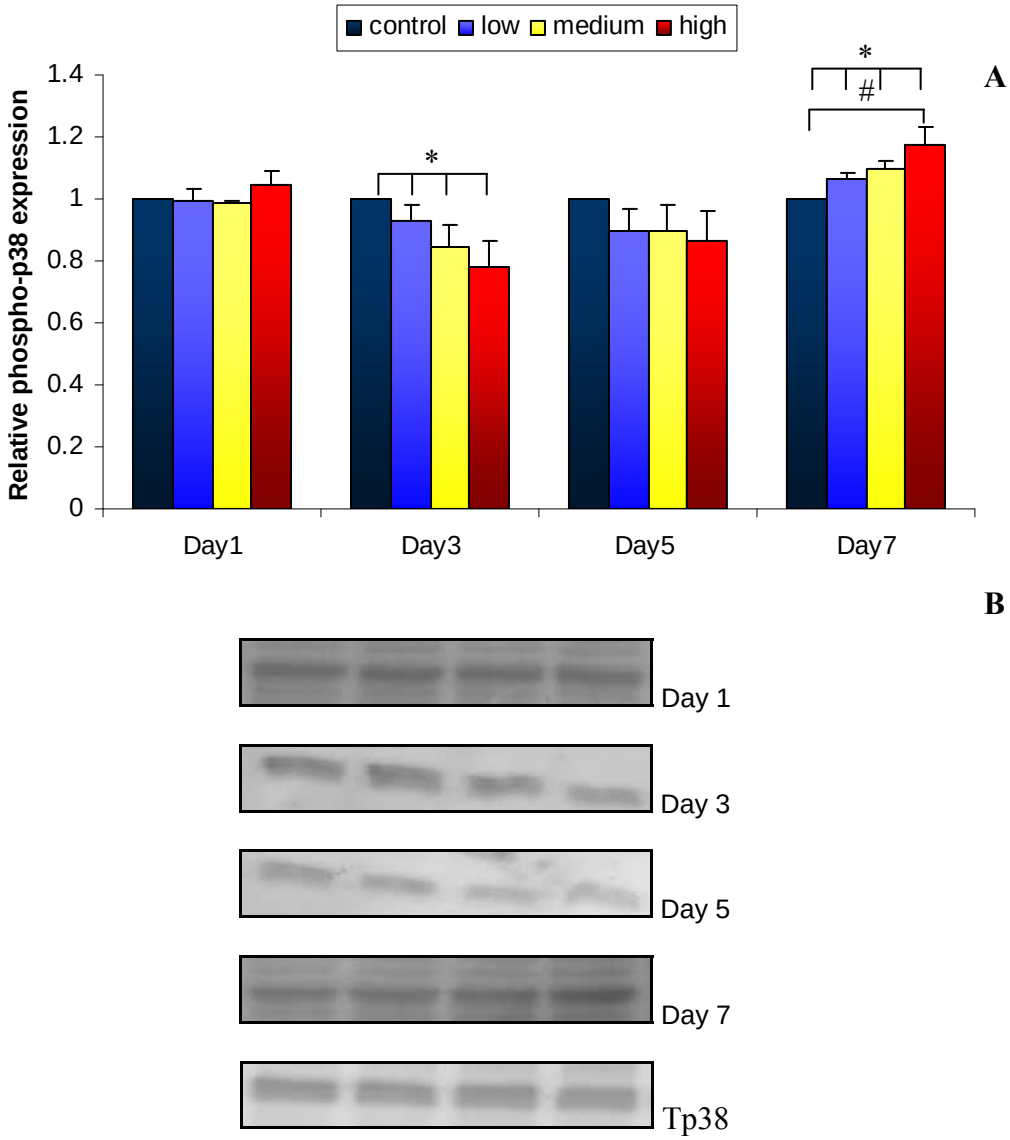



**Figure 3.9** Schematic representation of the ratio of phospho-p38α/β relative to harvest day control levels (Mean ± SEM, day 1, n=3 day 7, n=4) (A). Statistical analysis: Kruskal Wallis ANOVA *$p<0.05$, # $p<0.05$ (Dunn post hoc test). Representative western blot (B). Tp38 = representative total p38 blot.

Myoblasts were allowed to differentiate in the presence or absence of TNFα at low (0.5ng/ml), medium (5ng/ml), or high (10ng/ml) doses for 7 days. Cultures were treated every 24 hours where fresh differentiation medium (controls) or medium supplemented with TNFα was administered. Whole cell lysates were analysed by standard western blotting techniques.

### 3.3 Immunohistochemistry

A subsequent set of experiments were conducted to visualise certain markers at days 1, 4, and 7.

Day 1 cells were stained for MyoD (Figure 3.10). A trend for the movement away from a diffuse cytosolic staining to a more nuclear staining of immunoreactive MyoD protein with increasing dose of TNFα was visualised (Figure 3.10 A-D). Otherwise stated, it appears that a dose dependent nuclear localisation of immunoreactive MyoD was evident at day1.

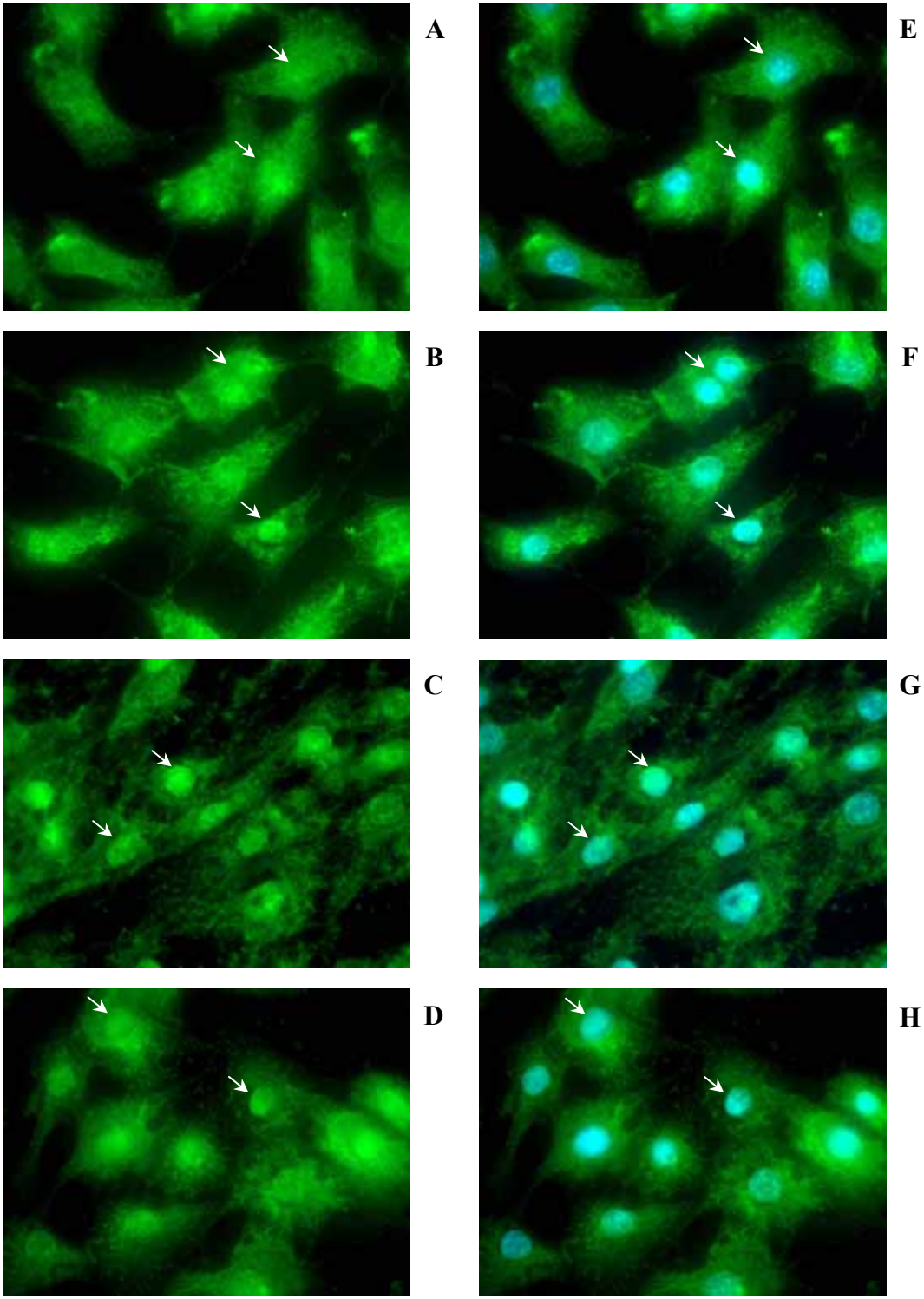



**Figure 3.10** MyoD staining. Immunohistochemistry of day 1 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left hand panel (A-D) shows cells stained for MyoD (FITC, green). The right hand panel (E-H) shows merged signals, MyoD (FITC), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

Day 4 cells were stained for MyoD (Figure 3.11) and myogenin (Figure 3.12). Merged signals of both markers plus the nuclear stain Hoechst are depicted in Figure 3.13.

Cells treated with a high dose of TNFα (Figure 3.11B) displayed a reduced amount of immunoreactive MyoD protein (particularly in the nucleus) when compared to control cells (Figure 3.11A) at day 4. Please note that where the secondary antibody to MyoD was FITC linked (green) for day 1 staining, the secondary antibody to MyoD at day 4 was Texas red linked, hence the incontinuity in MyoD immunofluorescence.

Less immunoreactive myogenin protein was detected in high dose treated cells (Figure 3.12 B) compared to controls (Figure 3.12 A).

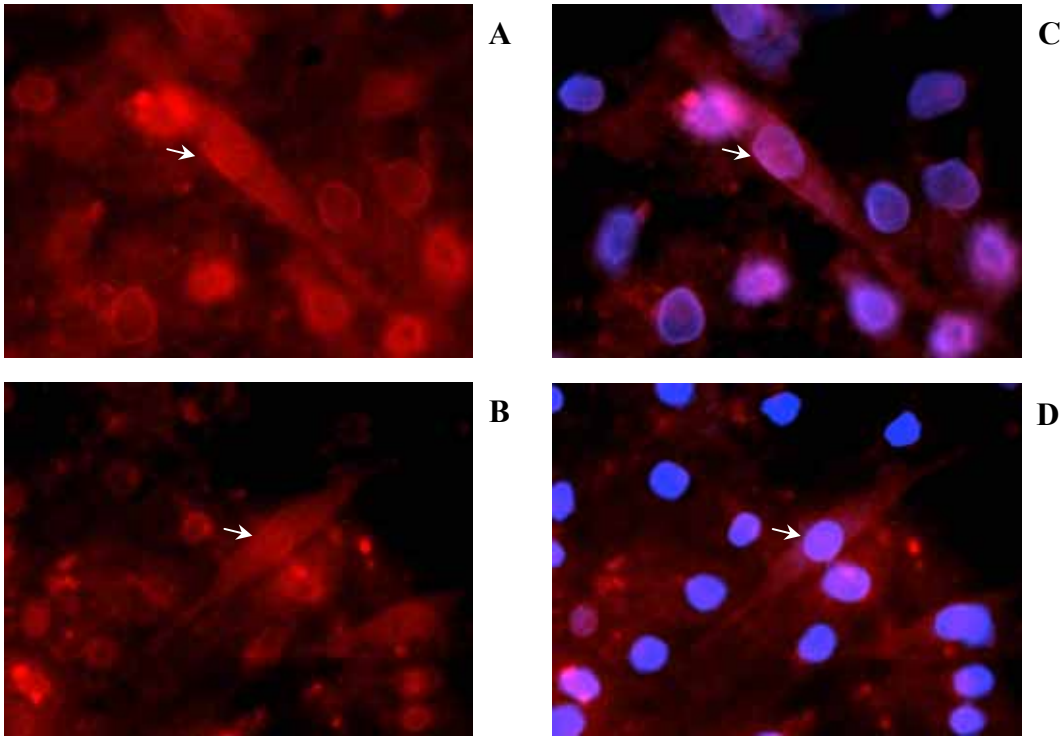



**Figure 3.11** MyoD staining. Immunohistochemistry of day 4 control- (A & C) and high dose (B & D) TNFα treated C2C12 cultures. The left panel (A & B) shows cells stained for MyoD (Texas red). The right panel (C & D) shows merged signals, MyoD (Texas red), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

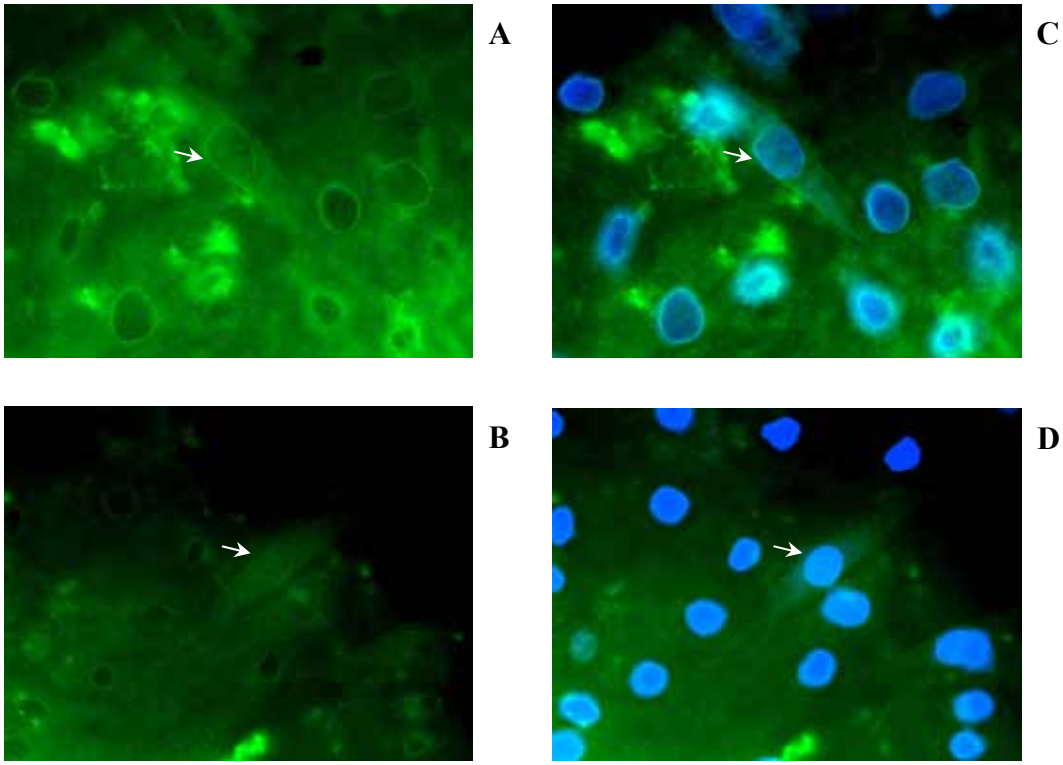



**Figure 3.12** Myogenin staining. Immunohistochemistry of day 4 control- (A & C) and high dose (B & D) TNFα treated C2C12 cultures. The left panel (A & B) shows cells stained for myogenin (FITC, green). The right panel (C & D) shows merged signals, myogenin (FITC), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

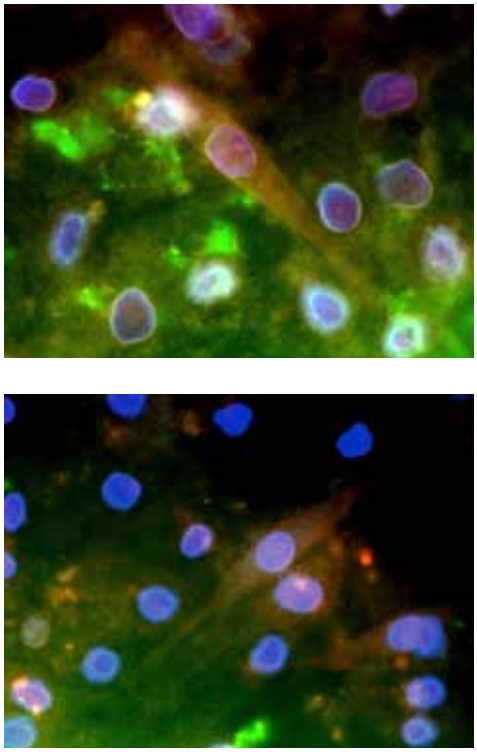



**Figure 3.13** Immunohistochemistry of day 4 control (A) and high dose (B) TNFα treated C2C12 cultures. Merged signals of MyoD (Figure 3.11), myogenin (Figure 3.12) and Hoechst (stains all nuclei blue) are shown. Images are at a 60X magnification.

Cells at day 7 were stained for myogenin (Figure 3.14) and phospho-p38α/β (Figure 3.15). Merged signals for both markers plus the nuclear stain Hoecsht are depicted in Figure 3.16.

Diffuse cytosolic staining of myogenin was visualised in high dose TNFα treated cells (Figure 3.14D). In contrast, notably more immunoreactive myogenin protein was localised to the nucleus in control cells (Figure 3.14A). Please note that where the secondary antibody to myogenin was FITC linked (green) for day 4 staining, the secondary antibody to myogenin at day 7 was Texas red linked, hence the incontinuity in myogenin immunofluorescence.

Immunoreactive phospho-p38α/β was localised to the nucleus at day 7 in all cultures (Figure 3.15).

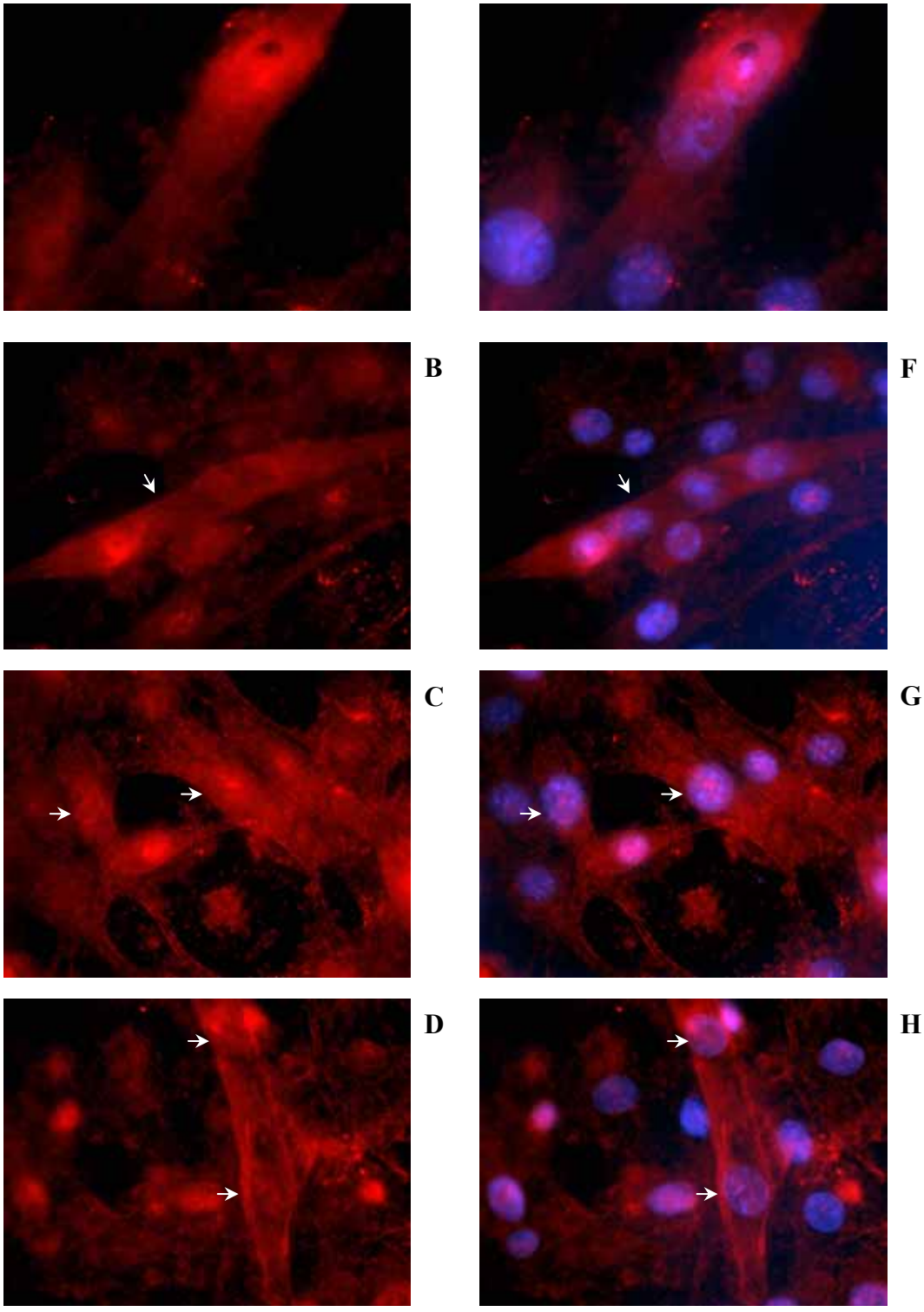



**Figure 3.14** Myogenin staining. Immunohistochemistry of day 7 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left panel (A-D) shows cells stained for myogenin (Texas red). The right panel (E-H) shows merged signals, myogenin (Texas Red), and Hoechst (stains all nuclei blue). White arrows indicate points of interest. Images are at a 60X magnification.

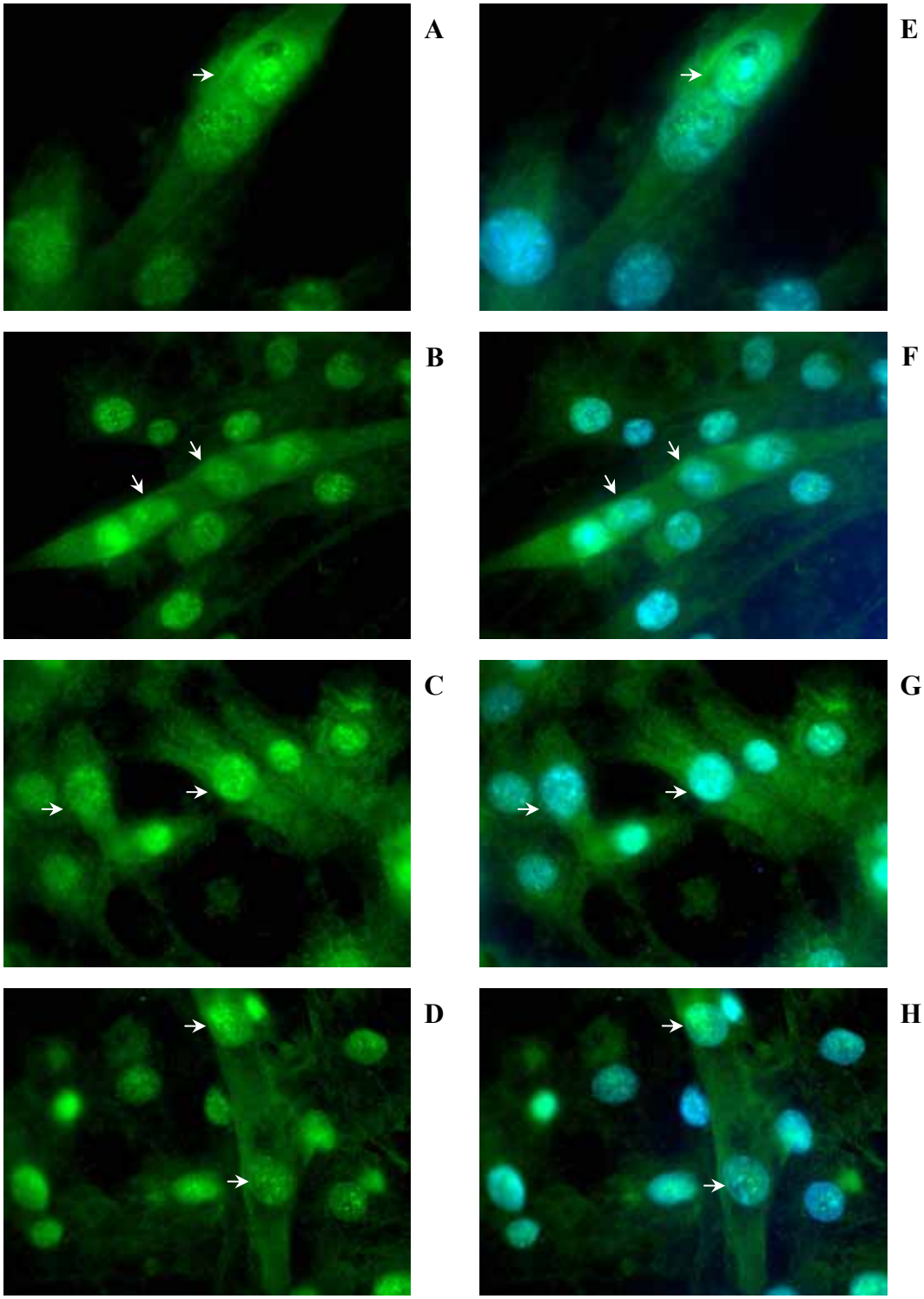



**Figure 3.15** phopsho-p38α/β staining. Immunohistochemistry of day 7 control (A & E), low (B & F), medium (C & G), and high dose (D & H) TNFα treated C2C12 cultures. The left panel (A-D) shows cells stained for phospho-p38α/β (FITC, green). The right panel (E-H) shows merged signals, phospho-p38α/β (FITC), and Hoechst (stains all nuclei blue). Images are at a 60X magnification.

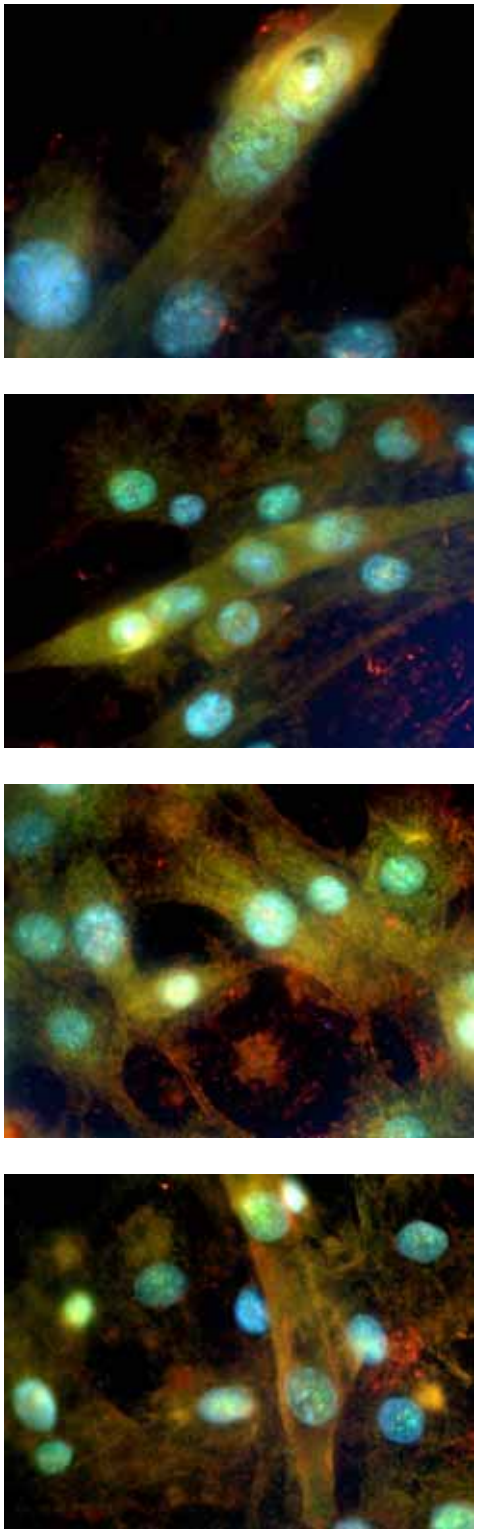



**Figure 3.16** Immunohistochemistry of day 7 control, low, medium and high dose (A-D) TNFα treated C2C12 cultures. Merged signals of myogenin (Figure 3.14), phospho-p38α/β (Figure 3.15) and Hoechst (stains all nuclei blue) are shown. Images are at a 60X magnification.

## CHAPTER 4: DISCUSSION

TNFα has long been implicated in muscle wasting disorders, and was initially referred to as cachectin in acknowledgement of its catabolic actions (Li *et al.*, 2000; Li *et al.*, 2001b). However, a physiological role for this pleiotropic cytokine in skeletal muscle regeneration has since been identified (Collins *et al.*, 2001; Li, 2003; Li *et al.*, 2001b; Warren *et al.*, 2002). It has become evident that the inflammatory response to muscle injury is functionally beneficial (Jarvinen *et al.*, 2005; Tidball, 2005). Following muscle damage, soluble factors released from infiltrating immune cells activate and chemotactically attract satellite cells to the injured area (as discussed in more detail in chapter 1). TNFα is, as such, released from infiltrating macrophages (Collins *et al.*, 2001; Tidball, 2005), as well as by injured muscle fibers (Collins *et al.*, 2001; Li, 2003), thereby conferring a relevant role for TNFα in skeletal muscle regeneration.

Much research on the effects of TNFα on satellite cell differentiation has been conducted in recent years. The outcomes of such research have been contrasting, suggesting both positive and negative outcomes. This can greatly be ascribed to different cell lines/models studied, employment of myoblasts vs. myotubes, varying doses of TNFα administered, and the duration of TNFα supplementation. It is therefore difficult to obtain a clear picture of it's mechanisms of action as all models slightly differ.

We therefore aimed at providing a comprehensive assessment of the effects of increasing doses of chronically supplemented TNFα on differentiating satellite cells.

The incorporation of various markers in a single model, allowed us to gain better insight into the dose dependent actions of TNFα. The doses of cytokine used in our study (0.5-10ng/ml) are physiologically relevant. Concentrations of 1-10ng/ml have been reported in the circulations of individuals with chronic inflammatory disease (Roubenoff *et al.*, 1992). TNFα levels following injury not associated with chronic disease states are dependent on the degree of damage.

*Model of satellite cell differentiation*

In the first part of the study we were successful in characterising a model of satellite cell differentiation in C2C12 cell cultures: The temporal expression patterns of a comprehensive range of markers characterising various phases in the differentiation program were investigated.

MyoD and myogenin are often used as markers of differentiation in studies on satellite cell differentiation. The aforementioned, as part of the MRF family of transcription factors, are necessary for the regulation of the myogenic differentiation process (as discussed in detail in chapter 1).

The dramatic increase in MyoD protein seen over the first 24 hours following the induction of differentiation was as expected, as MyoD is rapidly up-regulated in cells committed to the myogenic differentiation program (Sabourin *et al.*, 2000). *In vivo*, members of the myogenic transcription factor families (MRFs and MEF2 families) that regulate myogenesis are only expressed once quiescent satellite cells are activated and proliferating. Indeed, the levels of MyoD fluctuate within the cell cycle, peaking in G1

phase. However, the myogenic regulatory function of MyoD is repressed in cycling cells, and only 'activated' when differentiation cues are present (during G1 phase of the cell cycle). Following differentiation cues (removal of mitogenic signals), MyoD levels are further elevated (Dhawan *et al.*, 2005). This serves to explain the presence of MyoD at baseline in our model: Satellite cells in culture are already activated and cycling prior to the differentiation stimulus, and thus the basal level of MyoD also in our model. Once provided with the differentiation stimulus (serum removal), MyoD levels are further elevated, and its activity initiated concurrent with the removal of mitogenic stimuli. This underscores the value of the use of synchronised reversibly quiescent cultures as a more relevant model to an *in vivo* population of quiescent satellite cells.

The spike in MyoD protein expression at day 1 subsided by day 3, and increased again between days 5 and 7. This biphasic response implies that there was an increase in myoblasts initiating differentiation at day 7, a time point at which myotube formation was prominent. The C2C12 cultures used in this study were not synchronized. Therefore, there may be populations of cells within the culture at different stages of the cell cycle, some only responsive at later stages of the differentiation program.

The steady increase (up to day 3), and sustained (up to day 5) myogenin protein expression in our model is in accordance with the literature (Andres *et al.*, 1996; Langen *et al.*, 2001), and indicates that cultures in our model were progressing along the differentiation pathway. A drop in myogenin expression was seen by day 7. It appears from our model that sustained levels of myogenin are not required for the

continuity of satellite cell differentiation through to day 7. Other factors required for terminal differentiation and cell fusion such the secondary MRF, MRF4, are likely to be up-regulated in the advanced stages of differentiation thereby overseeing the completion of the differentiation program.

A steadily increased expression of the cell cycle inhibitor p21 for 3 days following the induction of differentiation where after it remained elevated for the remainder of the differentiation program was observed in our model. This indicates that the differentiating cells in our model steadily discontinue proliferation, exit the cell cycle, and commence differentiation. The formation of postmitotic multinucleated myotubes is, as such, a characteristic of the terminal differentiation (Andres *et al.*, 1996). One mechanism by which MyoD regulates myogenesis is through the promotion of cell cycle arrest in a mechanism involving induction of p21 (Song *et al.*, 1998). The marked increase in MyoD protein between days 0 and 1 was indeed followed by a steady increase in p21 protein in our model. Despite being a potent inducer of p21, MyoD is by no means the only inducer of p21. It has been reported that both MyoD and serum deprivation are required for induction of p21 (Guo *et al.*, 1995). Furthermore, p21 activity is proposed to be positively regulated by PI3-Kinase and MAPK (Ostrovsky *et al.*, 2003). A balance between various factors, and their temporal relationships, ultimately determine the total p21 protein levels in differentiating myoblasts (Ostrovsky *et al.*, 2003).

No marked increase in proliferation as measured by PCNA expression was detected over the first 5 days in our model with western blot analysis. An increase in PCNA

protein was however detected between days 5 and 7. This is contrary to what would be expected, particularly in light of the p21 data. The PCNA protein in our model was not decreased when p21 (and therefore cell cycle inhibition) was elevated. Data from our laboratory suggests that even though levels of PCNA protein are not altered, its localisation within a cell may be (E.Schabort, unpublished observation). This would indeed make sense, as PCNA is an accessory factor to DNA polymerase (Layne *et al.*, 1999), and it could only carry out its function once in the nucleus.

Employing various methods, recent publications have clearly identified a requirement for p38 MAPK (and specifically the α/β isoforms) in myogenic differentiation, (Cabane *et al.*, 2003; Chen *et al.*, 2005; Jones *et al.*, 2005; Wu *et al.*, 2000; Zetser *et al.*, 1999). A dramatic increase in phospho-p38 for 24 hours following serum removal was also evidenced in our model. This brings to light a very important consideration: the assumption that p38, once phosphorylated, is activated. The aforementioned is better verified by the assessment of its downstream substrates. p38 MAPK reportedly promotes myogenesis through mechanisms involving the induction and transcriptional activation of the MEF2 and MyoD families (Cabane *et al.*, 2003; Jones *et al.*, 2005; Wu *et al.*, 2000; Zetser *et al.*, 1999). Indeed, the pattern of MyoD protein expression in our model mimics that of phospho-p38. Both the aforementioned markers show a decline in protein expression between days 1 and 5 prior to an increase again between days 5 and 7. A requirement for p38 in the expression of both early (MyoD, p21) and late (MHC) markers of differentiation, as well as myotube formation and cytoskeletal organization has been reported (Cabane *et al.*, 2003). We propose that the biphasic mode of p38 phosphorylation in our model indeed suggests that the late increase in p38

phosphorylation plays a role in the attainment of the differentiation phenotype. p38 has been implicated in the control of myogenin and p21 expression as well (Cabane *et al.*, 2003). Incorporating a p38 inhibitor such as SB-203580 into future studies using our protocol would better ascertain such an involvement.

In the first phase of the study, the temporal patterns of expression of various factors that work in concert to bring about muscle gene expression, and a post-mitotic state characteristic of the differentiation phenotype, were investigated. The characterisation of such a control culture differentiation model, allowed us to subsequently investigate the effects of TNFα on the model.

*Dose-dependent effects of TNFα on differentiating C2C12 cells*

The trend towards increased MyoD protein in day 1 control cultures was even further elevated in TNFα treated cultures (all doses). This implies that cultures treated with cytokine for 24 hours following the induction of differentiation showed enhanced early differentiation. As mentioned in chapter 1, an important consideration is that of a positive, relevant role for TNFα in myogenesis. The increased MyoD protein content in TNFα treated cultures seen in the present study indicates that TNFα (0.5-10ng/ml) for 24 hours following the induction of differentiation appeared to enhance the initial stages of the differentiation program: the induction of a key primary MRF and thereby the commitment of cells to the myogenic lineage, and the induction of muscle specific gene transcription. Immunohistochemical analysis of day 1 cultures, illustrated a trend for immunoreactive MyoD to move away from a diffuse cytosolic- to a more nuclear

staining with increasing dose of TNFα (0.5-10 ng/ml), suggesting a dose dependent nuclear localisation of the transcription factor (Figure 3.10).

Our findings are in contrast with a study by Guttridge (Guttridge *et al.*, 2000), who observed a decreased MyoD protein content in C2C12 cultures after 24 hours of cytokine treatment. However the researchers did administer a 20ng/ml dose of cytokine, double the concentration of what is referred to as a high dose in our study. The contrasting results could be attributed to the fact that a dose of 20ng/ml is considered cytotoxic to cells (Dr. R. Smith, unpublished data).

With continually elevated TNFα supplementation in our study, this seemingly positive effect on differentiation was reversed. Cytokine treated cultures displayed a trend towards less MyoD protein with increasing doses of cytokine at days 3 and 5. Immunohistochemistry of day 4 cultures illustrated less immunoreactive MyoD protein in the nuclei of high dose treated cells compared to controls (Figure 3.11). This finding is in agreement with other reports by Guttridge (Guttridge *et al.*, 2000) and Langen (Langen *et al.*, 2004). Both the aforementioned studies did however only assess MyoD protein expression with continued TNFα supplementation up to 72 hours following serum withdrawal. To our knowledge, few studies assess biochemical markers of differentiation at time points beyond 72 hours following the differentiation stimulus. Furthermore, few published studies chronically supplement cultures to differentiate in the presence of TNFα for as long as 7 days. Our study provided an assessment of markers of differentiation at time points further down the myogenic differentiation pathway. The trend towards less MyoD protein in (particularly high dose cytokine

treated cells) was carried through till day 7. It therefore appears from our data that chronic supplementation of TNFα tends to negatively regulate a key early regulator of myogenic differentiation, and even more so with higher doses.

Chronic TNFα supplementation (and particularly the high dose of cytokine) negatively regulated the protein expression of myogenin, a secondary MRF, and further marker of differentiation. Myogenin expression was only assessed from day 3 onwards in the present study. There are however, studies in the literature reporting myogenin expression as early as day 1 following serum withdrawal (Guttridge *et al.*, 2000; Langen *et al.*, 2004). In such reports, cultures differentiating in the presence of TNFα (at between 10- and 20ng/ml) reduced both the protein and mRNA expression of myogenin at 24 and 48 hours. This is an interesting consideration in light of the possible positive effects 24 hours of TNFα supplementation (even at a 10ng/ml dose) seemed to have on early differentiation, as measured by MyoD in the present study. With continued TNFα supplementation in our study, our data corresponds to that of the aforementioned reports, in that myogenin expression tended to be reduced in TNFα treated cultures from day 3 onwards. Indeed, immunohistochemical analysis of day 4 cells indicated less immunoreactive myogenin protein in high dose treated cells compared to controls. The trend towards decreased myogenin protein, in particularly high dose TNFα treated cultures compared to controls at days 3 and 5, became significant at day 7, where a dose dependent decrease became evident. Immunohistochemistry of day 7 cells sufficiently illustrated this finding, as immunoreactive myogenin protein in control cells appeared to be concentrated in the nucleus, whereas less myogenin protein, stained in a diffuse fashion, was evidenced in

high dose treated cells (Figure 3.14). Furthermore, the faint nuclear myogenin staining compared to that of control cells illustrates that not only was there less myogenin protein, but less of the myogenin protein present in the cell appeared localised to the nucleus in high dose cytokine treated cells. This underscores the importance of addressing the localisation of proteins within a cell. As a transcription factor concerned with the induction of muscle specific genes during the conversion of myoblasts to mature myotubes, myogenin can only carry out its function if it is active and at the site it is needed, i.e. in the nucleus.

Phosphorylation of MRFs reportedly negatively regulates their activity (Sabourin *et al.*, 2000). PKC has been shown to phosphorylate myogenin on its DNA binding site, thereby preventing its binding to promoter regions of muscle specific genes (Sabourin *et al.*, 2000; Zhou *et al.*, 1994). PKC is stimulated through TNFR activation (MacEwan, 2002). This presents a possible mechanism through which TNFα negatively regulates myogenic differentiation, and warrants further investigation.

Similar results are found in the literature in study designs comparable to the one used in our study (Guttridge *et al.*, 2000; Langen *et al.*, 2001; Langen *et al.*, 2004), and in designs incorporating the roles and interactions of other factors relevant to myogenesis (Layne *et al.*, 1999). To our knowledge, few studies assess biochemical markers of differentiation at time points beyond 72 hours following the differentiation stimulus. Furthermore, few studies reported in the literature allowed cultures to differentiate in the presence of TNFα (at any dose) for as long as 7 days.

As discussed previously, cell cycle exit is vital to the attainment of the postmitotic state characteristic of differentiated muscle. The assessment of markers of the cell cycle provided further insight into the dose-dependent effects of TNFα on differentiating satellite cells up to 7 days following the induction of differentiation.

The protein expression of cell cycle inhibitor p21, as a broad specificity inhibitor of cdk's, was investigated in the present study. TNFα supplementation (with all doses administered) led to an elevated p21 protein expression compared to controls (a trend analogous to that of MyoD) at day 1. This is to be expected as mentioned previously, MyoD promotes the induction of p21. At day 3, as with MyoD, p21 protein expression in cytokine treated cultures tended to be reduced relative to controls, such that the seemingly positive effect TNFα supplementation had on myogenesis during the initial 24 hours post serum removal was reversed with continued cytokine supplementation. The dose dependent decrease in p21 that followed at day 5 indicates that TNFα significantly inhibited cell cycle inhibition of myoblasts at this advanced phase of the differentiation program. This implies therefore that continually elevated levels of TNFα negatively regulate cell cycle exit in a dose dependent fashion, and particularly so with high doses (10ng/ml) of cytokine at day 5. Otherwise stated, without cell cycle inhibition, continued proliferation will occur and differentiation cannot take place as (described in chapter 1) the aforementioned events are mutually exclusive. To our knowledge, no other study has assessed the effects of chronic TNFα supplementation on p21 mediated cell cycle inhibition *in vitro*.

Interestingly, it has been shown that p21 acts as a direct inhibitor of DNA polymerase by binding a subunit of PCNA (Roubenoff *et al.*, 1992), a marker of proliferation in our study. With the trend towards dose dependent decrease in p21 in cytokine treated cultures (significantly so at day 5), a similar, though inverted, pattern of PCNA protein expression would be seen. No significant difference in proliferation as determined by PCNA protein concentration resultant of cytokine treatment was, however, detected in our study. The dose dependent decrease in p21 at day 5 was accompanied by a slight trend towards a dose dependent increase in PCNA protein in TNFα treated cultures compared to controls. Although Western blot analysis showed no (significant) difference in PCNA protein between groups, an effect of TNFα on proliferation in differentiating myoblasts could be masked. As only whole cell lysates were analysed by western blotting in our study, PCNA protein both in the cytosol at its final site of production, and in the nucleus, where it would be involved with DNA replication, would be detected. Immunohistochemistry analysis would provide better insight to the localisation of PCNA protein (as discussed previously).

Cyclin D1, an integral part of the cell cycle progression machinery (for cell cycle review see (Schafer, 1998) presents an alternative marker of proliferation that could be included in future studies and thereby delineate a possible mitogenic role for TNFα in myogenesis as suggested by Li (Li, 2003). It has been found that TNFα, through the action of NFκB, regulates the expression of cyclin D1, thereby promoting cell cycle progression (Guttridge *et al.*, 2000). Alternatively, the MTT assay for cell viability and proliferation should be considered in future studies.

It is reported that p38 activity, required for myogenic differentiation, is induced by serum withdrawal via a pathway independent of that stimulated by cytokines and stress (Cabane *et al.*, 2003; Wu *et al.*, 2000). The marked increase in phospho-p38α/β between days 0 and 1 in our control cell differentiation model, correspond to such reports. Recently, Chen (Chen *et al.*, 2005), conducted a study in cardiotoxin injured, TNFα receptor double-knockout mice, which for the first time illustrated the importance of TNFα receptors in p38 activation during muscle regeneration. It was suggested in the aforementioned report that the elevated levels of TNFα present at the initiation of the muscle regeneration process, serve to initiate the regeneration process via p38.

From the abovementioned it would be implied that concurrent with TNFα supplementation, an increase in phospho-p38, over and above that induced by serum withdrawal, would be expected in our treated cultures. Over the first 24 hours post serum withdrawal, cultures treated with a high dose of cytokine showed a trend towards elevated phospho-p38α/β, while the other cytokine treated cultures did not seem to differ from controls.

In the normal physiological process of repair following injury, the concentrations of TNFα and growth factors are only elevated during the initial stages of muscle regeneration (and satellite cell activation). It is only after the removal of mitogenic stimuli has taken place that satellite cells can differentiate into new muscle cells to bring about repair (Andres *et al.*, 1996; Dhawan *et al.*, 2005; Wu *et al.*, 2000). Indeed,

growth promoting factors (mitogenic stimuli) have been shown to inhibit the actions of the MRFs (Dhawan *et al.*, 2005; Hawke *et al.*, 2001; Sabourin *et al.*, 2000).

In the current study, a trend existed for TNFα (0.5-10ng/ml) to positively regulate early markers of differentiation (MyoD, p21) after 24 hours of supplementation. Similarly, a 10ng/ml dose of TNFα after 24 hours of supplementation also fit this trend. With continued supplementation however, TNFα tended to negatively regulate markers of differentiation. The dose dependent decrease in phospho-p38 at day 3 of differentiation further illustrates this. It would appear therefore, that continually elevated levels of TNFα negatively affects the phosphorylation of the downstream signalling transducer, p38 MAPK. This effect seemed to be upheld till day 5 where all cytokine treated cultures still, displayed less p38 phosphorylation compared to controls.

Induction of MyoD expression, is one of the mechanisms through which p38 reportedly regulates myogenesis. The dose dependent decreases in MyoD protein, seen with continually supplemented TNFα in our study, could therefore be attributed to the decreased phosho-p38 detected (at days 3 and 5) in our study.

As mentioned previously, p38 activity is reportedly required for the expression (and transcriptional activation) of early and late markers of differentiation (Cabane *et al.*, 2003). As continually elevated levels of TNFα negatively regulated early- (MyoD), and later (myogenin) markers of differentiation, and cell cycle inhibition in our study, we propose that these effects are resultant of the decreased phospho-p38 levels in treated cells (at days 3 and 5) witnessed in our study.

Interestingly, a dose dependent increase in phospho-p38 followed at day 7. It is possible that in response to continued TNFα supplementation, a degree of receptor desensitisation, or receptor down regulation was occurring, with increasing dose of cytokine, after day 1 and up to around day 5. By day 7, it would appear therefore that some compensatory mechanism had occurred. This would need further investigation.

It is well known that ROS are generated in cultured skeletal myocytes in response to inflammatory cytokine exposure (Li *et al.*, 1999; Williams *et al.*, 1994). ROS are proposed to play a role in TNFα mediated cytotoxicity (Goossens *et al.*, 1995). We do not consider the doses of TNFα administered (0.5 – 10ng/ml) in our study to be cytotoxic. The assessment of caspase activity in future studies would serve to confirm this.

*Limitations to the study and future recommendations*

The C2C12 cultures used in this study were not synchronised, and a heterogenous population of cells were therefore studied. The use of synchronised reversibly quiescent cultures would be a more relevant model to an *in vivo* population of quiescent satellite cells.

In the present study, markers of differentiation were studied at protein level only. For a more comprehensive picture of the effects of TNFα on differentiating satellite cells, it would be useful to consider the events at mRNA level as well. Characterisation of the

post-transcriptional and post translational events resultant of TNFα supplementation could help better characterise the cytokine's mode of action.

Various trends exist in our data. By increasing our sample size, some of these trends may actually reach statistical significance.

Our protein samples were that of whole cell lysates. As a result, the protein expression levels in our samples were only a reflection of that in the entire cell. The growing importance of immunohistochemical techniques is becoming increasingly clear, as immunohistochemistry analysis illustrated that despite no differences in protein levels are found by western blotting, a difference in the localisation of proteins between samples may well exist. In future it would be advantageous to separate, for example, the cytoplasmic, nuclear, and membrane fractions of samples prior to analysis. 2D gel electrophoresis would be valuable as a further analytical tool.

## CHAPTER 5: SUMMARY AND CONCLUSIONS

The effects of TNFα are reportedly dose- and time dependent. Skeletal muscle is often affected by diseases of other organs. Indeed, TNFα is implicated in muscle wasting associated with chronic disease states such as cancer and AIDS, where its levels are chronically elevated. A physiological role for TNFα in muscle regeneration has also been reported. Many reports on the effects of TNFα on satellite cell differentiation *in vitro* are found in the literature. However, all models used for these purposes differ with regards to doses administered and duration of cytokine supplementation. Here, we provide a comprehensive characterisation of the dose dependent effects of chronically supplemented TNFα, on C2C12 cells. This research allows for better insight into the mechanisms by which TNFα regulates satellite cell differentiation. Markers of differentiation were assessed at both early (day 1), and later (days 3, 5, and 7) time points after the induction of differentiation. This allows us to draw conclusions of TNFα's differential effects after acute and chronic supplementation. Otherwise stated, this mode of TNFα supplementation allows conclusions of its effects on satellite cell differentiation in chronic disease states to be drawn as well.

We show here: TNFα supplementation appears to positively regulate early markers of differentiation after 24 hours of supplementation. However, with continually elevated levels of TNFα, this positive effect on C2C12 differentiation is reversed. Chronically elevated levels of TNFα at low, medium, and high doses negatively regulate markers of differentiation in a dose dependent fashion. We propose that these effects are mediated,

in part, by the attenuation of phospho-p38 MAPK, a recognised activator of myogenesis.

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## Appendix A: Table of satellite cell markers

**Table 1.1** List of satellite cell markers adapted and modified from (Hawke *et al.*, 2001) and (Charge *et al.*, 2004).

| Molecular marker | Expression pattern in satellite cells | Reference |
|---|---|---|
| CD34 | Quiescent | (Charge *et al.*, 2004) |
| CD56 | Quiescent, activated and proliferating | (Charge *et al.*, 2004; Hawke *et al.*, 2001) |
| Syndecan-3 & -4 | Cell surface heparin sulphate proteoglycans | (Cornelison *et al.*, 2001) |
| MNF | Quiescent, activated and proliferating | (Garry *et al.*, 1997) |
| Pax 7 | Quiescent, activated and proliferating | (Olguin *et al.*, 2004; Seale *et al.*, 2000b) |
| c-met | Quiescent, activated and proliferating | (Cornelison *et al.*, 1997) |
| M-cadherin | Quiescent, activated and proliferating | (Cornelison *et al.*, 1997) |
| VCAM-1 | Quiescent, activated and proliferating | (Charge *et al.*, 2004; Hawke *et al.*, 2001) |
| Desmin | Activated and proliferating | (Bockhold *et al.*, 1998) |
| Myf5 | Activated and proliferating | (Cornelison *et al.*, 1997) |
| MyoD | Activated and proliferating, in cells committed to myogenic program | (Cornelison *et al.*, 1997) |
| Myogenin | Early stages of differentiation | (Charge *et al.*, 2004; Hawke *et al.*, 2001) |
| MHC | Terminal differentiation | (Charge *et al.*, 2004; Hawke *et al.*, 2001) |
| MRF4 | Terminal differentiation | (Charge *et al.*, 2004; Hawke *et al.*, 2001) |

**Appendix B: Representative immunohistochemical protocol**

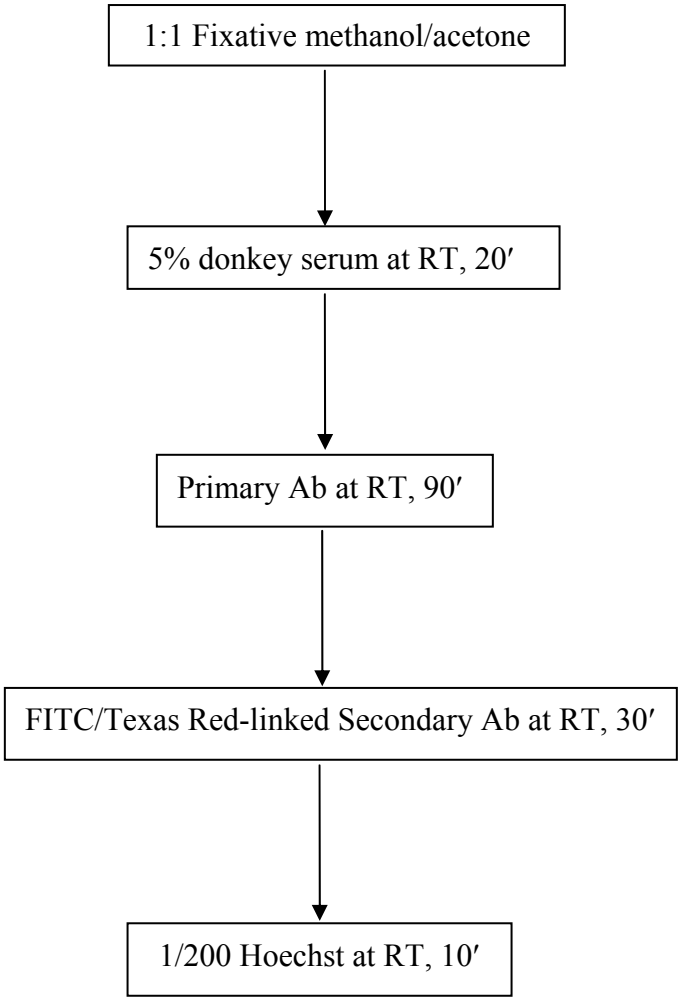


**Figure 1** Flow diagram of the immunohistochemical protocol followed in this study. See text for details.