DO INFLAMMATORY CYTOKINES DIFFERENTIALLY AFFECT MASSETER AND TIBIALIS ANTERIOR MUSCLE REGENERATION?

By

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To my parents, for all of their support throughout my education, and to my Aunt Gail, for her intellectual curiosity and inspiration

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LIST OF ABBREVIATIONS

AEC	3-amino-9-ethyl-carbazole
BrdU	Bromodeoxyuridine
JUN	c-Jun-N-terminal kinase
DMEM	Dulbecco's modified eagle's medium
EDL	Extensor digitorum longus muscle
EMyHc	Embryonic myosin heavy chain
HRP	Horseradish peroxidase
IGF	Insulin-like growth factor
IL-1β	Interleukin one beta
IL-4	Interleukin four
IL-6	Interleukin six
MAS	Masseter muscle
MRF	Myogenic regulatory factor
MRF4	Myogenic regulatory factor four
MyoD	Myogenic determination factor one
Myf5	Myogenic factor five
NFκB	Nuclear factor kappa B
p38MAPK	p38 mitogen activated protein kinase
Pax7	Paired box protein seven
PBS	Phosphate buffered solution
PCR	Polymerase chain reaction
SOL	Soleus muscle
TA	Tibialis anterior muscle
TBS	Tris-phosphate buffered solution

TMD	Temporomandibular disorders
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor one
TNFR2	Tumor necrosis factor receptor two

Abstract of Thesis Presented to the Graduate School
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DO INFLAMMATORY CYTOKINES DIFFERENTIALLY AFFECT MASSETER AND TIBIALIS ANTERIOR MUSCLE REGENERATION?

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Masseter (MAS) has a prolonged healing response after injury compared to other muscles such as tibialis anterior (TA). However, it is not known if this diminished repair capacity is due to a decreased ability of myogenic stems cells in MAS to activate, proliferate and form myofibers or the result of a differential response to specific inflammatory mediators. The purpose of this study was to: 1) compare the myogenic potential of MAS and TA in an in vitro model; and 2) determine if there is a differential effect of three inflammatory cytokines, TNF-α, IL-1β and IL-4, on MAS and TA myogenic potential in vitro.

Single muscle fibers from MAS and TA of CD-1 female mice were harvested and placed on matrigel-coated coverslips in 24 well plates. To evaluate early events, fibers were cultured for six days in a proliferation medium. Late events were evaluated by culturing the myoblasts/myotubes an additional six days in differentiation media. Cytokines were tested individually for an effect on cell type proportions during the proliferation and differentiation phases. Four culture replicates were evaluated for each condition. Activation and proliferation were assessed by immunostaining for MyoD, a myoblast marker; myotube and myofiber formation were evaluated by immunostaining

for myogenin. Proportions of cells with distinct phenotypes were calculated for each condition.

In the in vitro model, myogenic stem cells derived from MAS and TA were capable of activation, proliferation and formation of myofibers. However, cultures derived from MAS and TA differed in myogenic cell phenotype proportions indicating differences in maturation. In both MAS and TA derived cultures, cytokine administration affected specific myogenic cell phenotype proportions, suggesting a differential effect on specific phases of myogenesis. However, when cell proportions were normalized to controls, the only significant difference between MAS and TA derived cultures exposed to cytokine was an increase in the proportion of supporting cells. These results indicate that cytokine exposure has similar effects on myogenic cells derived from both muscles. Thus, the diminished capacity of MAS to repair does not appear to be the result of an increased adverse effect of cytokines on MAS presumptive myogenic cells.

CHAPTER 1
INTRODUCTION

Temporomandibular disorders (TMD) is a term used to describe a group of musculoskeletal pain conditions involving the temporomandibular joint region. TMD pain involves the muscles of mastication (myofascial pain), the joint itself (arthralgia) or both (LeResche 1997). Factors associated with myofascial pain in TMD patients have been shown, through logistical regression analyses, to include: clenching, head/neck trauma, anxiety and being of the female sex. Within the spectrum of TMD, the masseter (MAS) muscle is involved in nearly 60% of cases (Dworkin et al. 1990). It has been suggested that this muscle may display poor healing, which could result in ineffective treatment in some patients with masticatory muscle induced TMD. Pavlath et al. (1998) used a mouse model to compare the MAS muscle to a limb skeletal muscle, the tibialis anterior (TA), and found that the masseter muscle showed greatly delayed and decreased healing after a standardized freeze injury. The differential healing of the MAS muscle is of great interest to those managing TMD and clarifications may result in enhanced therapeutic approaches to patient care and treatment.

While little is known about the cause for the differential healing, we suggest two hypotheses. The first is that there may be inherent differences in the ability of MAS muscle to regenerate when compared to other skeletal muscles. A second hypothesis is that there may be some component(s) of the environment, such as differences in the inflammatory milieu, which may differ between the MAS muscle and other skeletal muscles. This may involve the delicate balance between cellular and chemical mediators, concentrations and temporal peaks. Preliminary studies by Morris-Wiman and Widmer have shown that mast cells are increased in MAS muscle after injury and

could play a role in decreased healing (Morris-Wiman and Widmer 2006). More recently in the same laboratory, MAS and TA were freeze injured and cytokine expression was determined (Harris 2010). The MAS muscle had an overall blunted cytokine expression compared to TA. Treatment with cromolyn, a mast cell inhibitor, partially enhanced cytokine expression within the MAS. These data support the hypothesis that the differential regeneration may be due to variations in inflammatory milieu and the environment. The goal of the present study was to further examine the regenerative potential of MAS and TA in an in vitro model and determine what effect specific cytokines (TNF-α, IL-1β and IL-4) have on regenerative potential.

Muscle Regeneration: Satellite Cell

Within skeletal muscle, somatic or adult stem cell populations reside that are capable of regeneration and maintenance of the muscle (for review see Shi and Garry 2006). These mononuclear cells are known as satellite cells. Satellite cells reside between the basal lamina and plasma membrane of the adjacent myofiber (Muir et al. 1965). In their normal state, these cells are quiescent and in response to injury they become activated, proliferate and express myogenic markers. Once satellite cells express myogenic markers they are termed myoblasts. Myoblasts ultimately fuse, either to existing myofibers or to each other, to form new myofibers (Bischoff 1994).

Myogenesis of satellite cells is regulated by a family of muscle specific transcription factors, known as muscle regulatory factors (MRF) (Yablonka-Reuveni et al. 2008). These transcription factors include: myogenic determination factor 1 (MyoD), myogenic factor 5 (Myf5), myogenin and myogenic regulatory factor 4 (MRF4) (Ludolph and Konieczny 1995). The MRFs are expressed differentially over time and at different stages in the activation of satellite cells (Yablonka-Reuveni and Rivera 1994).

Two of the MRFs, MyoD and myogenin, are upregulated during different stages of satellite cell activation. MyoD is upregulated primarily in proliferating satellite cells (Yablonka-Reuveni and Rivera 1994) and occasionally in differentiating cells as well, depending on the extracellular matrix environment (Yablonka-Reuveni et al. 2008). Whereas MyoD is upregulated primarily in satellite cell proliferation, myogenin is associated with satellite cell differentiation and is associated with withdrawal from the cell cycle, fusion of myoblasts to multinucleated myotubes and a decline in MyoD (Yablonka-Reuveni and Rivera 1994; Yablonka-Reuveni et al. 1999; Yablonka-Reuveni and Paterson 2001).

Differential Regeneration of Muscle

Although all skeletal muscles contain satellite cells, and thus the potential for regeneration, it has been suggested that the MAS muscle displays poor healing compared to other skeletal muscles. Pavlath et al. (1998) examined differences in regenerative capacities between the TA muscle (a limb muscle) and the MAS muscle (a craniofacial muscle) and reported marked differences both in timing of regeneration and quantity of fibrous connective tissue (scar tissue) formed. In the first experiment conducted by Pavlath et al. (1998), MAS and TA muscles were injured using either freeze or crush injury in a mouse model. In the TA, proliferation of myoblasts was evident within 2 days, extensive myotube formation within 4 days, normal myofiber size by day 7 and nearly complete architecture restoration by day 12. However, the MAS muscle displayed a markedly different course of regeneration: at day 12 large areas of injured muscle remained with little evidence of regenerated muscle fibers. At days 19-21, extensive interstitial connective tissue remained between fibers and by day 45 regeneration of the MAS muscle was observed to be less effective than the TA muscle

30 days prior. Hence, in the mouse model, the quality of regenerated tissue post crush or freeze injury was significantly better in the TA at all time points (through 45 days post injury).

The quantity of regenerated muscle was also examined and it was concluded that MAS muscle displayed poor regeneration throughout all anatomical areas of the muscle with the most dramatic deficits being in the core of the muscle (Pavlath et al. 1998). In an attempt to understand the reason why MAS muscle regenerated poorly, Pavlath et al. (1998) proceeded to compare regeneration of MAS muscle to two other muscles of the head and neck, the anterior belly of the digastric (a masticatory craniofacial muscle) and the sternocleidomastoid (a non-masticatory craniofacial muscle). It was found that both of these muscles regenerated similar to the TA muscle, concluding that neither masticatory muscle nor embryologic origin alone could explain differences in masseter muscle regeneration. To further examine MAS muscle injury, Pavlath et al. (1998) next examined regeneration of masseter muscle in response to endogenous injury (muscular dystrophy). In this model, a mouse with muscular dystrophy experienced a period of 3 weeks of muscle necrosis at 8 weeks of age, which targeted muscle fibers and spared nerves and blood vessels, and was followed by active regeneration of muscle. Again, MAS muscle showed significantly delayed regeneration in comparison to TA muscle.

An additional analysis was performed by Pavlath et al. (1998) to examine the relative amount of myoblasts activated in damaged TA versus MAS muscle. Following similar injury, myoblast cultures were established and co-stained with Bromodeoxyuridine **(**BrdU) and MyoD (a MRF) to provide an estimate of the number of activated satellite cells. In both culture and tissue sections, TA displayed approximately

2.5 times more activated satellite cells than MAS muscle (Pavlath et al. 1998). Criticisms of this experiment are that they were not able to normalize for net wet weight or fiber number. In these experiments the number of activated satellite cells was compared within its native environment- either MAS or TA muscle. Therefore, one can hypothesize that the reason for decreased activation of myoblasts may be within the myoblasts themselves or may be a factor of the environment.

In the last of the experiments reported in Pavlath et al. (1998), the ability of myoblasts to fuse and express a differentiation-specific protein, embryonic myosin heavy chain (EMyHC), during regeneration was assessed. The time course of expression of EMyHc was found to be similar for both muscles, while the amount was decreased throughout in the MAS muscle. This suggests that MAS myoblasts may be able to fuse and differentiate similar to those of TA but may be decreased in number (Pavlath et al. 1998). The reduction in number and alteration in behavior may be inherent to MAS or may be a result of the environment. This may lead one to hypothesize that satellite cells from the MAS are capable of differentiation yet are decreased in number. However, because the experiments were conducted with cells in their native environment, one cannot rule out a possible environmental influence.

Acute Inflammation: A Response to Muscle Injury

The current understanding of muscle repair after acute trauma is that, first, there is rapid disruption of structural components in the muscle, then there is an inflammatory stage that includes removal of cellular debris and, lastly, there is regeneration or repair of muscle fibers (Tidball 1995). Throughout the inflammatory process there are numerous cytokines, chemokines and growth factors being released. Tumor necrosis factor-α (TNF-α) is a pleiotropic cytokine whose functions range from mediating shock in

sepsis to causing necrosis of tumors (Beutler and Cerami 1988). In a study by Warren et al. (2002), the specific role of TNF-α was examined in traumatic muscle injury. In particular, the levels of expression post injury were analyzed and the implications of blocking receptors for this cytokine examined. In the first part of the study, Warren et al. (2002) looked at wild type control mice to examine trends in TNF-α expression post freeze injury of the tibialis anterior muscle. Using real time PCR and immunocytochemical analysis, Warren et al. (2002) showed that TNF-α levels did increase post-injury and peaked at 24 hours. At early time points, staining was localized primarily to inflammatory cells and at later time points there was moderate staining of regenerating myofibers. To further test the role of TNF-α in muscle regeneration, knockout mice that were genetically deficient in the two main TNF-α receptors, TNFR1 and TNFR2, were subjected to a freeze injury of the TA muscle (Warren et al. 2002). The influx of inflammatory cells was not significantly different from control mice, suggesting redundancy of TNF-α. Similar results were seen for mice that had the receptors but were administered antibodies to TNF-α. The conclusion of Warren et al. (2002) was that TNF-α does play an important role in the process of muscle regeneration. However, there are likely redundant systems at work, since muscle repair was only slightly affected by deficiencies in TNF-α.

In another study, the role of TNF-α in muscle inflammation was examined by injecting the cytokine into muscle (Peterson et al. 2006). After administering the cytokine to mice for 7 days, Peterson et al. (2006) immunocytochemically stained muscle for detection of macrophages and neutrophils. Their results revealed that administration of TNF-α for 7 days did significantly increase inflammatory cells in

skeletal muscle (between two-fold and seven-fold), but did not result in overt signs of increased muscle atrophy, injury or inflammation. While these results provide insight into the role of TNF-α in skeletal muscle inflammation, Peterson et al. (2006) did not follow up long term to see if signs of neutrophil-induced muscle injury were present at subsequent time points. Additional studies conflict with Peterson's results and suggest that neutrophils do contribute to muscle injury during inflammation (Brickson et al. 2003; Pizza et al. 2005) and reperfusion of ischemic tissue (Rubin et al. 1996; Palazzo et al. 1998). In a study by Hodgetts et al. (2006), depletion of host neutrophils and inhibition of TNF-α with Etanercept was found to reduce muscle necrosis in mdx mice, a model for Duchenne Muscular Dystrophy. Lastly, recent studies by Al-Shanti et al. (2008) demonstrated that there is positive crosstalk between TNF-α and IL-6 which may act to enhance the IGF system and promote myoblast differentiation. Insulin growth factor I (IGF-1) is a cytokine that is essential for the differentiation of satellite cells and plays an important role the anabolism of muscle fibers (Broussard et al. 2004).

IL-1β is another ubiquitous pro-inflammatory cytokine. As with TNF-α, there is much debate regarding the role of IL-1β in muscle inflammation. Both TNF-α and IL-1β are elevated before the onset of muscular dystrophy in the murine model of this disease (Kumar and Boreik 2003). In addition, chronic administration of IL-1β to rats has been found to reduce protein synthesis, while the administration of IL-1β receptor antagonist prevented muscle mass deficiencies in an animal model (Cooney et. al 1999). The exact mechanism by which IL-1β may inhibit muscle regeneration remains unknown, but recent studies suggest that it may act by inhibiting IGF-1 and expression of myogenin (Broussard et al. 2004). Additional research has further demonstrated that the anti-

inflammatory cytokine IL-10 is capable of suppressing IL-1β, thereby ceasing its inhibition of IGF-1 (Strle et al. 2008).

The cytokine IL-4 also appears to have an important role in muscle regeneration. The results of a study by Horsley et al. (2003) suggest that IL-4 may act as a myoblast recruitment factor in the initial stages of myoblast fusion to form myofibers and in this way may promote the formation of new muscle fibers in muscle regeneration post injury. Harris (2010) used a mouse model to examine the differential expression of cytokines post injury. She found that IL-4 was increased in TA compared to masseter at baseline, decreased during early repair, and recovered in late repair. An explanation for the varying levels of expression could be that the initial decrease was an attempt by TA to prevent premature fusion of myoblasts and allow proliferation (Harris, 2010).

Given the complexity of their actions and the interactions of these inflammatory mediators, it is difficult to assign a role to any one cytokine. It is likely that each cytokine plays varying roles temporally to either prolong muscle damage or promote healing and that this role depends on the presence of other cytokines and environmental cues.

Summary

MAS muscle damage and regeneration are important areas of research and gaining a better understanding of this topic has wide ranging implications in the treatment of masticatory muscle pain. Inflammation plays an integral role in muscle repair, but just as inflammatory mediators help promote healing they can also induce further injury and a delicate balance is needed to ensure the normal healing process. Satellite cells are multipotent stem cells that reside in muscle. Following muscle injury these cells become activated and proliferate and differentiate into new myofibers. Inflammatory factors affect not only key inflammatory cells, such as neutrophils and

macrophages, but also regenerative cells, satellite cells. Studies by Pavlath et al. (1998), using a mouse model, have shown that MAS muscle displays delayed and reduced healing compared to TA muscle. This reduced healing may be due to inherent properties of the MAS muscle or may result from differences in the inflammatory milieu compared to other skeletal muscles. In Harris (2010) the inflammatory milieu after a freeze injury was compared between MAS and TA in a mouse model. An overall blunted cytokine expression in MAS muscle was observed. The aim of the present study was to examine, in an in vitro mouse model, the effect of specific cytokines on the regeneration of TA and MAS muscle. In particular, the effect of three cytokines, TNF-α, IL1β and IL-4, was examined at proliferation and differentiation stages of the regenerative process.

Significance

TMD is a prevalent condition that can be debilitating and chronic. Current therapy for myofascial pain is palliative and does not address the underlying pathophysiology. A better understanding of the role of inflammatory cytokines on regeneration of MAS muscle may lead to drug therapies that will enhance regeneration. Understanding the effect of individual cytokines is the first step in developing an accurate model of MAS muscle regeneration.

Hypotheses

Our hypotheses were that

- MAS and TA muscle fibers differ in their inherent ability to repair with the regenerative capacity of masseter muscle being significantly reduced;

- MAS and TA differ significantly in their response to cytokines (TNF-α, IL-1β and IL-4) present in the inflammatory milieu during repair, with masseter regeneration being significantly more adversely affected by the presence of these cytokines.

Specific Aims

Our specific aims were to

- evaluate inherent differences (satellite activation, myoblast proliferation, myotube and myofiber formation) between MAS and TA muscle regeneration in an in vitro model;

- examine the effects of TNF-α, IL-1β and IL-4 on satellite activation, myoblast proliferation, myotube and myofiber formation in an in vitro model of MAS and TA regeneration.

CHAPTER 2
MATERIALS AND METHODS

Overview of Experimental Design

Our general experimental design was as follows:

1. Masseter and tibialis anterior muscles were harvested, digested in collagenase and single myofibers isolated under a dissecting microscope and via gradient separation.

2. A 10 µl slurry of myofibers was placed on the center of a matrigel-coated glass coverslip in a 24 well plate.

3. For each culture replicate, a total of 4 plates were used. There were 4 plates per muscle type in early (proliferation) cultures and 4 per late (differentiation) cultures. Therefore, there were 16 plates total (Fig 2-1).

4. Cytokines (TNF-α, lL-1β, and lL-4) were added to wells either on the first day of culture (day 1) to examine early events or on day 6 to examine late events.

5. Cultures were fixed either on day 6 for early events or day 12 for late events and immunostained for MyoD and myogenin.

6. In a standardized microscopic field the number of cells corresponding to a specific cell phenotype for each experimental group or control was determined by a blinded investigator.

7. Statistical analyses were performed to assess significant differences between groups.

Myofiber Isolation

Female CD-1 mice (n=4) were sacrificed at 8 weeks of age and the MAS and TA muscles were harvested and cleaned of connective tissue under a dissecting microscope. Muscles were then transferred to a 35 mm dish with 0.2% collagenase solution and incubated for 2 hours at 37ºC. At the end of digestion, muscle was transferred to a 35 mm dish with 10% horse serum (HS) in DMEM. Under a dissecting scope, single fibers were liberated from muscles by pulled glass pipettes and by trituration in wide mouth Pasteur pipettes. Dissected fibers were transferred to 15 mL conical centrifuge tubes containing 10% HS (Shefer et al. 2004). In this 1g gradient

muscle fibers were separated from lighter fibroblasts and other supporting cells. Gradient separation was repeated 3 times and separated myofibers removed to a Petri dish with 10% HS. Coverslips were coated with a solution of matrigel in DMEM (1:6 ratio; prepared according to manufacturer's instructions) and incubated at 37ºC for at least 30 minutes. A 10 µl slurry of isolated myofibers was placed on the center of a coverslip in each well of the 24 well plates.

Myofiber Culture

Plates containing myofibers were placed in a 37º C incubator for 10-20 minutes to allow myofiber attachment to Matrigel. 500µl of proliferation medium (20% FBS, 10% horse serum, 1% chick embryo extract) was then added to each well (Shefer et al. 2004). To assess early events in muscle repair (proliferation), cytokines were added to the wells as follows: IL-1β (0.1, 0.4, 0.8 ng/ml, Strle et al. 2008; Broussard et al. 2004); TNF- α (0.02, 0.05, 1.0 ng/ml, Broussard et al. 2003); and IL-4 (0.5, 1 and 2 ng/ml, Horsley et al. 2003) (Fig 2-2). Cultures were maintained for 6 days in proliferation medium, with a change of medium at 72 hrs. After 6 days of culture, plates for analysis of cytokine influence on early events were rinsed in PBS and fixed in cold 4% paraformaldehyde in phosphate buffer. Plates were stored at 4º C until immunostained for MyoD and myogenin as described below.

For the assessment of cytokine effects on late events in muscle repair (myotube/myofiber formation), after 6 days of culture, proliferation medium was replaced with differentiation medium (2% FCS, 10% horse serum, 0.5% chick embryo extract in DMEM) and maintained for another 6 days (12 days total)(Shefer et al. 2004). Cytokine or vehicle was added at this time to assess effects on myoblast fusion and differentiation. Fresh medium with cytokine or vehicle was added every 48 hours. After

6 days (12 days total) cultures were fixed in paraformaldehyde and immunostained for MyoD and myogenin as described below.

Immunofluorescent Staining/ Evaluation

Coverslips were incubated in 2% normal goat serum (NGS) for 20 minutes to block non-specific staining and then overnight in the refrigerator with anti-MyoD primary antibody. Coverslips were rinsed in PBS and then incubated in a secondary antibody conjugated to alkaline phosphatase for 3 hours at room temperature. After rinsing in TBS, NBT/ BCIP alkaline phosphatase substrate was added to each well. The reaction was stopped by replacement of the substrate with TBS. After extensive rinsing in TBS, coverslips were incubated an anti-myogenin primary antibody overnight in the refrigerator. After rinsing in PBS, coverslips were incubated in an appropriate secondary antibody conjugated to biotin for 3 hours at room temperature, rinsed, then incubated for 1 hour in streptavidin conjugated to HRP. Coverslips were then rinsed and finally incubated in the HRP substrate AEC. The reaction was stopped by the substitution of PBS for the substrate. Wells were rinsed with PBS, and the coverslips were carefully removed and mounted onto glass slides for viewing and image acquisition.

Counting and Classification of Cells

Coverslips were viewed under brightfield using a Nikon FXA microscope and a 10x field acquired using a Zeiss Mr5 digital camera and Axiovision software. The field was chosen at the interface of the regions of greatest cell density radiating from the attached myofiber and the region of cell migration. Digital images were analyzed using JMicroVision (Nicolas Roduit, ver. 1.2.7) image analysis software. This software program allowed the categorization of cells into one of the following phenotypic groups:

For the early culture we categorized cells into the following 4 groups (Figure 2-3):

- Darkly stained cells, MyoD positive cells-presumptive myoblast
- Medium stained cells, myogenin-positive cells-late stage myoblasts
- Lightly stained cells, supporting cells
- Fibers

For the late culture we categorized cells into the following 6 groups (Figure 2-4):

- small, round cells not associated with myotube or myofiber (Class 6)
- thin myofibers (Class 1)
- medium thickness myofibers (Class 2)
- thick myofibers (Class 3)
- stained myofibers with central nuclei (Class 4)
- round myofiber-like (Class 5)

Intra-rater reliability of cell categorization was tested separately for early and late cultures. For both, the Pearson Product Moment correlation coefficient was 0.97.

Power Sample Size Estimation

Sample size for the proposed cytokine experiments was based on data from Broussard et al. (2004). Means and standard deviations of myosin heavy chain protein and myogenin protein levels from C_2C_{12} cells after exposure to 3 different doses of IL-1β were used in the calculations. Using an alpha level of 0.05 and a power of 0.9, the minimum number of animals required for detection of differences among 3 different doses of a cytokine was 4.

Statistical Analyses

Descriptive statistics were reported as means and standard errors. Inferential statistics were calculated using a repeated measures ANOVA with statistical significance set at 0.05. For all aims, four factors were examined: muscle, cytokine,

dose and cell classification. Post-hoc comparisons were made using the LSD test when significant main effects/interactions were identified in the ANOVA.

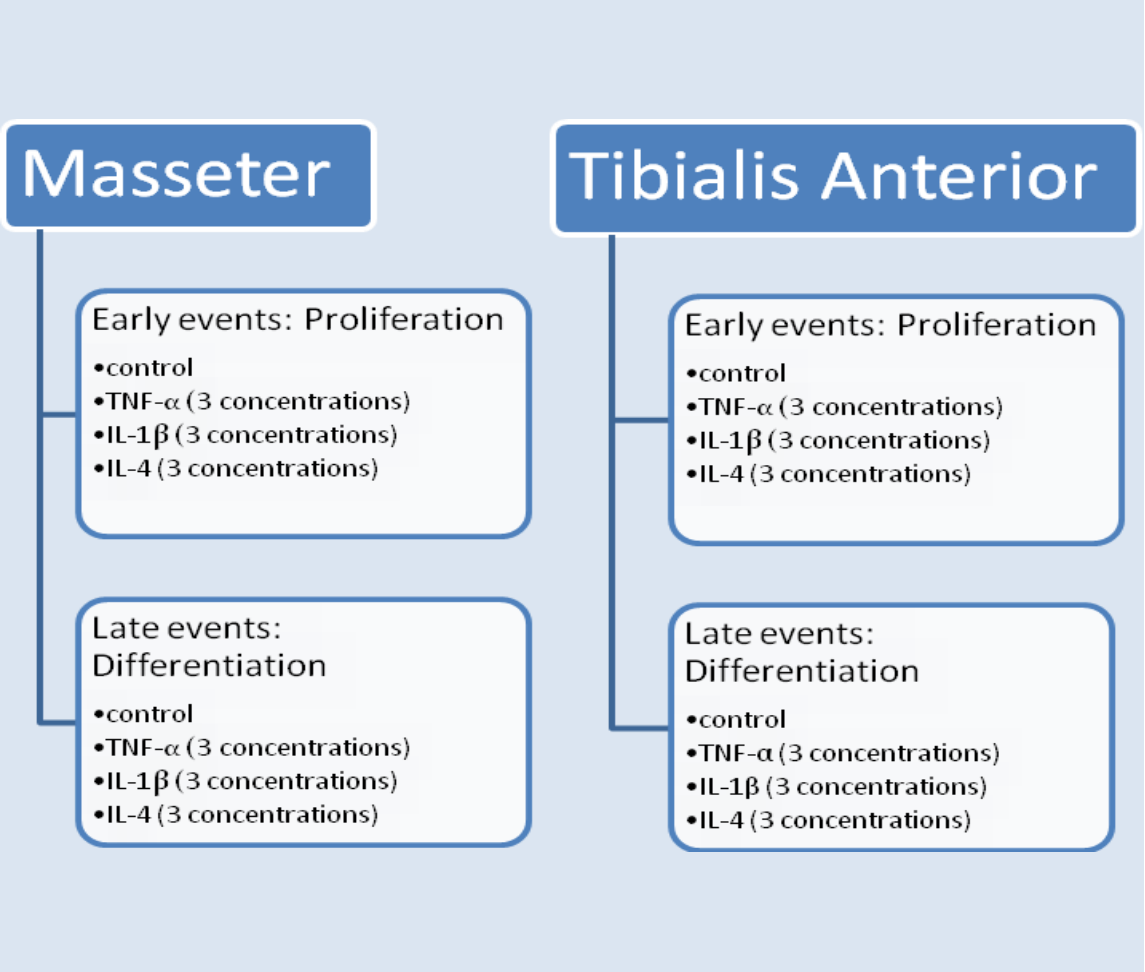


Figure 2-1. Visual representation of the study design and variables examined. Each of these 4 combinations was represented in a plate, and each combination was replicated 4 times (4 combinations x 4 replicates= 16 plates total).

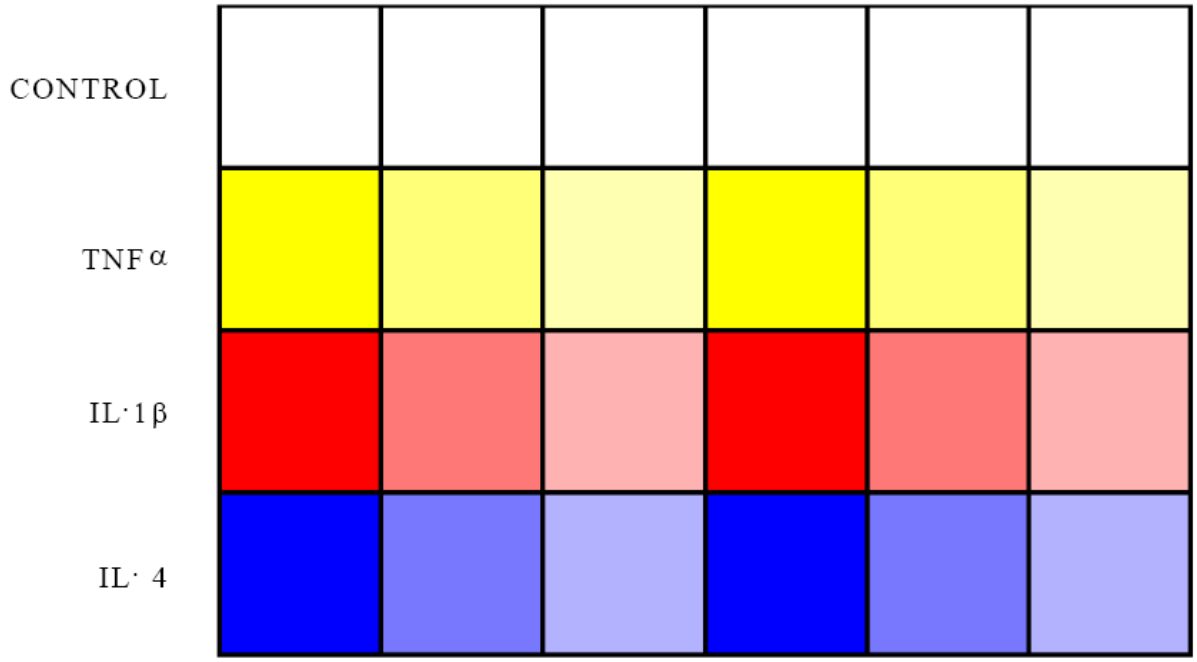


Figure 2-2. Visual representation of layout of each 24-well plate. Each plate contained either masseter or tibialis anterior muscle fibers. The top row was assigned to the controls and no cytokine was administered to these wells. The second, third and fourth rows were administered cytokines TNF-α, IL-1β and IL-4. Each cytokine was delivered in 3 concentrations, shown here by the intensity of the pigmentation of each box. Right and left sides of each well were duplicates.

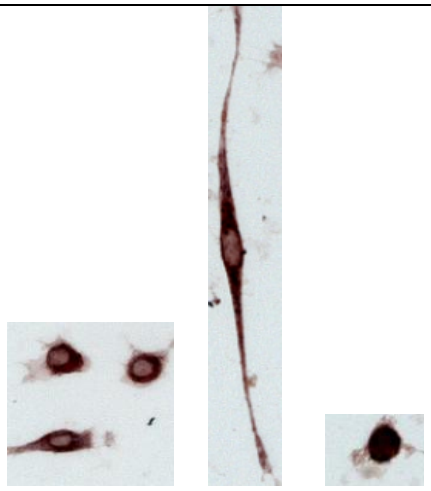
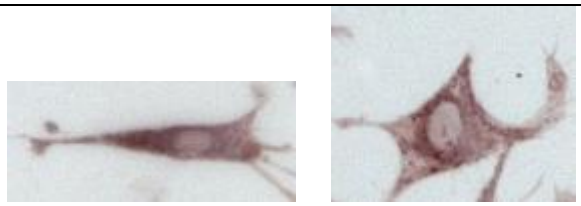
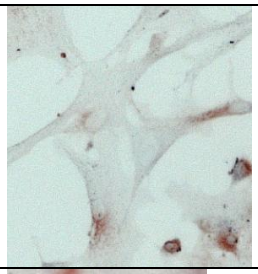
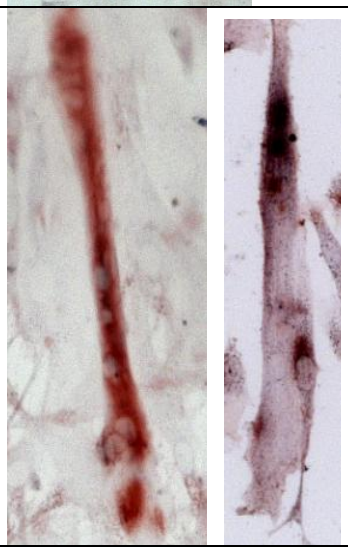
Darkly stained cells	**Immature myoblasts** MyoD-positive	
Medium stained cells	**Advanced myoblasts** Myogenin-positive	
Lightly stained cells	**Fibroblasts and other supporting cells**	
Fibers	**All fiber types**	

Figure 2-3. Cell phenotype categories for early cultures.

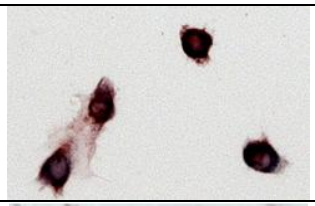
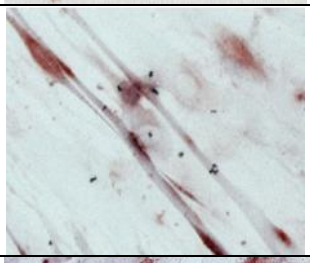
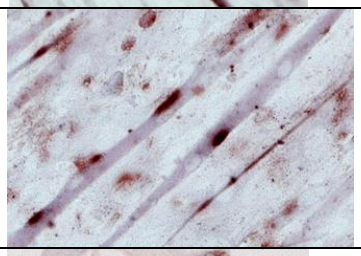
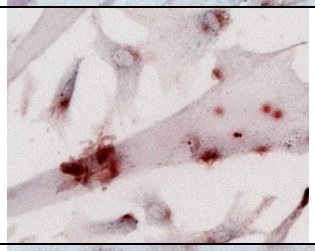
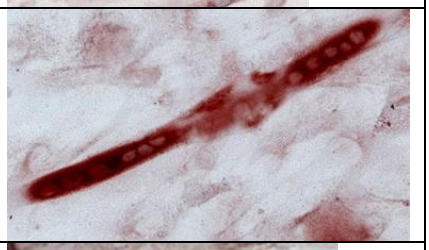
Class 6	**Immature myoblasts** Small dark cells not associated with myotube or myofiber MyoD positive	
Class 1	**Thin, multinucleate myofibers**	
Class 2	**Medium thickness myofibers with lateral nuclei**	
Class 3	**Thick myofibers with lateral nuclei**	
Class 4	**Myofibers with central nuclei** Blue/brown stained	
Class 5	**Round Myofiber-like cells with central nucleus**	

Figure 2-4. Cell phenotype categories for late cultures**.**

CHAPTER 3
RESULTS

Do Differences in Cell Proportions Exist Between MAS and TA Control Cultures?

Examination of MAS and TA early (proliferation) control cultures revealed distinct differences in cell phenotypes (Figure 3-1A-C). Generally, MAS control cultures had a higher cell density and a greater quantity of cells that were medium stained or MyoD/myogenin-positive. Results of the analysis of variance, shown in Table 3-1, identified a significant interaction (muscle x classification). Further post-hoc testing identified TA as having a statistically larger proportion of MyoD-positive darkly-stained cells, presumably young myoblasts (Figure 3-1C). MAS was found to have a significantly greater proportion of medium stained MyoD/myogenin-positive cells, presumably myoblasts/myotubes (Figure 3-1C).

Examination of late (differentiation) MAS and TA control cultures also revealed distinct differences in cell phenotype (Figure 3-2A-C). As was observed in proliferation control cultures, generally, late MAS control cultures had a higher cell density than TA control cultures. Additionally, MAS differentiation cultures appeared to have more highly developed myofibers that were well aligned with one another. Late TA control cells also possessed myofibers but in lower density and less aligned than in the late MAS controls. A significant interaction (muscle x classification) was identified by the analysis of variance, shown in Table 3-2. Post-hoc testing identified MAS as having a statistically significant greater proportion of Class 2 cells, medium thickness myofibers with lateral nuclei. TA had a significantly greater proportion of Class 6 cells, MyoD-positive cells or myoblasts (Figure 3-2C).

Do Differences in Cell Proportions Exist Between MAS and TA Proliferation Control Cultures and Cytokine-Exposed Cultures?

In MAS and TA proliferation cultures, in both controls and cytokine-exposed, the highest proportion of cells were darkly stained, MyoD-positive myoblasts, followed by medium stained MyoD/myogenin positive myoblasts and finally lightly stained supporting cells and myofibers. Statistical testing for differences among factors in MAS cytokine-exposed and control proliferation cultures identified a significant interaction (cytokine x classification x concentration) (Table 3-3). Significant differences in cell proportions between control and cytokine-exposed MAS proliferation cultures at different cytokine concentrations were found after post-hoc testing. Administration of TNF-α only at the low concentration resulted in a significantly greater proportion of darkly stained, MyoD-positive myoblasts (Figure 3-3A). No differences were observed at medium and high concentrations. The low concentration of IL-1β also resulted in significant differences; a significant increase in the proportion of medium-stained myogenin/MyoD-positive cells and a significant decrease in the proportion of darkly-stained MyoD-positive cells were identified. In contrast, the low concentration of IL-4 had no effect on cell proportions, but exposure to medium and high concentrations resulted in significant increases in the proportion of light-stained supporting cells and significant decreases in the proportion of darkly-stained MyoD-positive cells.

Statistical testing for factor differences in the TA cytokine-exposed and control proliferation cultures identified a significant interaction (cytokine x classification) (Table 3-4). However, significant differences based on cytokine concentration were not detected. Post-hoc testing identified significant differences between control and cytokine-exposed TA proliferation cultures (Figure 3-3B,D,F). TNF-α administration did

not significantly affect cell proportions in TA proliferation cultures. Exposure to IL-1β resulted in a significant increase in the proportion of darkly-stained, MyoD-positive cells and a decrease in the proportion of medium stained, MyoD/myogenin-positive cells. IL-4 exposure significantly affected only the proportion of darkly stained, MyoD-positive cells.

Do Differences in Cell Proportions Exist Between MAS and TA Differentiation Control Cultures and Cytokine-Exposed Cultures?

A significant interaction of factors was identified in the ANOVA, shown in Table 3-5, (cytokine x classification x concentration x muscle). Further post-hoc testing identified significant differences in cell proportions between control and cytokine-exposed MAS differentiation cultures (Figure 3-4A,C,E). Exposure to TNF-α at the highest concentration resulted in a significant increase in the proportion of Class 6 cells, MyoD positive myoblasts. TNF-α exposure at all concentrations resulted in a decrease in the proportion of Class 4 myofibers. Exposure to high concentrations of IL-1β also decreased the proportion of Class 4 myofibers, but this decrease was concomitant with an increase in medium-sized myofibers (Class 2). IL-4 exposure resulted in a decrease in the proportion of Class 4 cells, but only at the lowest concentration. At high IL-4 concentrations, the proportion of Class 5 cells was increased.

Significant differences in cell proportions between control and cytokine-exposed TA differentiation cultures were also identified (Figure 3-4B,D,F). Exposure to TNF-α at the highest concentration resulted in a significantly increased proportion of Class 5 cells and a concomitant decrease in the proportion of Class 4 myofibers. In contrast, exposure to TNF-α at the medium concentration resulted in a significant increase in Class 6 cells, MyoD-positive myoblasts. IL-1β exposure at the highest concentration

also resulted in a significant increase in the proportion of Class 6 cells. Significant effects with IL-4 exposure were only identified at the medium concentration. Exposure resulted in an increased proportion of Class 2 and Class 3 myofibers and a decrease in Class 6 MyoD-positive myoblasts.

Do Differences Exist in the Effects of Cytokine Exposure on Cell Proliferation and Myofiber Formation Between MAS and TA Cultures?

To compare the effects of cytokine exposure on MAS and TA cultures, the means of cell proportions for MAS and TA for each cytokine were normalized to cell proportion means of control cultures to eliminate any differential growth effects. A significant interaction of factors, shown in Table 3-6, was found in proliferation cultures (cytokine x classification x concentration x muscle). Exposure to medium concentrations of IL-1β and medium and high concentrations of IL-4 resulted in significant differences between MAS and TA in the proportion of light cells (Figure 3-5B,C). No significant differences in the proportion of different cell/ fiber types were detected between MAS and TA late, differentiation cultures with any cytokine exposure (Table 3-7; Figure 3-6A-C).

Table 3-1. ANOVA: MAS and TA controls, early cultures

Effect	df	F	p
Muscle	1	2.5	0.17
Classification	3	104.7	0.00*
Muscle x Classification	3	6.3	0.00*

* $p < 0.05$

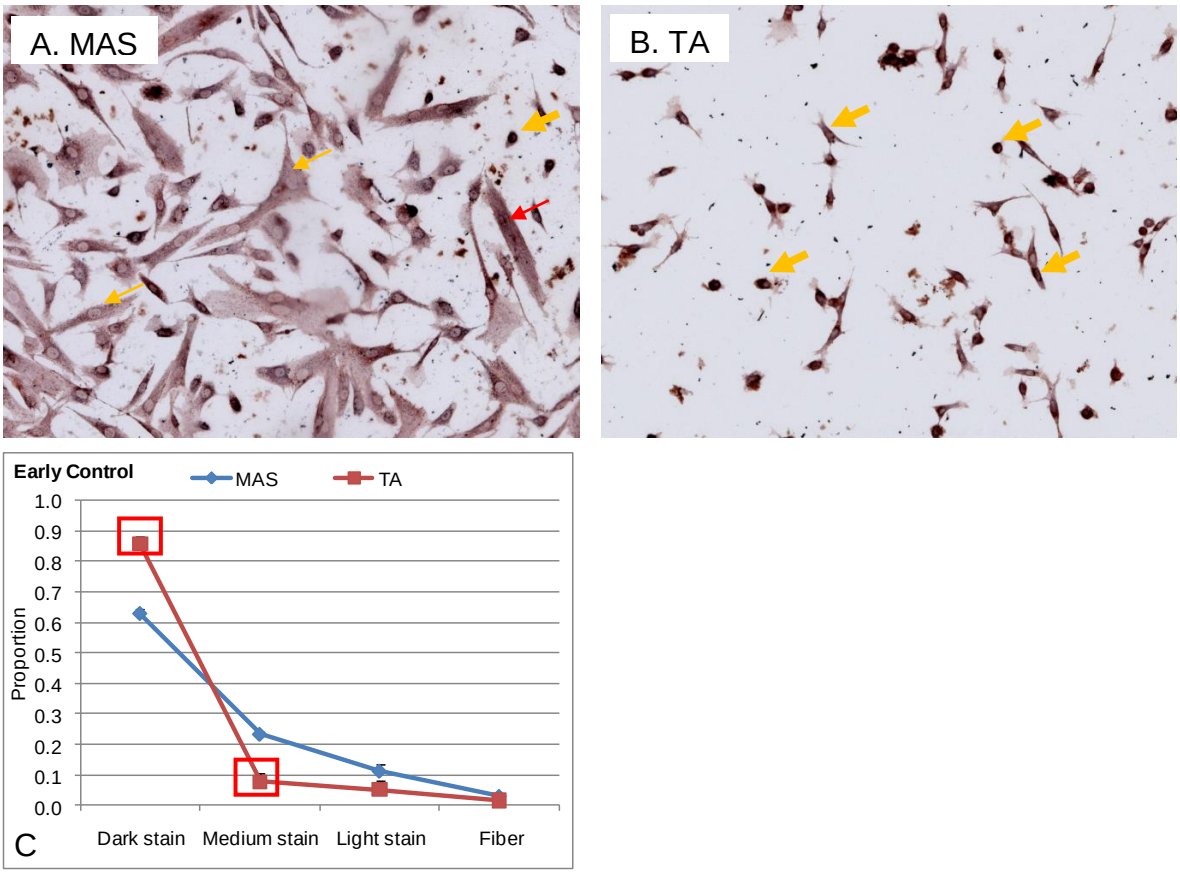


Figure 3-1. Photomicrographs of MAS and TA proliferation (early) cultures and graph of cell phenotypes (mean±SE). A,B) The upper panels are representative fields analyzed for MAS and TA. Thick arrows (yellow) label darkly blue-stained MyoD positive cells. Thin arrows (yellow) label medium blue-brown stained MyoD/myogenin positive cells. A myofiber is labeled with a red arrow in MAS. C) In the graph in the lower left panel differences in cell proportions between MAS and TA control proliferation (early) cultures are shown. Statistically significant differences in cell proportions between muscles are denoted by a red box.

Table 3-2. ANOVA: MAS and TA controls, late cultures

Effect	df	F	p
Muscle	1	-9.00	1.00
Classification	5	5.75	0.00*
Muscle x Classification	5	1.33	0.00*

* $p < 0.05$

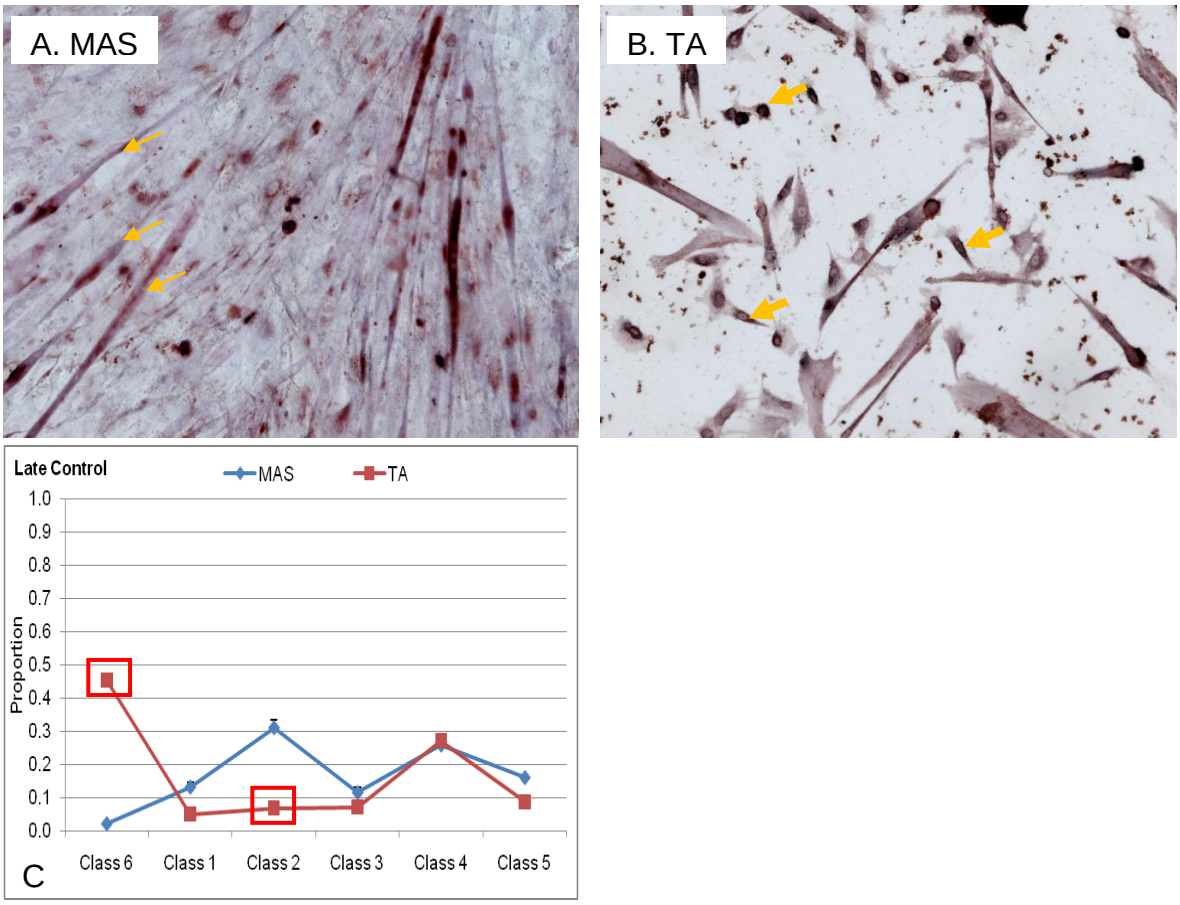


Figure 3-2. Photomicrographs of MAS and TA differentiation (late) cultures and graph of cell phenotypes (mean±SE). A,B) The upper panels are representative fields analyzed for MAS and TA. Thick arrows (yellow) label darkly blue-stained MyoD positive cells. Thin arrows (yellow) label medium myofibers. C) In the graph in the lower left panel differences in cell proportions between MAS and TA control proliferation (early) cultures are shown. Statistically significant differences in cell proportions between muscles are denoted by a red box.

Table 3-3. ANOVA: Early MAS Controls vs. treated with cytokines

Effect	df	F	p
Cytokine	3		
Concentration	2		
Classification	3	2.25	0.00*
Cytokine x Concentration	6	1.57	0.00*
Cytokine x Classification	9	3.92	0.00*
Concentration x Classification	6	1.67	0.98
Cytokine x Concentration x Classification	18	2.38	0.01*

* $p < 0.05$

Table 3-4. ANOVA: Early TA controls vs. treated with cytokines

Effect	df	F	p
Cytokine	3	1.0	0.44
Concentration	2	8.4	0.02*
Classification of cells	3	88.9	0.00*
Cytokine x Concentration	6	-8.1	1.00
Cytokine x Classification	9	2.9	0.02*
Concentration x Classification	6	0.7	0.62
Cytokine x Concentration x Classification	18	1.1	0.37

* $p < 0.05$

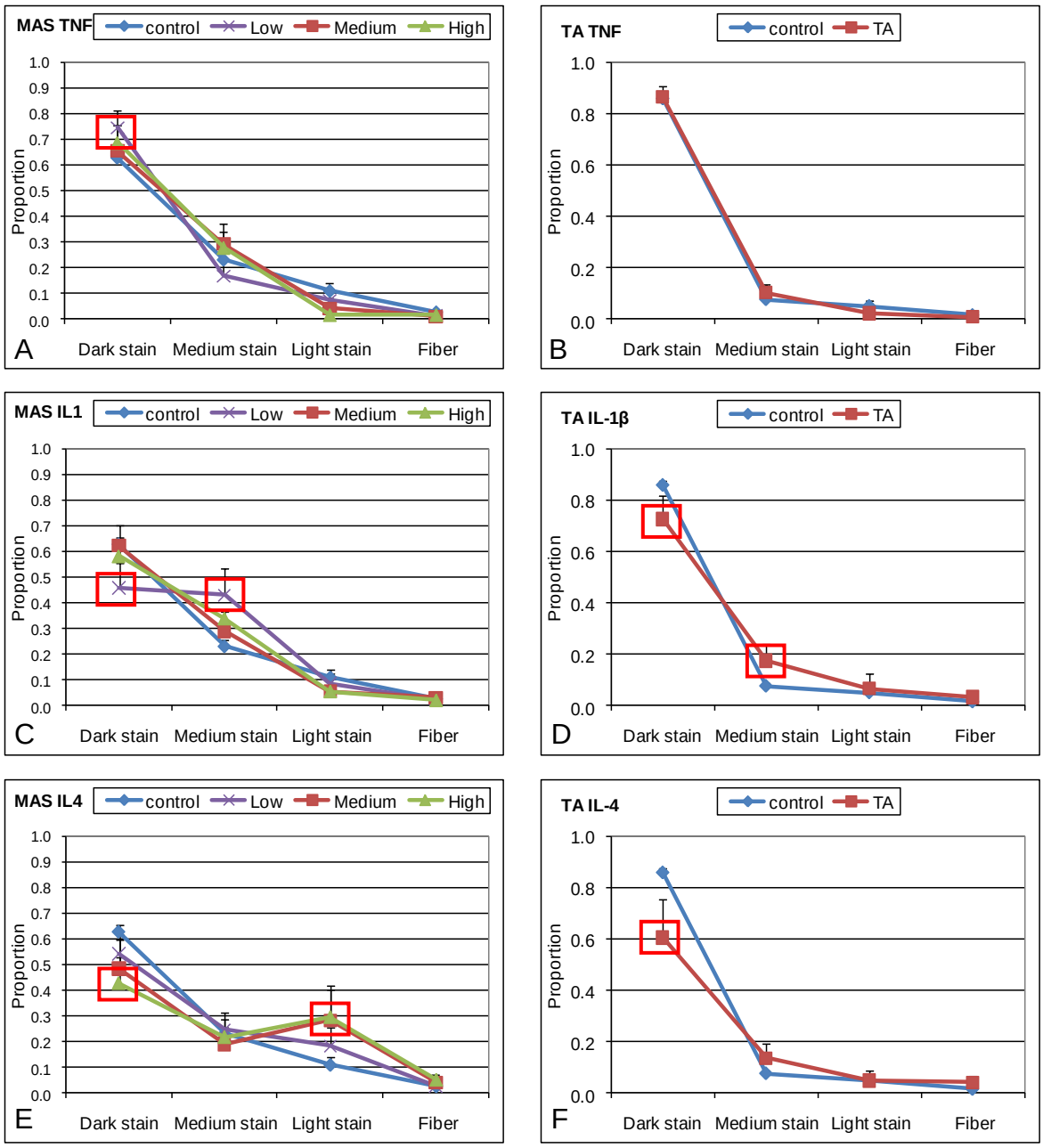


Figure 3-3. Graphs illustrating differences in controls vs. treated proliferation (early) cultures exposed to cytokines (mean±SE). A, B) MAS and TA exposed to TNF-α. C, D) MAS and TA exposed to IL-1β. E, F) MAS and TA exposed to IL-4. Statistically significant differences in cell proportions are denoted by a red box.

Table 3-5.ANOVA: Late controls vs. treated with cytokines

Effect	df	F	p
Muscle	1	0	0.80
Cytokine	3	3	0.09
Muscle x Cytokine	3	0	0.97
Concentration	2	3	0.09
Concentration x Muscle	2	0	0.83
Classification	5	10	0.00*
Classification x Muscle	5	27	0.00*
Cytokine x Concentration	6	3	0.02*
Cytokine x Concentration x Muscle	6	0	0.98
Cytokine x Classification	15	2	0.02*
Cytokine x Classification x Muscle	15	2	0.09
Concentration x Classification	10	1	0.44
Concentration x Classifications Muscle	10	0	0.95
Cytokine x Concentration x Classification	30	1	0.20
Cytokine x Concentration x Classification x Muscle	30	2	0.01*

* $p < 0.05$

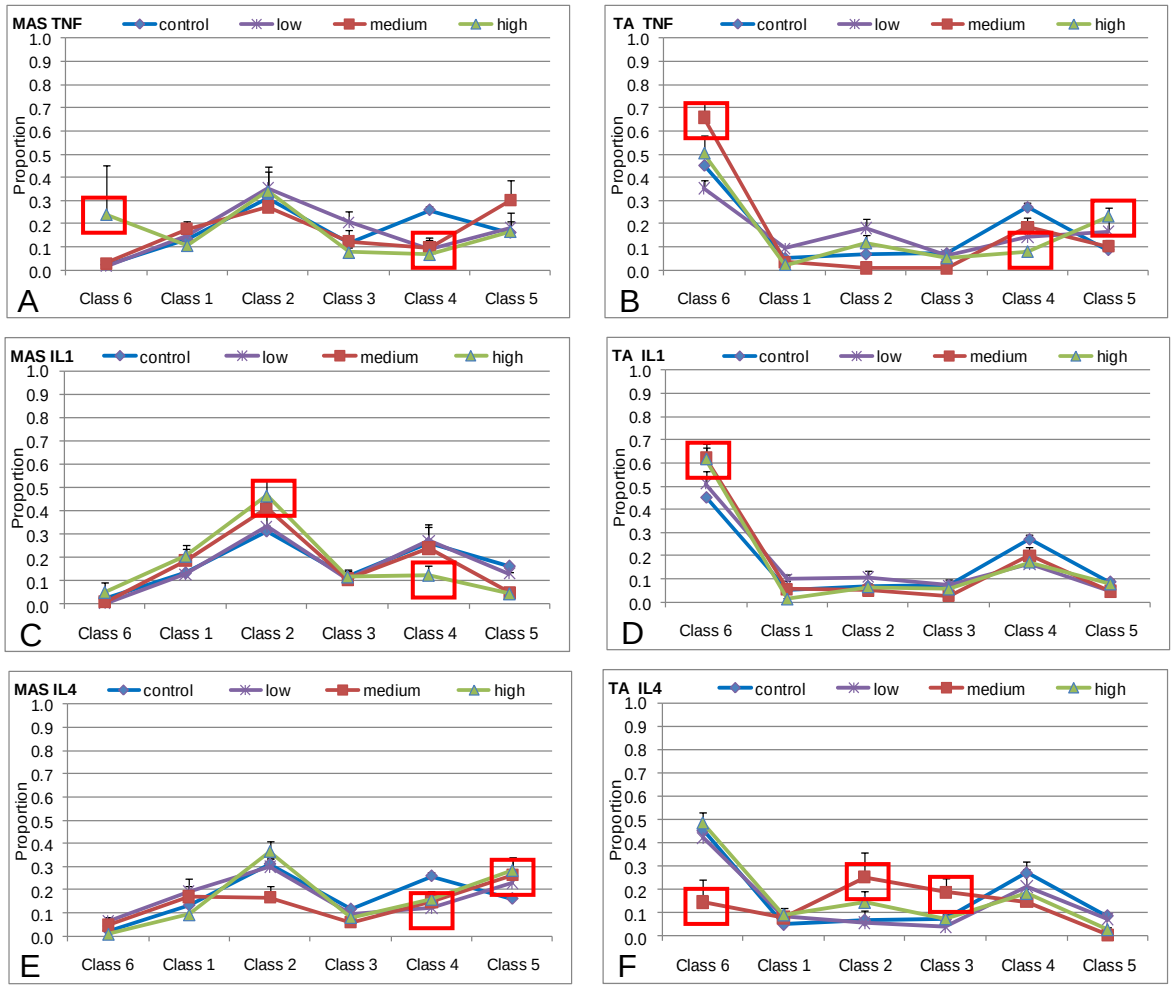


Figure 3-4. Graphs illustrating differences in controls vs. treated differentiation (late) cultures exposed to cytokines (mean±SE). A, B) MAS and TA exposed to TNF-α. C, D) MAS and TA exposed to IL-1β.E, F) MAS and TA exposed to IL-4. Statistically significant differences in cell proportions are denoted by a red box.

Table 3-6. ANOVA: Early MAS vs. TA treated with cytokines and normalized to means of controls

Effect	df	F	p
Muscle	1	0.32	0.61
Cytokine	2	2.52	0.16
Muscle x Cytokine	2	1.40	0.32
Concentration	2	1.09	0.39
Concentration x Muscle	2	0.55	0.60
Classification	3	1.63	0.25
Classification x Muscle	3	0.07	0.97
Cytokine x Concentration	4	1.06	0.42
Cytokine x Concentration x Muscle	4	2.23	0.13
Cytokine x Classification	6	1.70	0.18
Cytokine x Classification x Muscle	6	1.06	0.42
Concentration x Classification	6	1.46	0.25
Concentration x Classification x Muscle	6	0.63	0.71
Cytokine x Concentration x Classification	12	0.53	0.88
Cytokine x Concentration x Classification x Muscle	12	2.45	0.02*

* $p < 0.05$

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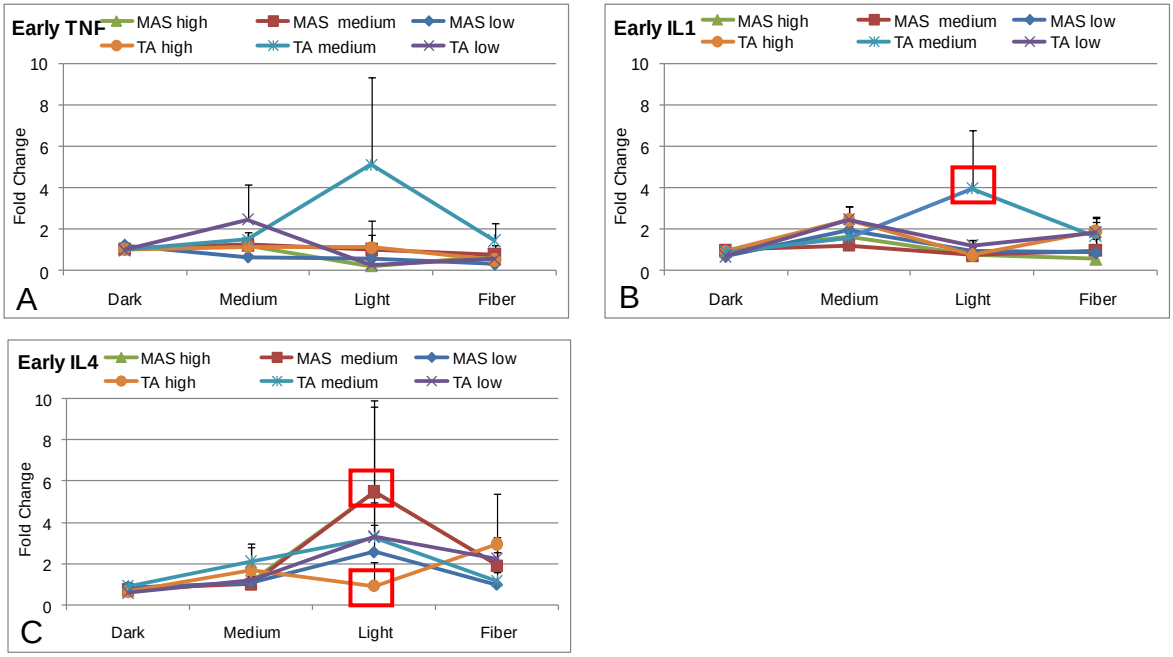

Figure 3-5. Graphs illustrating differences in MAS and TA proliferation (early) cultures exposed to cytokines (mean±SE). A) Exposed to TNF-α. B) Exposed to IL-1β. C) Exposed to IL-4. For analyses, means for each muscle and cytokine exposure were normalized to control means. Statistically significant differences in fold changes between muscles at the same concentrations are denoted by a red box.

Table 3-7. ANOVA: Late MAS vs. TA treated with cytokines and normalized to means of controls

Effect	df	F	p
Muscle	1	0.58	0.50
Cytokine	2	0.63	0.56
Cytokine x Muscle	2	1.98	0.22
Concentration	2	1.39	0.32
Concentration x Muscle	2	1.33	0.33
Classification	5	0.57	0.72
Classification x Muscle	5	0.93	0.49
Cytokine x Concentration	4	0.53	0.71
Cytokine x Concentration x Muscle	4	1.67	0.37
Cytokine x Classification	10	1.47	0.20
Cytokine x Classification x Muscle	10	1.18	0.34
Concentration x Classification	10	0.79	0.63
Concentration x Classification x Muscle	10	0.72	0.70
Cytokine x Concentration x Classification	20	1.12	0.36
Cytokine x Concentration x Classification x Muscle	20	0.95	0.53

* $p < 0.05$

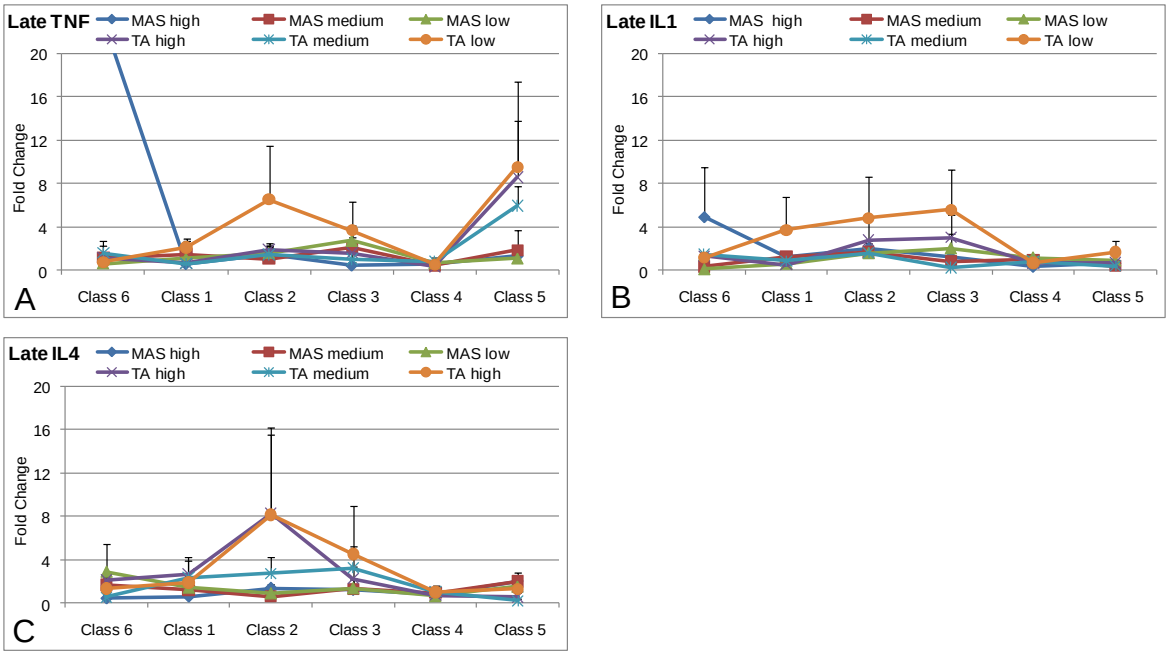

Figure 3-6. Graphs illustrating differences in MAS and TA differentiation (late) cultures exposed to cytokines (mean±SE). A) Exposed to TNF-α. B) Exposed to IL-1β. C) Exposed to IL-4. For analyses, means for each muscle and cytokine exposure were normalized to control means. No statistically significant differences in cell proportions were identified.

CHAPTER 4
DISCUSSION

Previous studies have demonstrated that injured MAS and TA differ in their regenerative response with the MAS muscle requiring three times longer to repair (Harris, 2010; Pavlath et al. 1998). However, in these studies it was not determined if the flaw in repair capacity lies in the ability of satellite cells to activate, proliferate and form myofibers, or was the result of a hostile environment. Previous work in the laboratory has provided evidence that the cytokine/chemokine milieu differs between MAS and TA both at baseline and after injury (Harris, 2010). The present study sought to examine differences in an in vitro model and using this model to determine if responses to specific cytokines differed between MAS and TA.

Differences in the In Vitro Proliferative and Differentiation Potential between Presumptive Myogenic Stem Cells Isolated from MAS and TA Myofibers

Initially we established baseline growth characteristics for cells emanating from myofibers isolated from MAS and TA both during early events in myogenesis (satellite cell activation, migrating, and proliferation) and during late differentiation events (myotube and myofiber formation). We observed significant differences between MAS and TA in the proportion of cells representing different stages of myoblast maturation and muscle fiber formation. Early proliferation MAS cultures had a significantly greater proportion of myogenin positive cells than TA cultures. Additionally, late differentiation MAS cultures had significantly greater proportion of medium sized myofibers than TA cultures. Late MAS cultures consistently had myofibers that were more advanced, organized and more closely resembled muscle fibers. In contrast, in both early proliferation and late cultures, TA had a significantly greater proportion of darkly stained blue-cells, presumably proliferating MyoD-positive myoblasts. These results suggest

that, at least in vitro, satellite cells in isolated myofibers from both MAS and TA are capable of activation and proliferation and the formation of myofibers. However, the data also suggest that, in vitro, cells derived from myofibers isolated from MAS and TA may differ in their time course towards myofibers formation.

Few previous studies have examined in vitro the relative myogenic potentials of cells isolated from MAS and limb muscle. Pavlath et al. (1998) examined the proliferative potential of myoblasts isolated from freeze-damaged MAS and TA and observed a decreased potential in myoblasts isolated from MAS. However, it could not be determined if the decreased potential was inherent to the MAS myoblast, the result of fewer satellite cells in MAS (Ono et al. 2010) or the result of prior exposure to the inflammatory milieu. Ono et al. (2010) compared the ability of clonally-derived satellite cells from MAS, extensor digitorum longis (EDL) and soleus (SOL) to proliferate and differentiate and reported that cells isolated from MAS proliferated more but differentiated later than cells isolated from either limb muscle. These results would appear to contradict ours. However, more likely they are the result of differences in the in vitro models. The in vitro model used by Ono et al. examined the potential of cells enzymatically isolated from myofibers and maintained quiescent for extensive periods prior to placement in a culture medium that promotes proliferation. Our in vitro model was developed to better approximate conditions during muscle repair after injury. In our model, presumptive myogenin cells (satellite cells) were isolated in situ on myofibers and allowed to activate in situ before migration, proliferate and finally proliferation. Thus the behavior of satellite cells in our model was not influenced by enzyme mediated changes in adhesion characteristics or relationships with their associated basal lamina

ECM. The differences observed between our results and Ono et al. most likely reflect differences in the behaviors of isolated presumptive myogenic stems cells in an artificial environment and cells allowed to undergo responses to myofibers disruption in situ.

Differences in the Response to Cytokine Exposure In Vitro between Presumptive Myogenic Stem Cells Isolated from MAS and TA Myofibers

The effects of the addition of each cytokine to proliferation and differentiation cultures are discussed under separate headings. However, there is a wealth of data from both in vivo and in vitro studies that each of these cytokines can elicit the expression of the other cytokines as well as increase their own expression in myogenic cells. Significant effects of exposure to each cytokine on myogenesis were detected in our in vitro model, but assays to specifically assess the types and quantities of cytokines present in our cultures are needed to clarify potential confounding variables and help solidify our interpretation of the roles of each cytokine.

TNF-α

Previous studies have demonstrated the complex role of TNF-α in inflammation and muscle regeneration (Palacios et al. 2010, Broussard et al. 2003, Grounds et al. 2008, Li et al. 2003, Langen et al. 2004). TNF-α can modulate muscle regeneration through its inactivation of Pax7, a gene expressed by quiescent satellite cells, through the activation of p38 MAPK (Palacios et al. 2010). Inactivation of Pax7 allows the satellite cell to exit the quiescent state and become activated, and activates MRFs, in particular MyoD and myogenin, promoting proliferation and early stages of differentiation (Palacios et al. 2010). Therefore, through its activation of p38 MAPK, TNF-α promotes proliferation and early stages of differentiation of satellite cells (Palacios et al. 2010). TNF-α also activates NFκB which positively influences

proliferation but inhibits differentiation (Grounds et al. 2008). TNF-α also inhibits IGF signaling through activation of c-Jun N-terminal kinase (JUN) (Broussard et al. 2003; Grounds et al. 2008). In summary, research has shown that TNF-α enhances proliferation (Li et al. 2003) and has varying effects on differentiation of satellite cells in vitro (Langen et al. 2003, 2004).

Based on these studies we would expect to see an increased proportion of dark, MyoD-positive cells in TNF-α exposed cultures. We did observe significant increases in the proportion of dark MyoD-positive cells in all TNF-α exposed MAS cultures (early and late) as well as in exposed late TA. The concentrations at which this effect was seen varied between proliferation and differentiation cultures, suggesting that sensitivity to the cytokine between early presumptive myoblasts and later derivations of these stem cells differed. Cell proportions in TA early proliferation cultures were not affected by TNF-α exposure. Considering the delicate effect of concentration and minimal data detailing appropriate concentrations, it is likely that we did not deliver the optimal concentration of TNF-α needed to see an effect on TA early cultures. Overall, the increase in proportion of MyoD-positive myoblasts in both early and late MAS and late TA cultures after exposure to TNF-α supports previous findings that TNF-α enhances proliferation. In addition to increasing the proportion of presumptive myoblasts, treatment with TNF-α significantly decreased the proportion of more differentiated MyoD/myogenin positive multinucleated myofibers (Class 4) in late MAS (at all concentrations) and TA (at high concentration). Therefore, our results suggest that in our in vitro model TNF-α has a negative effect on myoblast differentiation which would

be in accordance with TNF-α's inhibitory role on differentiation through disruption of IGF (Broussard et al. 2003).

In late TA cultures, when TNF-α was delivered at high concentration, there was a significant increase in the proportion of small, round, myogenin positive myofibers with central nuclei (class 5 cells). This cell phenotype has not been previously described either in vivo or in vitro. The cells appear to be budding off established myofibers. It is unclear if these cells represent a myofiber phenotype unique to our in vitro model or are a prelude to apoptosis (Sishi et al. 2011; Andrianjafiniony et al. 2010). Several studies of the effects of TNF-α in vivo and in vitro have established its role in signaling apoptosis. However, it has been demonstrated that the concentrations of TNF-α delivered in this study do not promote apoptosis in vitro (Broussard et al. 2003).

IL-1β

As with TNF-α, IL-1β activates p38 MAPK, and in doing so promotes proliferation of satellite cells. In addition, IL-1β inhibits myogenin and myosin heavy chain expression, hindering differentiation events possibly by inhibition of IGF-1 (Strle et al. 2010; Broussard et al. 2004). However, high doses of IL-1β have been shown to result in IL-6 production by myofibers, which promotes myogenesis (Strle et al. 2008). Taken together these findings suggest that IL-1β enhances proliferation and has a dose dependent effect on differentiation, with enhancement of differentiation and fusion when delivered at high doses.

In our early cultures, IL-1β had a positive influence on differentiation of presumptive myogenic stem cells derived from MAS and TA fibers. In early proliferation MAS and TA cultures we observed an increase in the proportion of medium stained, MyoD/myogenin positive myoblasts and a decrease in dark, MyoD- positive

presumptive proliferating myoblasts. In TA, this effect was seen at all concentrations, suggesting that our concentrations were on the high end and pushed myofibers towards production of IL-6 and hence differentiation. In MAS enhanced differentiation was only seen when IL-1β was delivered at its lowest concentration. This would suggest that the low concentration delivered might actually be high for the muscle and promoted IL-6 production. However, higher concentrations may have induced IL-1β- mediated myoblast apoptosis and resulted in the minimal effect observed on differentiation when it was delivered at medium and high concentrations.

An increase in the proportion of dark, MyoD positive myoblasts was observed in late differentiation cultures of TA myofibers when IL-1β was delivered at medium and high concentration suggesting a positive effect on myoblast proliferation at these levels. In MAS, delivery at its highest concentration resulted in an increase in the proportion of medium myofibers (Class 3 cells). These results reiterate the varying role of IL-1β depending on muscle type and concentration. We predict that the exposure of differentiation TA cultures to higher concentrations of IL-1β would further enhance differentiation and result in increased proportions of larger myofibers. Likewise, we predict that delivery of lower concentrations of IL-1β to late differentiation MAS cultures would promote proliferation and result in a greater proportion of MyoD-positive cells.

IL-4

IL-4 acts as a myoblast recruitment factor that promotes fusion of myoblasts to form myofibers (Horsley et al. 2003). Prior in vivo studies have demonstrated that, after a freeze injury, levels of IL-4 decrease in early stages of regeneration in TA but not MAS, suggesting that levels may need to remain low in order to prevent premature fusion of myofibers (Harris 2010). Based on these studies, we would anticipate

increased fusion of myofibers with the addition of IL-4. Our results support this prediction. In early cultures of MAS and TA we saw a decrease in the proportion of dark, MyoD-positive, presumptive myoblasts. In TA this effect was seen at all concentrations administered, whereas in MAS it occurred only at medium and highest concentrations.

Whereas in early cultures we saw a decrease in MyoD-positive cells, in late cultures IL-4 appeared to push cultures towards differentiation and regeneration. The effect of IL-4 on cell phenotype in MAS differentiation cultures varied significantly with concentration. When the cytokine was delivered at its lowest concentration, an increase in the proportion of MyoD/myogenin positive myofibers (Class 4 cells) was seen, whereas delivery at highest concentrations caused an increase in the proportion of small, round myofiber-like cells (Class 5 cells). As there are no previous reports of IL-4 involvement in apoptosis, the increased presence of this cell phenotype would suggest that these myofiber-like cells are a unique phenotype and not a prelude to apoptosis. In late differentiation TA cultures, exposure to the medium concentration of IL-4 resulted in increased proportion of medium and large myofibers (Class 2 and 3 cells) and a decreased proportion of MyoD- positive myoblasts. Therefore, it appears that IL-4 enhanced differentiation in late MAS cultures (when delivered at its lowest concentration) and in late TA cultures (at medium concentration). These findings are in accordance with previous studies suggesting the positive influence of IL-4 on differentiation and fusion of myogenic stem cells (Horsley et al. 2003).

When delivered at medium and highest concentrations, there was a significant increase in MAS proliferation cultures in the proportion of lightly stained cells, which included supporting cells such as fibroblasts. The finding that IL-4 increases the

proportion of supporting cells suggests that, in addition to the effects of these cytokines on myoblasts, another key element in their role in myogenesis may be their effect on supporting cells. Inflammatory mediators may promote proliferation of fibroblasts and supporting cells and this could push a muscle towards a state of scarring rather than regeneration (Li et al. 2004). However, the presence of supporting cells may also promote myogenic cells proliferation and differentiation (Joe et al. 2010). Clearly additional studies are needed to elucidate this effect, but the unique effect of IL-4 on lightly stained cells was an unexpected observation in the present study and suggests a novel mechanism for differential muscle repair.

Overall our findings suggest that IL-4 promotes differentiation and fusion of myotubes and myofibers and this is in accordance with recent studies that have demonstrated the importance of IL-4 in myogenesis (Charvet et al. 2006). In addition, we saw an effect on lightly stained supporting cells, which may be indicative of another key factor in muscle repair.

Differential Effects of Cytokine on Presumptive Myogenic Stem Cells Derived from MAS and TA

As cell proportions differed significantly between MAS and TA control cultures, in order to compare the effects of cytokines between MAS and TA, data was normalized to control means for each group. This eliminated any differences that might be the result of differences in normal growth between MAS and TA.

In proliferation cultures significant differences were observed between MAS and TA in the effects of lL1β and IL-4 on the proportion of light cells. Previous studies have demonstrated that formation of myofibroblastic cells is an adverse effect of chronic inflammation (Li et al. 2004) and phenotypic changes from myoblast to non-myogenic

cell types have been reported in several in vitro models in response to a variety of environmental cues including cytokines and growth factors (Joe et al. 2010; Payne et al. 2010; Shefer et al. 2004).

No other significant effects were observed on cell phenotype proportion between MAS and TA cultures indicating that exposure to cytokines had similar effects on myogenic cells derived from both MAS and TA. These results would suggest that the diminished capacity of masseter to repair is not the result of an increased adverse effect of examined cytokines on presumptive myogenic cells in masseter.

Conclusion

The results of this study indicate that, in our in vitro model, presumptive myogenic stem cells derived from the fibers of MAS and TA are capable of activation, proliferation and formation of myofibers. However, cultures derived from MAS and TA differed in cell phenotype proportions suggesting differences in maturation. Cytokine administration affected both proliferation and differentiation of presumptive myogenic stem cells. In general, TNF-α and IL-1β enhanced proliferation. IL-1β and IL-4 positively influenced proliferation and differentiation and IL-4 promoted fusion. These effects varied significantly with concentration of cytokines. Exposure to these cytokines has similar effects on myogenic cells derived from TA or MAS. Overall, these results suggest that the diminished capacity of MAS to repair is not the result of an increased adverse effect of these cytokines on presumptive myogenic cells in MAS.

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BIOGRAPHICAL SKETCH

Camden Doughtie was born in Savannah, Georgia. She grew up in Hilton Head Island, SC, with her parents, Collins and Allison, and her younger brother, Logan. At the age of 12 she moved to Tampa, FL where she attended middle school and high school. Upon graduation she enrolled in the University of Florida. While at the university, Camden majored in neuroscience and completed research on spinal cord regeneration at the McKnight Brain Institute under the mentorship of Dr. Dena Howland.

In 2004, she graduated and moved to Boston, MA. There, Camden attended Harvard School of Dental Medicine and completed her dental degree. She completed research on implant supported restorations with Dr. German Gallucci. During dental school, she developed a strong interest in the field of orthodontics and applied for residency. In 2008, she enrolled again at the University of Florida, this time for a 3 year orthodontic residency. While in Gainesville, she worked with Drs. Joyce Morris-Wiman and Charles Widmer on masseter muscle regeneration for her thesis.

During her spare time Camden enjoys cooking, traveling and watching foreign films. After graduation in May 2011, she plans to move to a new city and begin work as a private practice orthodontist while her fiancé, Andrew Brown, attends graduate school for architecture.