A Dissertation

entitled

Immunobiology of ICAM-1 in Skeletal Muscle Growth Processes

by

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Prior work in our laboratory demonstrated that β2 integrins, adhesion molecules expressed exclusively by inflammatory cells, contribute to the hypertrophic response to muscle overload. A potential mechanism for β2 integrin-mediated skeletal muscle hypertrophy involves its ligation to intercellular adhesion molecule-1 (ICAM-1), a major ligand for the β2 integrin CD11b/CD18. We find ICAM-1 to be expressed by skeletal muscle cells (satellite cells/myoblasts and myofibers) following mechanical overload, and its expression augmented the ensuing muscle regenerative and hypertrophic responses, as indicated by increased formation of regenerating myofibers, as well as elevated levels of protein synthesis in the muscles of wild-type compared to ICAM-1-/- mice, respectively. These results are corroborated by findings from our cell culture model, which demonstrated that skeletal muscle cell expression of ICAM-1 expression augments critical events of myogenesis, specifically those pertaining to myoblast fusion and myotube hypertrophy. Furthermore, we identify the adhesive function of the ICAM-1 extracellular domain to mediate early events of myogenesis leading to the formation of nascent myotubes, whereas subsequent events of myotube hypertrophy occur through increased myonuclear number, rates of protein synthesis, and Akt/p70s6k signaling.
associated with the cytoplasmic domain of ICAM-1. Our collective findings demonstrate that ICAM-1 expression facilitates vital myogenic processes at multiple stages of skeletal muscle development and growth, and establish a novel role for adhesion molecules of the inflammatory response in regulating skeletal muscle growth processes.
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Chapter 1

Introduction

1.1 Background

Skeletal muscle growth involves a series of complex cellular and molecular events associated with the development, formation and subsequent maturation of muscular tissue. Myogenic processes serve a critical role in vertebrate embryogenesis, whereby progenitor cells located in different somitic regions of the mesoderm give rise to trunk and limb musculatures\(^1\). Postnatal skeletal muscle demonstrates a remarkable plasticity for adapting to a myriad of physiological demands, such as exercise-induced myotramua, or dysfunction associated with clinical pathologies. In this capacity, myogenic processes serve to restore structure and function to skeletal muscle injured by exercise or disease (repair/regeneration), as well as facilitate the increase in muscle size (hypertrophy) after increased muscle use (i.e., overload).

The prescription of exercise is considered a therapeutic approach to improve skeletal muscle structure and function, as well as to address clinical dysfunctions in skeletal muscle. In particular, resistance exercise is valued for its integral role in promoting muscle protein synthesis\(^2\), which enhances the size and strength of skeletal muscles involved. In addition to elevated rates of muscle protein synthesis, muscle stem
cells (satellite cells), and cellular signaling pathways also contribute to the hypertrophic response to muscle overload (e.g. Akt/mTOR).

The inflammatory response, characterized by the accumulation of inflammatory cells (e.g., neutrophils and macrophages) within the affected skeletal muscle, cytokine production, as well as the expression of leukocyte adhesion molecules, has been identified as a crucial regulator of myogenic processes. Specifically, the inflammatory response has been demonstrated to augment skeletal muscle hypertrophy after mechanical overload, and regeneration after injury. However, underlying cellular and molecular mechanisms by which the inflammatory response augments these processes remain to be determined. 

Therefore, the central objective of this dissertation research is to establish greater insight on the immunobiology of skeletal muscle, as it pertains to the regulation of myogenic processes.

In light of this, our laboratory has been investigating the role of adhesion molecules, specifically β2 integrins (leukocyte-exclusive adhesion molecules) and intercellular adhesion molecule-1 (ICAM-1), in facilitating regenerative and hypertrophic processes in skeletal muscle. The primary function of β2 integrins is to promote cell communication between leukocytes and other cell types, leading to the tight adhesion between leukocytes and their target sites. This process is complemented by target cell expression of ICAM-1, a major ligand for the B2 integrin CD11b/CD18, which is known to function as a site of attachment for leukocytes. Based on preliminary and ongoing studies, our overarching hypothesis is that skeletal muscle cell expression of ICAM-1 augments myogenic processes, including regenerative and hypertrophic responses to muscle overload.
1.2 Specific Aims

To characterize the function of ICAM-1 expression by skeletal muscle cells, as well as to investigate its contributions to myogenic events, we utilize an *in vivo* and *in vitro* experimental design that incorporates genetically engineered mice, cultured skeletal muscle cells, and pharmacological tools.

The dissertation research first seeks to establish ICAM-1 expression in whole muscle after mechanical overload, and assess its role in facilitating the ensuing regenerative and hypertrophic processes (*Specific Aim 1*). These findings are complemented by the determination of phenotypic alterations associated with skeletal muscle cell expression of ICAM-1 at different stages of myogenic development (myogenesis), which provides an *in vitro* approach to explore potential underlying mechanisms of ICAM-1 mediated regulation of myogenic processes, including the function of specific domains of ICAM-1, and as well as identification of pertinent signaling pathways (*Specific Aim 2*).

**Specific Aim 1:** Determine the extent to which ICAM-1 is expressed by skeletal muscle cells after mechanical loading, and assess the contribution of ICAM-1 expression to ensuing muscle regeneration and hypertrophy.

We characterized ICAM-1 expression by skeletal muscle cells by assessing its localization to satellite cells and terminally differentiated muscle fibers (myofibers) in control and overloaded muscles of wild-type mice, as well as utilized wild-type and genetically engineered ICAM-1−/− mice to investigate the contributions of mechanical load-induced expression of ICAM-1 to ensuing regenerative and hypertrophic processes in whole muscles and myofibers.
Specific Aim 2: Determine the involvement of ICAM-1 expression by skeletal muscle cells in the events of in vitro myogenesis, and investigate underlying mechanisms associated with ICAM-1 mediated myogenic processes.

We established a cell culture model through the stable transfection of ICAM-1 to characterize ICAM-1 expression at critical stages of skeletal muscle cell development, and incorporated pharmacological tools, including cell permeable peptides and neutralizing antibodies, to assess the specific functions of the different domains of ICAM-1, as well as reveal relevant cell signaling pathways associated with ICAM-1 mediated growth processes.
1.3 Significance

Skeletal muscle dysfunction is a deleterious outcome stemming from physical inactivity, aging, as well as cardiovascular, pulmonary, and metabolic diseases. It is associated with increased protein degradation and reduced protein synthesis, which leads to a loss of skeletal muscle mass (atrophy). Reductions in skeletal muscle mass significantly compromise muscle function, performance in activities of daily living, and functional independence. These impairments ultimately contribute to an increased risk for injury, disability, and clinical frailty, thereby resulting in higher rates of mortality and morbidity\textsuperscript{13,14}. The scope of this problem is magnified due to an increasingly aging population, as aging exacerbates the reduction in skeletal muscle mass. Therefore, there is an urgent need for novel strategies to improve the structure and function of skeletal muscle.

Resistance exercise is often prescribed by established health organizations as a therapeutic approach to increase skeletal muscle mass and strength\textsuperscript{15}. Recent studies demonstrated that the inflammatory response plays a critical role in regulating the myogenic response to muscle overload\textsuperscript{7,8}, which mimics resistance training. However, the underlying mechanisms by which the inflammatory response confers such beneficial adaptations to skeletal muscle are unclear. Our research on the function of ICAM-1 expression by skeletal muscle cell is significant because it will establish greater insights on the cellular and molecular events by which the inflammatory response promotes muscle growth and recovery from exercise-induced injury. Understanding such events and mechanisms is essential in designing effective strategies to maximize beneficial adaptations of resistance exercise, as well as to overcome skeletal muscle
dysfunction/atrophy, and enhance the quality of life in individuals affected by cardiovascular, pulmonary, and metabolic diseases.

Findings from this study are expected to advance the field of exercise immunology by offering novel perspectives on the contributions of the inflammatory response in augmenting exercise-induced skeletal muscle hypertrophy, and adding to existing knowledge on mechanisms involved in the regulation of cell signaling pathways associated with the different stages of skeletal muscle development. Information generated from our work may also facilitate the development of new approaches to rehabilitate musculoskeletal injuries and promote the maintenance and/or growth of skeletal muscle. Furthermore, it may lead to the development of novel translational strategies, such as targeted pharmaceuticals, to promote muscle growth and address skeletal muscle dysfunction associated with aging, and/or inflammatory muscle diseases.
Chapter 2

Specific Aim 1

2.1 Specific Aim 1: Background

Myogenic processes after muscle injury and overload are accompanied by a local inflammatory response, characterized by the accumulation of leukocytes within the affected muscle\textsuperscript{7,8,9,10,16}. Prior investigators have demonstrated that leukocyte accumulation in skeletal muscle contribute to protection from subsequent injury\textsuperscript{17}, as well as regeneration and hypertrophy\textsuperscript{7,8}. The effector functions of leukocytes are activated upon their adhesion to membrane structures and/or the extracellular matrix of target cells. This adhesion is facilitated by β2 integrins, which are transmembrane glycoproteins expressed exclusively by leukocytes and cells of the hematopoietic lineage\textsuperscript{12,18}. β2 integrins exist as heterodimers when the common β subunit (CD18) is non-covalently bound to one of four α subunits (CD11a, CD11b, CD11c, or CD11d)\textsuperscript{12,19}. In addition to promoting leukocyte adhesion\textsuperscript{11}, β2 integrins function as signal transducing molecules that facilitate the production of reactive oxygen species (ROS) and cytokines from leukocytes, as well as promote neutrophil diapedesis\textsuperscript{11,19}. These functions are controlled by ligation of the CD11b/CD18 β2 integrin with one of several ligands, including: ICAM-1 (CD54), compliment protein iC3b, and fibrinogen\textsuperscript{18,20}. 
Intercellular adhesion molecule-1 (ICAM-1) is a glycoprotein expressed on the plasma membrane of leukocytes and endothelial cells, and serves as a major ligand for the β2 integrin CD11b/CD18\textsuperscript{11,12}. As a member of the immunoglobulin superfamily of adhesion molecules, ICAM-1 consists of five extracellular domains, a transmembrane segment, and an intracellular/cytoplasmic domain\textsuperscript{11}. In addition to its primary role as a site of attachment for leukocyte adhesion\textsuperscript{12}, ICAM-1 has been shown to promote cell survival\textsuperscript{21}, motility\textsuperscript{22}, and cytokine production\textsuperscript{23,24,25} in various cell types.

Earlier work in our laboratory demonstrated, via a muscle overload model of mice deficient in CD18 (CD18\textsuperscript{-/-}), that β2 integrins contribute to hypertrophy, and influence the temporal patterns of satellite cell/myoblast proliferation and skeletal muscle differentiation\textsuperscript{8}. We speculate that β2 integrins regulate the hypertrophic response to muscle overload by facilitating the binding of inflammatory cells to and initiating their communication with skeletal muscle cells. The viability of this mechanism requires the identification of a specific ligand that is expressed on skeletal muscle cells, which interacts with β2 integrins after muscle overload. As ICAM-1 serves as a primary ligand for β2 integrins\textsuperscript{11,12}, we hypothesize that if skeletal muscle cells express ICAM-1 after muscle overload, then β2 integrin-ICAM-1 interactions could serve to enhance the hypertrophic response through leukocyte adhesion-induced release of cytokines and/or the activation of intracellular ICAM-1 signaling in skeletal muscle cells.

Hence, the overall objectives of this aim are to first, establish skeletal muscle cell expression of ICAM-1 after muscle overload, and subsequently, assess its contributions to pertinent skeletal muscle growth processes, specifically regeneration and hypertrophy.
2.2 **Specific Aim 1 Hypothesis 1:** Mechanical Loading Induces Skeletal Muscle Cell Expression of ICAM-1.

Prior investigators have reported that ICAM-1 is constitutively expressed at low levels on leukocytes and vascular endothelial cells\(^{11,26}\). However, ICAM-1 expression can be induced in many different cell types after treatment with inflammatory stimuli, such as cytokines and free radicals\(^{11,27}\). Of interest, induced expression of ICAM-1 by skeletal muscle cells have been reported following treatment of cultured human skeletal muscle cells with cytokines\(^{28,29,30,31}\), and in patients with inflammatory myopathies\(^{32,33}\). To date however, the *in vivo* expression of ICAM-1 in skeletal muscle cells under non-pathological conditions, such as after mechanical loading, has not been described.

Recent work in our laboratory characterized the expression of ICAM-1 in skeletal muscle\(^{34}\). Synergistic ablation of gastrocnemius and soleus muscles of wild-type and β2 integrin deficient (CD18\(^{-/-}\)) mice exposed the plantaris muscles to mechanical overload. Real-time PCR analysis revealed 28, 21, and 11 fold increases in gene expression of ICAM-1 in overloaded muscles of wild-type mice at 3, 7, 14 d of overload, respectively. Increased gene expression was accompanied by significant elevations in ICAM-1 protein expression (4 – 6 fold) in overloaded compared to control muscles of wild-type mice. As gene and protein expressions of ICAM-1 were elevated to similar levels in wild-type and CD18\(^{-/-}\) mice, we interpret these findings to indicate that the increased expression of ICAM-1 after muscle overload occurred via a β2 integrin independent mechanism.

Importantly, our preliminary findings demonstrate that whole muscle expression of ICAM-1 is inducible following mechanical loading. Hence, we extend these findings by assessing ICAM-1 localization to satellite cells and myofibers of overloaded muscles.
2.2.1 Specific Aim 1 Hypothesis 1: Methods

*Animals:* Wild-type (C57BL/6; Jackson Laboratory) mice were exposed to a control condition, or 3, 7, 14, or 21 d of muscle overload. Experimental procedures were performed at 15 – 20 weeks of age (~26 – 30 g body mass). Before all surgical procedures, mice were anesthetized with intraperitoneal injections of 1.25% avertin (tribromoethanol; 0.02 ml/g body mass), with supplemental doses (0.05 – 0.1 ml) given as needed. Post tissue extraction, anesthetized mice were sacrificed by cervical dislocation. All experimental procedures were approved by The University of Toledo Animal Care and Use Committee. Mice were housed in a pathogen free environment on a 12 h light/dark cycle, and fed standard laboratory chow with water ad libitum.

*Mechanical Loading:* Muscle overload of the plantaris muscles was achieved through synergistic ablation of soleus and gastrocnemius muscles in wild-type mice with aseptic techniques. The soleus and gastrocnemius muscles of anesthetized mice were first exposed by making an incision on the posterior-lateral aspect of the lower limb. The distal and proximal tendons of the soleus, lateral and medial gastrocnemius were subsequently cut and carefully excised. The plantaris muscle was left intact to function as the primary plantar flexor during normal cage ambulation. Incisions were then closed with sterile suture, and mice monitored for recovery from anesthesia before being returned to their cages. Mice were also kept warm during the entire duration of anesthesia, including surgery and recovery, via a heated platform (37°C).
**Muscle Collection:** Recovered mice were free to ambulate and resume normal cage activities for 3, 7, 14, or 21 d post-surgery. An additional set of mice, which had normal cage activity and did not undergo surgery, were used as controls. Mice were killed by cervical dislocation under anesthesia. Plantaris muscles were dissected out, blotted dry, and weighed. Muscles were subsequently coated with optimal cutting temperature compound (Fisher Scientific), frozen in melting isopentane cooled on dry ice, and stored at −80°C for histological and confocal analyses.

**Confocal Microscopy:** Transverse sections (25 mm) of muscles were fixed in 50% acetone/50% methanol, blocked, and incubated with an ICAM-1 antibody (R&D Systems, #AF796; 1:20), or an antibody that recognizes the CD31 (endothelial cell marker; BD Pharmingen, #550274; 1:50) or CD11b antigen (myeloid cell marker; clone M1/70; BD Pharmingen product, #550282; 1:50).

Myofibers were delineated using fluorescent wheat germ agglutinin (WGA; Alexa Fluor® 633; 1:500)\textsuperscript{35}, which binds to sialic acid and N-acetylglucosaminyl residues of glycoproteins that are on the sarcolemma of myofibers, and links the sarcolemma to the basal lamina\textsuperscript{36,37}. Prior work has also demonstrated that WGA co-localizes with both laminin and dystrophin\textsuperscript{36,37}, which are commonly used markers of the basal lamina and sarcolemma of myofibers, respectively. Due to the ubiquitous expression of glycoproteins, WGA will delineate other cells residing in skeletal muscle, as well as connective tissue. Sections were mounted with Fluoromount-G containing DAPI and analyzed using a TCS SP5 multi-photon laser scanning confocal microscope (Leica Microsystems) at The University of Toledo’s Advanced Microscopy and Imaging Center.
**Immunohistochemistry:** Acetone fixed transverse sections (10 mm) were quenched in hydrogen peroxide (1:100), and treated with a primary antibody that recognizes an extracellular domain of mouse ICAM-1 (R&D Systems, # AF796; 1:20). Slides serving as negative controls received PBS in place of the primary antibody. After incubations initially with a biotinylated anti-goat secondary antibody (1:200), and then with horseradish peroxidase (1:1000), sections were subsequently developed with 3-amino-9-ethylcarbazole.

ICAM-1+ myofibers in one entire section of a muscle were manually counted and results were expressed as a percentage of the total number of myofibers within the section. The cross sectional area of ICAM-1+ and ICAM-1 negative myofibers were quantified using image analysis software (Image Pro Plus). The number and size of ICAM-1+ myofibers were determined only if myofiber membrane expression of ICAM-1 could be clearly delineated.

**Skeletal Muscle Cell Cultures:** Expression of ICAM-1 was evaluated in C2C12 cells (ATCC, a murine line of skeletal muscle cells), and in primary skeletal muscle cells. Primary cultures were established by digesting hindlimb muscles of 5 d old wild-type mice as described below. To enrich the purity of our primary myoblast population, a pre-plating method modified from Blau et al\textsuperscript{38,39} was adopted to provide a selective growth advantage for satellite cells/myoblasts over fibroblasts. Pre-plating consisted of resuspending cells digested from muscles into uncoated dishes for 2 h in isolation medium consisting of 80% Ham’s F-10 nutrient mixture (Sigma-Aldrich) and 20% fetal bovine serum (FBS; Sigma-Aldrich) supplemented with 2.5 ng/ml of bFGF (R&D Systems, # AF796; 1:20).
systems) and antibiotic-antimycotic reagents (100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B; Sigma-Aldrich).

Unattached cells were aspirated and centrifuged (800 g, 4°C, 5 min), subsequently plated in Matrigel® (BD Biosciences) coated dishes, and allowed to proliferate in isolation medium at 37°C/5% CO₂, with fresh media fed every 2 days. At ~60% confluence, cells were dislodged with Trypsin (Sigma-Aldrich) and exposed to a second bout of pre-plating for 1 h, using the techniques described above. Immunolabeling for Pax7 (an established marker of satellite cells/myoblasts)⁴⁰, revealed that > 90% of cultured primary cells were Pax7+.

Both C2C12 cells and primary myoblasts were seeded at 2,500 cells/cm² in growth medium (DMEM and 10% FBS; 1:1 DMEM/Ham’s F10, with 20% FBS and 2.5 ng/ml of bFGF, respectively), and allowed to proliferate to confluence. C2C12 cells were seeded in non-coated wells, whereas primary cells were seeded in Matrigel® coated wells. For differentiating cultures, C2C12 and primary cells were treated with differentiation medium (DMEM and 2% horse serum) for up to 6 d to induce the formation and maturation of multi-nucleated myotubes. Growth and differentiation media for both cell lines contained antibiotic-antimycotic reagents, and were changed every 2 d.

To induce ICAM-1 expression, cultures containing proliferating myoblasts or differentiated myotubes (4 d in differentiation medium) were treated with 10 ng/ml of rmTNF-a (R&D Systems) for 24 h. ICAM-1 expression in proliferating cultures was assessed via flow cytometry and western blot; whereas western blotting and immunofluorescence were used to detect ICAM-1 expression in differentiating cultures.
**Western Blotting:** Cultured skeletal muscle cells were homogenized with a cell sonicator (Misonix; Model S-4000) in reducing sample buffer (2% sodium dodecyl sulphate, 1.5% dithiothreitol, 1M Tris-HCL and 10% glycerol), containing protease inhibitors (1 mM EDTA, 5mg/ml leupeptin, 5mg/ml aprotinin, and 11 mM 4-(2-aminoethyl) benzenesulfonyl fluoride; Sigma-Aldrich). Cell lysates were then boiled and separated on 10% SDS-PAGE gels, and transferred to PDVF-FL membranes using a semi-dry protocol (20 V for 1 h in a solution containing 192 mM glycine, 25 mM Tris, and 10% methanol). Membranes were subsequently blocked with a Tris-buffered saline (TBS) solution containing 50% Odyssey® blocking buffer, cut at ~75 kDa, and incubated overnight at 4°C with an antibody for ICAM-1 (R&D Systems, # AF796; 1:500), or α-tubulin (Cell Signaling, # 3873; 1:750), which served as a control for sample loading. Membranes were then washed and incubated with an Alexa Fluor® 680 anti-goat (ICAM-1) or anti-mouse (α-tubulin) secondary antibody (Invitrogen; 1:5,000 – 1:10,000). The relative abundance of ICAM-1 and α-tubulin was quantified using the Odyssey® infrared detection system (LI-COR; Biosciences). Cell lysates from a line of murine monocytes/macrophages (RAW 264.7 cells; Santa Cruz) were included as positive controls.

**Immunocytochemistry:** ICAM-1 localization in cultured skeletal muscle cells was assessed by immunofluorescence. Wells were fixed in 70% methanol/30% acetone, permeabilized with 0.2% Triton X-100, blocked, and incubated overnight at 4°C with an antibody for ICAM-1 (R&D Systems, # AF796; 1:50). After incubation with an Alexa Fluor® 488 secondary antibody (Invitrogen; 1:100), cells were mounted with Fluoromount-G containing 4’,6-Diamidino-2- phenylindole (DAPI, Southern Biotech).
**Flow Cytometry:** Flow cytometry was performed on cells isolated from digested plantaris muscles and in cultured C2C12 and primary myoblasts. Plantaris muscles were pooled, minced, centrifuged, and then incubated at 37°C in DMEM containing 0.1% pronase (Calbiochem) for 1 h. Cell suspensions were subsequently filtered (60 μm; Millipore), centrifuged, and suspended in staining buffer. Cultured cells were collected from tissue culture dishes using StemPro® Accutase® according to the manufacturer’s protocol (Invitrogen). The number of cells isolated was determined using a hemacytometer.

Isolated cells were treated (30 min at 4°C) with Fc block (BD Biosciences) and 200–500,000 cells were incubated for 30 min with one or more fluorophore-conjugated antibodies to identify leukocytes (0.5 ug of FITC-CD45; BD Biosciences), ICAM-1 (0.4 ug of PE-CD54 clone YN1/7.4; eBiosciences), satellite cells/myoblasts (0.5 ug of Alexa Flour® 649-integrina7; kindly provided by Fabio Rossi, University of British Columbia), or endothelial cells (0.5 ug of FITC-CD31; BD Biosciences). Isotype control antibodies were used in antibody titration experiments to control for non-specific binding. Cells were analyzed using the BD Biosciences FACSaria (for digested muscle) or FACSCalibur (for cultured cells) at the University of Toledo Flow Cytometry core facility.
2.2.2 Specific Aim 1 Hypothesis 1: Results

ICAM-1 is Localized to Endothelial Cells of Control Muscles

To follow up on our preliminary findings of ICAM-1 gene and protein expression, immunofluorescent labeling was performed to characterize the cellular location of ICAM-1 in whole muscles (Figure 1). During control conditions, ICAM-1 was expressed in neighboring cells adjacent to myofibers, of which ~ 95% co-localized to CD31+ endothelial cells (Figure 1D), and in blood vessels (Figure 1E). This is consistent with earlier findings that ICAM-1 is constitutively expressed by endothelial cells in the vasculature of skeletal muscles. Of importance, ICAM-1 is not expressed in myofibers of control muscles.

![Image of confocal microscopy images showing ICAM-1 localization in control muscles of wild-type mice. Representative images from confocal microscopy: A) ICAM-1 (green), B) WGA (membrane marker; red) and ICAM-1 (green), C) CD31 (endothelial cell marker; purple), and D) Merged image of ICAM-1 (panel A), and CD31 (panel C). Co-localization analysis revealed that the majority (90–95%) of the ICAM-1 labeling in control muscles was expressed by CD31+ endothelial cells. ICAM-1 was not found on the membrane of myofibers in control muscles. E) Representative image from immunohistochemistry detection. In muscle sections of control muscles, ICAM-1 was found only in presumptive blood vessels (arrowheads). Scale bar = 50 μm.]
ICAM-1 is Not Constitutively Expressed by Cultured Skeletal Muscle Cells

The constitutive expression of ICAM-1 in skeletal muscle cells was assessed via flow cytometry of integrin α7, a marker of satellite cells/myoblasts\textsuperscript{38}, as well as western blotting in control cultures of C2C12 and primary cells (Figure 2). Both flow cytometry and western blot analysis revealed that ICAM-1 was not expressed by control cultures of C2C12 and primary skeletal muscle cells, including proliferating myoblasts, differentiating myoblasts, as well as terminally differentiated myotubes. These results demonstrated ICAM-1 is not constitutively expressed in skeletal muscle cells throughout the different stages of skeletal muscle cell development.

Figure 2: ICAM-1 expression in cultured C2C12 and primary skeletal muscle cells under control conditions. 

A) Flow cytometry analysis of ICAM-1 expression in cultured myoblasts. ICAM-1 was detected using a phycoerythrin (PE) conjugated antibody, whereas myoblasts were identified using an AlexaFlour\textsuperscript{®} 649 (AF649) α7 antibody. ICAM-1 was not expressed by C2C12 and primary myoblasts in control cultures.

B) Western blot analysis of ICAM-1 expression in cultured myotubes treated with differentiation medium for up to 6 days.
ICAM-1 is Co-localized to Myofibers of Overloaded Muscles

In comparison to control whole muscles, confocal microscopy revealed that ICAM-1 was expressed by a variety of cell types after muscle overload. Specifically, ICAM-1 was co-localized to endothelial cells (CD31+), myeloid cells (CD11b+), and mononuclear cells in the interstitium (CD45+/CD31\textsuperscript{neg}) (Figures 3–4). Significantly, muscle loading was observed to induce ICAM-1 expression in 9, 18 and 20% of myofibers at 3, 7 and 14 d of overload, respectively. In addition, myeloid cells (CD11b+) were found to be closely associated with ICAM-1+ myofibers (Figure 4). The ICAM-1+ myofibers in our findings did not exhibit hallmark features of injury, necrosis, or regeneration. Hence, our observations demonstrate that induced expression of ICAM-1 by myofibers can occur in non-pathological conditions and in the absence of overt muscle damage.

**Figure 3:** ICAM-1 localization in 7 and 14 d overloaded muscles of wild-type mice. Representative images from confocal microscopy. A) ICAM-1 (green) was found to be co-localized to CD31+ endothelial cells (cyan) and to be associated with the membrane of myofibers (WGA+; red). Cells (DAPI+; blue) in the interstitium were also found to express ICAM-1 (arrow). Column labeled as “MERGED” represents an overlay of the ICAM-1, CD31, WGA and DAPI images. Scale bar = 25 μm (7 d), and 50 μm (14 d). B) Higher magnification clearly revealed the colocalization of ICAM-1 (green) with the membrane marker WGA (red) in overloaded muscles. Scale bar = 10 μm.
To determine the contribution of ICAM-1 expression in overloaded myofibers, histological analyses revealed that the mean cross-section area of ICAM-1+ myofibers was 20% larger than myofibers that did not express ICAM-1 at 14 days of overload (Figure 5).

**Figure 4:** ICAM-1 and CD11b localization in 7 d overloaded muscles of wild-type mice. Representative images from confocal microscopy. ICAM-1 (green) and CD11b (red) co-localized on the membrane of myofibers (arrow) and CD11b+ cells were closely associated with ICAM-1+ myofibers (arrowhead). Numerous CD11b+ cells residing in overloaded muscles expressed ICAM-1. Scale bar = 50 µm.

To determine the contribution of ICAM-1 expression in overloaded myofibers, histological analyses revealed that the mean cross-section area of ICAM-1+ myofibers was 20% larger than myofibers that did not express ICAM-1 at 14 days of overload (Figure 5).

**Figure 5:** Cross-sectional area of ICAM-1 positive and ICAM-1 negative myofibers from control and overloaded (3, 7, and 14 d) wild-type mice. **A)** Mean cross-sectional area for control and overloaded mice. Statistical analysis was performed on mean data from overloaded muscles. **B)** Frequency distribution of myofiber cross-sectional area in 14 d overloaded muscles. * = significantly greater for ICAM-1 positive compared to ICAM-1 negative myofibers at 14 d of overload. n = 115 and 406 for ICAM-1 positive and ICAM-1 negative myofibers, respectively. Mean ± SEM.

We interpret these findings to indicate that overload-induced expression of ICAM-1 by myofibers serves as a mechanism for facilitating myofiber hypertrophy after mechanical overload.
ICAM-1 is Co-localized to Satellite Cells/Myoblasts of Overloaded Muscles

Presumptive satellite cells (a specialized population of myogenic stem cells), normally residing between the sarcolemma and basal lamina of myofibers, were also observed to express ICAM-1 in overloaded muscles via confocal analysis (Figure 6A). To substantiate overload-induced expression of ICAM-1 by satellite cells, flow cytometry analysis revealed a 5 fold increase in ICAM-1 expression of satellite cells/myoblasts (CD45+/CD31+/α7+) in 7 day overloaded muscles compared to control muscles (Figures 6B–C). Our findings demonstrate a population of satellite cells in overloaded muscles that express ICAM-1. As satellite cells play a crucial role in regulating skeletal muscle regeneration\(^41,42\)), we speculate that ICAM-1 expression may augment regenerative processes in skeletal muscle.
TNF-α treatment induces ICAM-1 expression in cultured skeletal muscle cells

In contrast to control cultures, both proliferating and differentiated skeletal muscle cells (C2C12 and primary wild-type) treated with TNF-α showed a prominent ICAM-1 band appearing at 110 kDa (Figure 7).

Flow cytometry on proliferating cultures revealed TNF-α treatment to induce ICAM-1 expression in 30% and 15% of C2C12 and primary myoblasts, respectively (Figure 7A). In differentiated cells, immunolabeling localized ICAM-1 expression to multinucleated myotubes and non-fused myoblasts, following TNF-α treatment (Figure 8).

Figure 7: ICAM-1 expression in cultured C2C12 and primary skeletal muscle cells treated with TNF-α (10 ng/ml for 24 h). A) Flow cytometry analysis of ICAM-1 expression in cultured myoblasts treated with TNF-α. ICAM-1 was detected using a phycocerythrin (PE) conjugated antibody; whereas, myoblasts were identified using an AlexaFluor® 649 (AF649) α7 antibody. TNF-α treatment caused ICAM-1 to be expressed by 30% and 15% of C2C12 and primary myoblasts, respectively. B) Western blot analysis of ICAM-1 expression in proliferating myoblasts or differentiated myotubes (5 d) under control conditions and after TNF-α treatment.

Figure 8: ICAM-1 localization in differentiated cultures of C2C12 and primary cells. Treatment of differentiated cultures with 10 ng/ml TNF-α for 24 h resulted in the expression of ICAM-1 by multinucleated myotubes and in non-fused myoblasts in both C2C12 and primary cells. ICAM-1 was not detected in control cultures (not shown). Scale bar = 50 µm.
Of significance, the molecular weight of ICAM-1 in our cell cultures (Figure 7B) was identical to the 110 kDa band found in control and overloaded whole muscles from our preliminary findings. As confocal analysis of overloaded muscles revealed several cell types to express ICAM-1, we interpret our in vitro findings as further indication that skeletal muscle cell expression of ICAM-1 contributes to enhanced ICAM-1 protein expression in whole muscles after mechanical loading.
2.2.3 Specific Aim 1 Hypothesis 1: Interpretations

Our current findings correspond with preliminary observations of increased gene and protein expression of ICAM-1 in whole muscles with mechanical overload, and conclusively demonstrate that mechanical loading induces skeletal muscle cell expression of ICAM-1. However, the mechanism through which mechanical loading induces ICAM-1 expression in skeletal muscle cells remains to be determined. As indicated by our preliminary studies, β2 integrins do not contribute to the expression profile of ICAM-1 after muscle overload. In cultured skeletal muscle cells, several cytokines shown to induce myoblast or myotubes expression of ICAM-1 (e.g., TNF-α, IL-1β, IL-6, and IL-4)\textsuperscript{28,29,30,31} have also been reported to be elevated at the gene and/or protein level in skeletal muscle after mechanical loading\textsuperscript{43,44,45}. In addition to inflammatory stimuli, cyclic strain\textsuperscript{46} and fluid shear stress\textsuperscript{47} have also been demonstrated to induce endothelial cell expression of ICAM-1. Thus, the increased expression of ICAM-1 after muscle overload may have been the result of mechanical loading-induced cytokine production and/or through a mechanosignal transduction mechanism.

Significantly, our results demonstrate that ICAM-1 expression is substantially up-regulated in the satellite cells/myoblasts and myofibers of overloaded muscles. Consequently, we speculate that satellite cell expression of ICAM-1 contributes to the ensuing muscle regeneration by augmenting regenerating myofiber formation; whereas myofiber expression of ICAM-1 augments the hypertrophic response to overload in whole muscles and myofibers by augmenting muscle protein synthesis.
2.3 Specific Aim 1 Hypothesis 2: Skeletal Muscle Cell Expression of ICAM-1

Contributes to Muscle Regeneration after Mechanical Loading.

As the resident stem cells of skeletal muscle, satellite cells function as the primary source for postnatal progeny with myogenic potential\textsuperscript{41}, and possess the intrinsic capacity for self-renewal\textsuperscript{48}. In the unperturbed state, satellite cells reside between the basal lamina and sarcolemma of myofibers, where they remain in mitotic quiescence as a reserve population of cells\textsuperscript{41}. However, in response to a variety of physiological stimuli, including mechanical overload and/or injury, satellite cells are recruited back into the cell cycle where they proliferate, and subsequently migrate to the damaged site via chemotaxis\textsuperscript{1}. This results in the generation of a population of muscle precursor cells (myoblasts) within the skeletal muscle, which commences repair and/or regenerative processes to prevent the necrosis of damaged fibers, and replace necrotic fibers, respectively, thereby restoring structure and function to injured muscles\textsuperscript{41}.

While the extent to which satellite cells contribute to overload-induced hypertrophy remains controversial, recent studies utilizing recombinant-based technology have firmly established satellite cells as indispensable in the process of skeletal muscle regeneration\textsuperscript{41,42}. Specifically, through the use of \textit{Cre} recombinase on genetic mouse models, Pax7 has been identified as the major regulator of satellite cell activity and proliferation, and its expression is required for new and regenerating fiber formation\textsuperscript{40,49}.

As we have demonstrated muscle overload to induce ICAM-1 expression in a population of satellite cells, we hypothesize that ICAM-1 enhances the formation of regenerating myofibers through its regulation of Pax7-expressing satellite cells.
2.3.1 Specific Aim 1 Hypothesis 2: Methods

Animals: Wild-type and ICAM-1 knock-out (ICAM-1\(^{-/-}\), ICAM1tm1Cws; C57BL/6 background; kindly provided by C. Wayne Smith, Baylor University) were exposed to control conditions or 7 and 3, 7, and 14 d of muscle overload described above. ICAM-1\(^{-/-}\) mice were generated by replacing the entire coding region of the ICAM-1 gene with a puromycin cassette\(^{50}\), which yields no alternatively spliced ICAM-1 isoforms\(^{51}\).

Histology: To investigate the role of ICAM-1 in facilitating regenerating myofiber formation, transverse sections (10 μm) of 7 and 14 d overloaded plantaris muscles from wild-type and ICAM-1\(^{-/-}\) mice were stained with hematoxylin and eosin and examined for signs of injury, necrosis, and regeneration as described above. The total number of myofibers in hematoxylin and eosin stained sections of overloaded muscles were manually counted, with centrally nucleated regenerating myofibers expressed as a percentage of the total number of myofibers within a muscle section.

Western Blot: To gain insight on the role of satellite cells in augmenting overload-induced regeneration, muscle homogenates were blotted for Pax7 expression, as a readout of the temporal pattern of satellite cell activity and proliferation. Briefly, control and overloaded samples (3, 7, 14 d) were homogenized with a bead homogenizer (TissueLyser; Qiagen) in reducing sample buffer containing protease inhibitors as described above. Muscle homogenates were then boiled and separated on 10% SDS-Page gels, and transferred to nitrocellulose membranes via a semi-dry protocol described above. Membranes were subsequently blocked in Tris-buffered saline (TBS) containing 1% casein, cut at ~ 40 kDa, and incubated overnight at 4°C with an antibody for Pax7.
(University of Iowa Developmental Studies Hybridoma Bank; 1:200), or GAPDH (Cell Signaling Cat# 2118; 1:5,000), which served as a control for sample loading. Membranes were then washed and incubated with an Alexa Fluor® 680 anti-mouse IgG (Pax7) or anti-rabbit secondary antibody (Invitrogen; 1:5,000 – 7,500). The relative abundance of Pax7 and GAPDH was quantified using the Odyssey® infrared detection system (LI-COR; Biosciences).
2.3.2 Specific Aim 1 Hypothesis 2: Results

*ICAM-1 expression influences regenerating myofiber formation after muscle overload*

At 7 d of overload, the percentage of regenerating myofibers was 3.5 fold higher for wild type compared to ICAM-1−/− mice (Figure 9). Conversely, the percentage of regenerating myofibers showed a higher trend (p = 0.11) in ICAM-1−/− compared to wild-type mice at 14 d of overload. These findings demonstrate that ICAM-1 expression facilitates muscle regeneration in response to mechanical overload.

![Figure 9](image_url)

*Figure 9:* Regenerating myofiber formation. **A)** Representative cross sections of overloaded muscles from wild-type and ICAM-1−/− mice at 7 d of overload. Centrally nucleated (regenerating) myofibers were higher in wild-type (35%) compared to ICAM-1−/− mice (11%). Scale bar = 100 μm. **B)** Regenerating myofibers expressed as a percentage of the total number of myofibers. # = significant interaction at 7 d of overload. Mean ± SEM.

Hence, we interpret our results to indicate that skeletal muscle cell expression of ICAM-1 augments the kinetics of regenerating myofiber formation and their subsequent maturation into normal myofibers with peripheral nuclei after muscle overload.

*ICAM-1 expression acutely regulates the temporal pattern of satellite cell proliferation*

To corroborate the altered kinetics of ICAM-1 mediated regenerating fiber formation, western blot analysis was performed to determine the relative abundance of
Pax7, a marker of satellite cells/myoblasts. At 3 d of overload, Pax7 expression increased by 2 fold in ICAM-1\(^{-/-}\) compared to wild-type mice (Figure 10). However, at 7 and 14 d of overload, the relative abundance of Pax7 increased above control levels by a similar magnitude in wild type and ICAM-1\(^{-/-}\) mice. These results demonstrate that the expression of ICAM-1 contributes to the acute regulation of the number of satellite cells/myoblasts in overloaded muscles.

**Figure 10:** Satellite cells/myoblasts abundance. **A)** Representative western blot (30 µg of protein/lane) of Pax7 (60 kDa) in control (CT) and overloaded (3, 7 and 14 d) muscles of wild type (WT) and ICAM-1\(^{-/-}\) (IC) mice. **B)** Quantitative analysis of the relative abundance of Pax7 protein. # = significant interaction at 3 d of overload. * = significantly elevated at each overload time point compared to control levels for both wild type and ICAM-1\(^{-/-}\) mice. n = 6 – 8/group. Mean ± SEM.
2.3.3 Specific Aim 1 Hypothesis 2: Interpretations

Taken together, our findings demonstrate that skeletal muscle cell expression of ICAM-1 influences the temporal patterns of regenerating fiber formation and satellite cell/myoblast activity after muscle overload. We interpret our collective findings as a strong indication of ICAM-1’s role in facilitating regenerative processes in skeletal muscle. ICAM-1 expression on satellite cells/myoblasts in overloaded muscles could serve to accelerate the formation of regenerating myofibers by acutely enhancing the adhesion/fusion of myoblasts to one another, and/or contributing nuclei to existing myofibers through myonuclear accretion. This would result in a decrease in satellite cell/myoblast activity, as indicated by the reduction in the relative abundance of Pax7 in wild-type mice at 3 d of overload. Significantly, our results demonstrate that ICAM-1 expression augments critical stages of skeletal development and formation.
2.4 **Specific Aim 1 Hypothesis 3:** ICAM-1 Facilitates Skeletal Muscle Hypertrophy after Mechanical Loading by Augmenting Rates of Muscle Protein Synthesis.

The primary objective of this aim was to determine the contributions of skeletal muscle cell expression of ICAM-1 in facilitating the hypertrophic response to mechanical overload. Load-induced hypertrophy is a desired adaptation of exercise, and represents an increase in skeletal muscle mass, thereby enhancing overall structure and function of the muscles involved. Fundamentally, skeletal muscle hypertrophy occurs when the rates of muscle protein synthesis exceed those of protein degradation. The enhanced capacity of skeletal muscle to generate and synthesize important proteins (e.g., actin, myosin) leads to a higher total protein yield within the affected muscle, as well as other morphological enhancements, such increased cross-section area of individual muscle fibers.

Besides muscle protein synthesis, satellite cells have also been proposed as a prerequisite for hypertrophy. The contribution of satellite cells was initially justified through the concept of myonuclear domain\(^52\), which postulates a coordinated increase between myonuclei number and cytoplasm volume, in order to maintain a constant domain size\(^53,54,55,56\). Post-mitotic myonuclei accretion is thought to occur through the fusion of satellite cells with existing myofibers following exercise-induced trauma or injury\(^57,58\). However, the effectiveness of satellite cells in facilitating hypertrophy is controversial. Recent studies utilizing Pax7\(^{cre}\) mice have demonstrated that effective muscle hypertrophy can still occur in the absence of satellite cells\(^49\). Furthermore, recent studies have also reported that myonuclei accretion precedes any discernible changes in skeletal muscle size\(^54\). Hence, the extent to which satellite cells contribute to skeletal muscle hypertrophy is under much debate.
Based on our preliminary findings of larger cross-sectional area in ICAM-1+ myofibers after mechanical overload (Figure 5), we speculate that skeletal muscle cell expression of ICAM-1 serves to augment hypertrophic responses to mechanical loading, as they pertain to the hypertrophy of whole muscles, as well as individual myofibers. In addition, as skeletal muscle hypertrophy is dependent on protein synthesis, we hypothesize that ICAM-1 expression enhances rates of whole muscle protein synthesis, as a mechanism by which ICAM-1 mediated muscle hypertrophy occurs.
2.4.1 Specific Aim 1 Hypothesis 3: Methods

**Hypertrophy:** Wet muscle mass, total protein content, and myofiber size in the plantaris muscles of wild-type and ICAM-1$^{-/-}$ mice served as outcome measures of hypertrophy. To determine total protein content, control and overloaded (14, 21 d) plantaris muscles were homogenized with a bead homogenizer (TissueLyser; Qiagen) in reducing sample buffer containing protease inhibitors as described above. Muscle homogenates were subsequently centrifuged (5,000 x g, 4°C, 10 min), and the amount of protein in supernatants determined via filter paper dye-binding assay$^{59}$. The cross-sectional area of normal myofibers (no signs of injury or central nucleation) in hematoxylin and eosin stained sections of control and 14 d overloaded plantaris muscles were quantified using image analysis software (Image Pro Plus).

**Protein synthesis:** To corroborate observations from our outcome measures of hypertrophy, in vivo rates of muscle protein synthesis were determined via nonradioactive western blot, known as surface sensing of translation (SUnSET)$^{60}$. This novel technique has been validated in skeletal muscle cell cultures and whole muscle preparations$^{61}$. It utilizes antibiotic puromycin (an analog of tyrosyl-tRNA – an inhibitor of protein translation), and anti-puromycin antibodies to detect the amount of puromycin incorporation into nascent peptide chains, which directly reflects the rate of mRNA translation and hence, the rate of protein synthesis$^{60}$.

Control and 7 d overloaded plantaris muscles of wild-type and ICAM-1$^{-/-}$ mice were incubated in vitro in DMEM (Hyclone) supplemented with 1 µM puromycin (Calbiochem; EMD Chemicals) for 30 min at 37°C/5% CO$_2$. Post-incubation, whole
muscles were homogenized and determined for protein content as described above. Muscle homogenates were subsequently boiled, separated on 10% SDS-PAGE gels (50 µg/lane), and then transferred to PDVF-FL membranes using a wet transfer protocol (200 mA for 1 h in 192 mM glycine, 25 mM Tris, and 10% methanol). Membranes were blocked in 5% non-fat dry milk, incubated overnight at 4°C with mouse anti-puromycin (clone 12D10; 1:5,000 in TBS-T/5% BSA; kindly provided by Dr. Philippe Pierre, Centre d'Immunologie de Marseille-Luminy), and then incubated with an isotype specific (IgG2A) anti-mouse secondary antibody (Alexa Fluor® 680; 1:10,000 in TBST/5% BSA). The relative abundance of puromycin in muscle homogenates were quantified by measuring the density of the entire lane using the Odyssey® infrared detection system (LI-COR; Biosciences)61. Coomassie blue stained gels served as loading controls.
2.4.2 Specific Aim 1 Hypothesis 3: Results

ICAM-1 augments outcome measures of skeletal muscle hypertrophy

Synergistic ablation of gastrocnemius and soleus muscles exposed the plantaris muscles of both wild type and ICAM-1−/− mice to 14 and 21 d of mechanical overload. In control muscles, the wet plantaris mass, total protein content, and myofiber cross-section area were similar between wild-type and ICAM-1−/− mice (Figure 11). Consistent with our β2 integrin findings, several markers of hypertrophy were significantly elevated in wild-type compared to ICAM-1−/− mice after muscle overload. In overloaded wild type mice, muscle mass and protein content were elevated above control levels by 2 fold; whereas myofiber size was 30% larger than control values. Significantly, overload failed to increase total protein content and myofiber size above control levels in ICAM-1−/− mice. Hence, the increase in wet plantaris mass of overloaded ICAM-1−/− mice observed could be
attributable to local edema, as opposed to authentic muscle growth. We therefore interpret our findings to indicate that the loss of ICAM-1 resulted in a blunted hypertrophic response after muscle overload.

**ICAM-1 augments whole muscle protein synthesis**

The specific contribution of ICAM-1 expression on skeletal muscle cells to synthesize proteins was explored as an underlying mechanism through which ICAM-1 augments the hypertrophic response to overload. Specifically, through nonradioactive detection of puromycin incorporation, whole muscle protein synthesis was elevated 10 fold in wild-type compared to ICAM-1−/− mice after 7 d of overload (Figure 12). Consistent with several outcome measures of hypertrophy, muscle overload generated a significant increase in muscle protein synthesis above control levels in wild-type mice; whereas in ICAM-1−/− mice, protein synthesis remained at control levels after overload. These findings indicate that ICAM-1 expression facilitated a greater synthesis of proteins in whole muscles after 7 d of overload, which manifested in the elevated markers of skeletal muscle hypertrophy observed at 14 and 21 d of overload.
2.4.3 Specific Aim 1 Hypothesis 3: Interpretations

Overall, our findings demonstrate a robust increase in whole muscle hypertrophy, as well as myofiber size, associated with skeletal muscle cell expression of ICAM-1 after mechanical overload. ICAM-1 mediated hypertrophy is attributable to the increased capacity of ICAM-1 to generate and synthesize proteins in whole muscles, as increases in whole muscle and myofiber size were preceded by elevated levels of protein synthesis. While underlying mechanisms by which ICAM-1 expression augments skeletal muscle hypertrophy are not well defined, a potential function for myofiber expression of ICAM-1 in overloaded muscles involves its dimerization, which enhances binding affinity for β2 integrins and activates downstream ICAM-1 signaling, thereby serving as a means by which myeloid cells adhere to myofibers via a β2 integrin dependent mechanism. Hence, ligation-induced activation of β2 integrins and ICAM-1 could putatively serve as a unifying mechanism by which myeloid cells\textsuperscript{7}, β2 integrins\textsuperscript{8}, and ICAM-1 (present study) contribute to hypertrophy after muscle overload. In this model, ligation-induced activation of β2 integrins and ICAM-1 initiates the release of cytokines and ROS from myeloid cells, which activates intracellular signaling pathways involved in the regulation of myogenic processes (e.g., MAPK and PI3K/Akt)\textsuperscript{27,62,63}, as well as cytokine production in ICAM-1 expressing cell types\textsuperscript{11,23,27,62}. Thus, myeloid cell adhesion to myofibers via a β2 integrin-ICAM-1 dependent mechanism could contribute to overload-induced hypertrophy via adhesion-induced release of cytokines from myeloid cells and/or activation of ICAM-1 signaling in myofibers\textsuperscript{63}. In this context, the reported clustering of ICAM-1 on the membrane of overloaded myofibers plays a physiologically relevant role in augmenting the ensuing hypertrophic response.
2.5 Specific Aim 1: Summary

Our results clearly demonstrate the expression of ICAM-1 by skeletal muscle cells (satellite cells/myoblasts and myofibers) following mechanical overload. Induced expression of ICAM-1 subsequently augmented the ensuing regenerative and hypertrophic responses to overload, which manifested in the increased formation of regenerating myofibers, and elevated levels of protein synthesis, respectively. Moreover, as ICAM-1\(^{-/-}\) mice exhibited minimal signs of regeneration and hypertrophy, we interpret our findings to indicate that ICAM-1 expression serves a fundamental and necessary role in augmenting crucial myogenic processes. Critically, our findings of increased whole muscle and myofiber hypertrophy, along with our reports of greater regenerating myofiber formation, demonstrate that ICAM-1 expression augments distinct and vital stages of skeletal muscle growth pertaining to the formation, development and maturation of muscle tissue \textit{in vivo}. Hence, to establish greater insight on the function of ICAM-1 in skeletal muscle, we extend our findings to a cell culture model to characterize phenotypic alterations associated with ICAM-1 expression at various stages of skeletal muscle cell development.
Chapter 3

Specific Aim 2

3.1 Specific Aim 2: Background

Skeletal muscle growth is regulated by an extensive and highly ordered sequence of cellular and molecular processes associated with the development, formation and subsequent maturation of muscular tissue (myogenesis) (Figure 13). Adult myogenesis is preceded by the activation of a specialized population of myogenic stem cells, known as satellite cells, which results in the generation and proliferation of muscle precursor cells (myoblasts) within the skeletal muscle$^{64}$. During myogenesis, myoblasts differentiate, adhere to each other (homotypic adhesion), and fuse to form multinucleated myotubes, which are terminally differentiated skeletal muscle cells$^{65,66,67,68,69,70}$. Nascent myotubes subsequently align with other myotubes and mononucleated myoblasts, and undergo

Figure 13: Schematic depiction of the sequence of events involved during \textit{in vitro} and \textit{in vivo} myogenesis.
hypertrophy via mechanisms involving myonuclear accretion (through the fusion of myoblasts with myotubes and/or fusion of myotubes to one another), as well as increased levels of protein synthesis\textsuperscript{65,66,67,68,69,70}. Myotubes are eventually assembled into well-aligned myofibers, of which parallel bundles span the entire length of the skeletal musculature\textsuperscript{71,72}. Hence, myogenesis \textit{in vivo} is indicated by the formation and maturation of centrally nucleated (regenerating) myofibers, which represent the end stages of myogenesis in which myotubes are forming and hypertrophying into normal myofibers with peripherally located nuclei\textsuperscript{70}.

We have demonstrated in Aim 1 that ICAM-1 expression can be induced by skeletal muscle cells through \textit{in vivo} mechanical overload, and by \textit{in vitro} treatment with cytokines (TNF-\textalpha). Significantly, ICAM-1 expression by satellite cells/myoblasts, as well as regenerating and normal myofibers, contributed to the ensuing regenerative and hypertrophic processes after mechanical overload. Further knowledge of underlying cellular and molecular mechanisms by which ICAM-1 expression confers such adaptations is required to better elucidate the role of the inflammatory response in augmenting skeletal muscle growth. Therefore, in Aim 2, we aspire to extend our findings to critical regulatory processes of \textit{in vitro} myogenesis, and develop greater insight on underlying events leading to ICAM-1 mediated skeletal muscle growth. Hence, our central objective in Aim 2 is to establish a cell culture model to assess phenotypic alterations associated with skeletal muscle cell expression of ICAM-1 at distinct stages of \textit{in vitro} myogenesis, as well as to investigate underlying mechanisms involved in ICAM-1 mediated myogenesis.
Prior studies which characterized the function of ICAM-1 have identified its extracellular domain to possess adhesive properties, whereas its intracellular/cytoplasmic domain is implicated in signal transduction, thereby suggesting distinct regulatory functions for the two domains of ICAM-1\textsuperscript{25,73}. Hence, we utilize our cell culture model to scrutinize the functions of the extracellular and cytoplasmic domains as potential mechanisms by which ICAM-1 augments critical events of \textit{in vitro} myogenesis. Specifically, we seek to delineate the roles of the extracellular and cytoplasmic domains in ICAM-1 mediated myogenesis.

Our objectives are achieved by establishing a cell culture model which incorporates genetic and pharmacological tools to determine the contributions of extracellular and cytoplasmic domains of ICAM-1 in distinct stages/events of \textit{in vitro} myogenesis. Our overarching hypothesis is that skeletal muscle cell expression of ICAM-1 augments critical events of \textit{in vitro} myogenesis, through mechanism involving its extracellular and cytoplasmic domains. Specifically, we hypothesize that the adhesive function of the extracellular domain of ICAM-1 regulates homotypic adhesion of myoblasts, leading to the initial formation of myotubes. Furthermore, we speculate that the signal transducing function of the ICAM-1 cytoplasmic domain facilitates subsequent events of myogenesis that ultimately lead to the hypertrophy of mature myotubes.
3.2 **Specific Aim 2 Hypothesis 1:** ICAM-1 Expression by Cultured Skeletal Muscle Cells Augments Nascent Myotube Formation and Myonuclear Accretion, and Contributes to the Subsequent Hypertrophy of Mature Myotubes.

Our results in Aim 1 demonstrate that ICAM-1 expression by skeletal muscle cells contribute to the formation of regenerating fibers and increased levels of hypertrophy in whole muscles. Significantly, ICAM-1 expression enhanced the formation of regenerating myofibers in the absence of an overt increase in satellite cells/myoblasts proliferation. Furthermore, ICAM-1 expression augmented the size of individual myofibers, as part of the hypertrophic response to muscle overload. We therefore interpret our findings to indicate that ICAM-1 augments preceding myogenic events leading to the formation and subsequent maturation of skeletal muscle *in vivo*. Specifically, the altered kinetics of satellite cell/myoblast activity, along with an accelerated response in regenerating myofiber formation, suggest a putative role for ICAM-1 expression in promoting events of myoblast-myoblast fusion to generate myofibers, as well as the fusion of myoblasts to existing myofibers. Hence, we expect our cell culture model to parallel the results of our *in vivo* findings, and hypothesize that ICAM-1 expression distinctly augments events of *in vitro* myogenesis that occur after the proliferation and differentiation of myoblasts; specifically the initial fusion of myoblasts to generate multi-nucleated myotubes, and the ensuing fusion of myoblasts to nascent myotubes. We further hypothesize that ICAM-1 expression augments subsequent events of myogenesis, which manifest in the hypertrophy of mature myotubes, thereby corresponding to our *in vivo* findings of larger ICAM-1+ myofibers.
### 3.2.1 Specific Aim 2 Hypothesis 1: Methods

**Skeletal Muscle Cells:** C2C12 myoblasts (ATCC) were stably transfected with a murine pHβa-ICAM1 expression vector\(^3\) (ICAM-1+ cells; kindly provided by Dr. Stephen Hedrick, University of California, San Diego) under transcriptional regulation of the human β-actin promoter. Transfection quality plasmid DNA was prepared using a commercially available kit (Qiagen), and transfected using Lipofectamine\textsuperscript{TM} 2000 according to the manufacturer’s protocol (Life Technologies). Transfected cells were placed under G418 (800 μg/ml) selection for a total of 24 d to create a stable line of transfected cells. A population of C2C12 myoblasts stably transfected with an empty pHβAPr-1 expression vector\(^4\) (EV cells; kindly provided by Dr. Gunning, University of New South Wales), as well as non-transfected C2C12 myoblasts (CT cells), served as controls.

Cultured cells from all 3 lines of skeletal muscle cells (CT, EV, ICAM-1+) were grown to confluence in growth medium (DMEM + 10% FBS), and then switched to differentiation medium (DMEM + 2% horse serum) to induce myotube formation for a time course of 6 d.

**Western blotting:** Cultured skeletal muscle cells were lysed with a cell sonicator (Misonix; Model S-4000) in reducing sample buffer (2% sodium dodecyl sulphate, 1.5% dithiothreitol, 1M Tris-HCL and 10% glycerol), containing protease inhibitors (1 mM EDTA, 5mg/ml leupeptin, 5mg/ml aprotinin, and 11 mM 4-(2-aminoethyl) benzenesulfonyl fluoride; Sigma-Aldrich). Cell lysates were then boiled and separated on 10% SDS-PAGE gels (25 μg of protein per lane), and transferred to PDVF-FL.
membranes (Millipore) using a semi-dry protocol (20 V for 1 h in Towbin’s transfer buffer containing 192 mM glycine, 25 mM Tris, and 10% methanol). Membranes were subsequently blocked in 5% non-fat dry milk, and incubated overnight at 4°C with an antibody that recognizes myogenin (clone FD5, BD Pharmingen, # 556358; 1:250), or α-tubulin (Cell Signaling Technology, # 3873; 1:500). Detection of antibody binding was achieved by using an Alexa Fluor® 680 anti-mouse secondary antibody (Invitrogen; 1:5000 – 1:7500), and the Odyssey® infrared detection system (LI-COR; Biosciences). Data is presented in raw scanning units.

**Immunofluorescence:** To assess myoblast proliferation, cultured cells were fixed in 70% methanol/30% acetone and permeablized with 1 N hydrochloric acid. To assess myoblast differentiation, cells were fixed in 2% formaldehyde followed by 70% methanol/30% acetone. To assess the formation of myotubes, cells were fixed in 70% methanol/30% acetone and permeablized with 0.05% Triton X-100. All cells were subsequently blocked in a buffer containing 3% bovine serum albumin, 0.05% Tween-20, and 0.2% gelatin mixed in PBS. Cells were then incubated for 2 h with an antibody that recognizes anti-5-bromo-2′-deoxyuridine (BrdU) (clone G3G4, Developmental Studies Hybridoma Bank; 1:500), anti-myogenin (clone FD5, Developmental Studies Hybridoma Bank; 1:200), or anti-sarcomeric myosin heavy chain (MHC) (clone MF20, Developmental Studies Hybridoma Bank; 1:20) for 2 h. All cells were washed with PBS and then incubated with a fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and mounted with Fluoromount-G™ containing 4′,6-Diamidino-2-phenylindole (DAPI; Southern Biotech).
Image capture was performed using a 10X objective on an epifluorescence microscope (Olympus IX70; B&B Microscopes) that was equipped with a CCD camera (RT KE SPOT™; Diagnostic Instruments). Fluorescent images were captured in six fields per well. The first image was taken at the center of the well and the second image was taken two fields of view (400 µm) to the right of the first image. The remaining four images were captured four fields of view (800 µm) from the preceding image, and collectively depicted a square in the center of the well. The same camera settings (i.e., exposure, gain, and gamma) were used for all images captured for a data set. The standardized approach to image capture was used to avoid investigator bias.

**Creatine Kinase Activity:** Cultured cells at 1, 2, and 3 d of differentiation were collected in a non-reducing/non-denaturing lysis buffer (1% Igepal®; Sigma-Aldrich) containing protease inhibitors listed above, and pelleted by centrifugation. The resulting supernatant (10 µl) was incubated at 25°C with a creatine kinase assay reagent (1 ml, Sigma Diagnostics). The rate of absorbance at 340 nm was measured at 30 second intervals for 2 minutes with a spectrophotometer (Spectronic®). Creatine Kinase activity (U/L) was subsequently calculated from changes in the rate of absorbance according to manufacturer’s instruction, and normalized to total protein concentration (mU/mg) as determined by filter paper assay described above.

**Myoblast Proliferation:** Myoblast proliferation in growth medium was quantified via hemocytometer count at 2 – 4 d of proliferation. Myoblast proliferation in differentiation medium was assessed by pulse labeling cells with BrdU and quantifying the incorporation of BrdU into nuclei as described above. Cells were pulse labeled with BrdU (100 µM) for 2 h at 1 – 3 d of differentiation. Fluorescent images were captured in six
standardized fields of view, and the number of BrdU+ nuclei was counted using image analysis software (Image Pro 7: Media Cybernetics Inc.) and expressed as a percentage of the total number of nuclei. On average, ~5,000 nuclei per well were analyzed.

*Myoblast Differentiation:* Protein expression and nuclear localization of myogenin were determined via western blotting and immunolabeling, respectively, at 1, 2, and 3 d of differentiation. The relative abundance of myogenin and α-tubulin (loading control) were quantified using the Odyssey® infrared detection system (LI-COR; Biosciences). Fluorescent images of myogenin and nuclei were captured in six standardized fields of view and the percentage of nuclei containing myogenin was quantified with Image Pro 7. The activity of creatine kinase was quantified via an activity assay as a biochemical marker for the later stage of differentiation.

*Myotube Formation and Size:* Cell cultures were fixed and immunolabeled with a mouse monoclonal anti-sarcomeric myosin heavy chain antibody throughout 6 d of differentiation. Fluorescent images of MHC and nuclei were captured in six standardized fields of view, and were analyzed using macro functions written for image analysis software (Image Pro 7; Media Cybernetics Inc.). Briefly, blue (DAPI) and green (FITC) colors were extracted from a merged image to create separate luminance images of nuclei and MHC, respectively. These images were then calibrated to the 10X objective. Using the luminance image of nuclei, the number of nuclei was counted using the cluster function. An outline of MHC labeling was created in the luminance image of MHC and then merged with the luminance image of nuclei. This merged image was used to quantify dependent measures.
Differentiated myoblasts were operationally defined as MHC+ mononuclear cells with an area greater than 100 µm$^2$, whereas myotubes were operationally defined as MHC+ cells with 2 or more nuclei and an area greater than 200 µm$^2$. Using these definitions, the software counted the number of nuclei, myotubes, and nuclei within a myotube (myonuclear number). The fusion index, which reflects myotube formation and myonuclei accretion, was calculated by expressing the number of nuclei within myotubes as a percentage of total nuclei. Indices of myotube size were also determined via digital image software analysis of the total myotube area, mean myotube diameter, maximum myotube length, and maximum myotube width in MHC labeled cell cultures through all 6 d of differentiation. On average, ~8,000 nuclei per well were analyzed.
3.2.2 Specific Aim 2 Hypothesis 1: Results

ICAM-1 Expression

Flow cytometry revealed that 95% of transfected myoblasts expressed ICAM-1 (Figure 14A), whereas immunolabeling confirmed its expression in terminally differentiated cultures (myotubes and non-fused myoblasts) (Figure 14C). Western blot analyses failed to reveal ICAM-1 expression in CT and EV myoblasts, whereas the ICAM-1 band observed in ICAM-1+ myoblasts was of the same size (110 kDa) as that observed in C2C12 myoblasts treated with 10 ng/ml TNF-α for 24 h (Figure 14B), as well as in our whole muscle findings reported in Aim 1. This expression was sustained throughout 6 d of differentiation (Figure 14D).

Taken together, these results confirmed the creation of a stable line of ICAM-1 expressing skeletal muscle cells.

Figure 14: ICAM-1 expression by cultured skeletal muscle cells. A) Flow cytometry analysis of ICAM-1 expression in cultured myoblasts. B) Protein expression of ICAM-1 (110 kDa) and GAPDH (loading control) in cultured myoblasts. CT = C2C12 (15 ug), EV = Empty Vector (15 ug/lane), CT+T= C2C12 cells treated with TNF-α (10 ng/ml for 24 h; 15 ug), and IC = ICAM-1+ (2.5 ug/lane). C) Immunofluorescence detection of ICAM-1 (red) and nuclei (blue) in ICAM-1+ cells treated with differentiation medium for 3 d. D) Western blots for ICAM-1 and α-tubulin (loading control) in ICAM-1+ cells treated with differentiation medium for up to 6 d (5 ug/lane).
ICAM-1 Expression does not Influence Myoblast Proliferation

The influence of ICAM-1 on myoblast proliferation was assessed in both growth medium and differentiation medium. Myoblast number was similar between cell lines at 2 – 4 d of treatment with growth medium. (Figure 15A). Proliferation in differentiation medium was assessed by pulse labeling cells with BrdU and quantifying the incorporation of BrdU into nuclei (Figure 15B). The percentage of BrdU+ nuclei was similar between the cell lines at 1 – 3 d of treatment with differentiation medium (Figure 15C). These data demonstrate that ICAM-1 expression by myoblasts does not influence their proliferation in either growth or differentiation medium.

**Figure 15:** ICAM-1 expression in myoblast proliferation. **A)** Myoblast number after 2 – 4 d of treatment with growth medium. **B)** Representative images of BrdU (red) incorporation into nuclei (blue) of control (CT), empty vector (EV), and ICAM-1+ cells at 2 d of differentiation (scale bar = 100 µm). **C)** Quantitative analysis of the percentage of nuclei that incorporated BrdU at 1 – 3 d of differentiation. n = 4 – 6 replicates/group for each dependent measure. Mean ± SEM.
ICAM-1 Expression does not Influence Myoblast Differentiation

The influence of ICAM-1 expression on myoblast differentiation was assessed by quantifying the protein expression and nuclear localization of myogenin. The relative abundance of myogenin protein and the percentage of nuclei expressing myogenin were similar between the cell lines through 3 d of differentiation (Figures 16A–D). The activity of creatine kinase was also quantified as a biochemical marker for the later stage of differentiation\(^{76}\). Levels of creatine kinase activity did not differ between cell lines through 3 d of differentiation (Figure 16E). These data demonstrate that the expression of ICAM-1 by myoblasts does not influence their ability to differentiate.
ICAM-1 Expression Augments Myotube Formation and Myonuclear Accretion

The extent to which ICAM-1 expression influences the initial formation of nascent myotubes and subsequent myonuclear accretion was determined through analysis of MHC and nuclei staining (Figure 17). The number of myotubes was 2 fold higher in ICAM-1+ cells compared to CT and EV cells at 2 and 3 d of differentiation (Figure 18A). Furthermore, the number of nuclei within myotubes was 2 – 3 fold higher in ICAM-1 cells compared to CT and EV cells at 2 and 3 d of differentiation (Figure 18B). Consequently, there was a 2 – 3 fold increase in fusion index in ICAM-1 cells compared to CT and EV cells at corresponding time points (Figure 18C). Taken together, our
findings indicate that ICAM-1 expression facilitates initial myoblast fusion to form multi-nucleated myotubes, as well as subsequent myonuclear accretion via the fusion of myoblasts to nascent myotubes.

**Figure 18**: ICAM-1 expression augments nascent myotube formation, subsequent myonuclear accretion, and eventual myotube fusion. Quantitative analysis of: **A**) total number of myotubes, **B**) average number of nuclei within myotubes, and **C**) fusion index (percentage of nuclei fused into myotubes). # = significantly higher for ICAM-1+ compared to CT and EV cells throughout 6 d of differentiation (main effect for cell line). * = significantly different compared to CT and EV cells at indicated day of differentiation (interaction effect). n = 6 replicates/group for each dependent measure. Mean ± SEM.

**ICAM-1 Expression Augments Fusion of Myotubes**

ICAM-1 mediated myotube formation reached a plateau at 3 d of differentiation, and declined throughout the rest of the time course, as the number of myotubes was ~2 fold lower in ICAM-1+ cells compared to CT and EV cells at 5 and 6 d of differentiation (**Figure 18A**). In contrast, the average number of nuclei within myotubes was 2 – 4 fold higher in ICAM-1+ cells compared to CT and EV cells at 5 and 6 d of differentiation (**Figure 18B**), which led to a similar increase in fusion index at the corresponding time points (**Figure 18C**). We attribute our findings of the reduced number of myotubes within a field of view, along with concomitant increases in the number and percent of nuclei fused in myotubes, to indicate that ICAM-1 expression augments nascent myotube fusion to generate mature myotubes.
ICAM-1 Expression Augments Myotube Size

The extent to which ICAM-1 expression influences myotube size was determined through software analysis of MHC and nuclei staining (Figure 17). The total area, mean diameter, and maximum length and width of myotubes were 1.3 – 3 fold higher in ICAM-1+ cells compared to CT and EV cells at 3 – 6 d of differentiation (Figures 19A–D). These findings suggest that ICAM-1 expression augments events of in vitro myogenesis, which culminate in the eventual hypertrophy of myotubes, as quantified by myotube size.

Figure 19: ICAM-1 augments myotube size. Quantitative analysis of: A) total area, B) mean diameter, C) maximum length, and D) maximum width in CT, EV, and ICAM-1+ cells at 1 – 6 d of differentiation. # = significantly higher for ICAM-1+ compared to CT and EV cells throughout 6 d of differentiation (main effect for cell line). * = significantly different compared to CT and EV cells at indicated day of differentiation (interaction effect). n = 6 replicates/group for each dependent measure. Mean ± SEM.
3.2.3 Specific Aim 2 Hypothesis 1: Interpretations

Findings from our cell culture model firmly establish the fundamental role of ICAM-1 expression by skeletal muscle cells in augmenting critical myogenic processes. Of significance, we observed ICAM-1 expression to augment distinct events associated with the preliminary, intermediate, and terminal stages of \textit{in vitro} myogenesis. Specifically, ICAM-1 expression augmented the initial fusion of myoblasts to generate nascent myotubes during the early stages (2 – 3 d) of myogenesis. We interpret this finding as further verification of our \textit{in vivo} reports of attenuated regenerating myofiber formation and altered satellite cell/myoblast activity in ICAM-1\textsuperscript{−/−} mice after mechanical overload. As skeletal muscle tissue is comprised of parallel bundles of myofibers \textit{in vivo}\textsuperscript{72}, ICAM-1 expression could serve to augment the myogenic process by acutely enhancing myoblast fusion to generate more myotubes, which are subsequently assembled into well-aligned myofibers. Taken together, our findings support a novel paradigm whereby ICAM-1 expression facilitates muscle regeneration through the enhanced fusion of myoblasts to form regenerating myofibers.

Following the initial formation of myotubes, ICAM-1 expression subsequently facilitated ongoing addition of nuclei via a mechanism involving the fusion of myoblast to nascent myotubes. The contributions of ICAM-1 in facilitating myonuclear accretion extended to the later stages (5 – 6 d) of myogenesis, at which point the fusion of existing well-aligned myotubes develops as an integral mechanism by which myotubes continue to add nuclei. To this end, we observed that ICAM-1 expression augmented the fusion of myotubes with one another to generate mature myotubes with substantially elevated myonuclei number, leading to dramatic increases in myotube size. As these results
parallel our *in vivo* report of larger ICAM-1+ myofibers after mechanical loading\textsuperscript{34}, we propose that skeletal muscle cell expression of ICAM-1 ultimately manifests in greater myotube and myofiber size at later stages of *in vitro* and *in vivo* myogenesis, respectively.
3.3 **Specific Aim 2 Hypothesis 2:** ICAM-1 Expression by Cultured Skeletal Muscle Cells Augments Myoblast Fusion through Mechanisms Involving Homotypic Myoblast Adhesion and the Adhesive Function of its Extracellular Domain.

Findings from our cell culture model firmly demonstrated significant phenotypic alterations associated with the expression of ICAM-1 by skeletal muscle cells at distinct stages of *in vitro* myogenesis. To gain deeper insight on the immunobiology of skeletal muscle growth processes, we investigated underlying mechanisms associated with ICAM-1 mediated myogenesis.

The formation of myotubes and the ongoing addition of nuclei are primarily dependent on membrane union between the plasma membranes of elongated myoblasts with other myoblasts, or multinucleated myotubes, respectively. Myoblast fusion entails a highly regulated set of cellular events, including cell migration, recognition, and adhesion. Skeletal muscle cells can migrate long distances prior to fusion, and this process of further complicated by the potential fusion among multiple cell types in skeletal musculature. Hence, accurate recognition of fusion competent myoblasts and their effective adhesion are paramount to successful myoblast fusion.

Among its varied role as a significant protein of the inflammatory response, ICAM-1 promotes cell-cell contact. Specifically, prior studies in endothelial cells have identified the extracellular domain of ICAM-1 to function as the primary ligand for α-subunits of β2 integrins (CD11a and CD11b), and a binding partner for fibrinogen. In this capacity, the extracellular domain is reported to possess adhesive properties that promote cell-to-cell adhesion.
Preliminary findings demonstrated that skeletal muscle cell expression of ICAM-1 augmented initial events of myogenesis, as indicated by increased formation of nascent myotubes (Figures 17–18). As myoblast fusion and initial myotube formation is dependent on homotypic myoblast adhesion\textsuperscript{67,69,70}, we hypothesize that ICAM-1 expression augments the homotypic adhesion of fusion competent myoblasts, which serves as an important preceding mechanism by which ICAM-1 augments ensuing events of myoblast fusion. Furthermore, we speculate that skeletal muscle cell expression of ICAM-1 augments the initial events of myogenesis, whereby myoblasts adhere and fuse to generate nascent myotubes, through a mechanism involving its extracellular domain.

To test our hypothesis, we perform a cell suspension assay to assess the degree of myoblast-to-myoblast adhesion in all cell lines. Additionally, we utilize a neutralizing antibody specific to ICAM-1 to determine the function of the extracellular domain of ICAM-1 in mediating myoblast adhesion and subsequent fusion. Lastly, we assess ligand binding of β2 integrins, fibrinogen, and other serum components to the extracellular domain of ICAM-1 as potential mechanisms that initiate events associated with ICAM-1 mediated myogenesis.
3.3.1 Specific Aim 2 Hypothesis 2: Methods

*Myoblast Adhesion:* Cell suspension adhesion assays were performed using published procedures\(^{83,84}\). Cells were seeded at high density (18,000 cells/cm\(^2\)) in p60 tissue culture dishes in growth medium and allowed to adhere for 2 h, and subsequently treated with differentiation medium for 24 h to generate fusion competent myoblasts\(^{83}\). These myoblasts were subsequently suspended and placed in a rotary shaking water bath (37°C and 80 rpm) for 2 h. The total number of cells at the beginning of the incubation, and the number of single cells at 15, 30, 45, 60, 90, and 120 min of incubation were counted using a hemocytometer to determine the percent of aggregated cells.

At 15, 60, and 120 min of incubation, 15,000 cells were added to a cytospin funnel (Thermo Scientific) using pipette tips that had been cut to an opening of greater than 5 mm. After centrifugation (Cytospin 4; Thermo Scientific), slides were fixed in 2.5% glutaraldehyde, and mounted with Fluoromount-G containing DAPI (SouthernBiotech). Images of glutaraldehyde-induced fluorescence (FITC setting) were captured in six standardized fields of view. The number of cells within an aggregate of cells will be quantified using the cluster function of Image Pro 7.

*Antibody Neutralization of ICAM-1:* To further assess the role of the extracellular domain in mediating homotypic adhesion of myoblasts, fusion competent ICAM-1+ myoblasts were treated differentiation medium supplemented with vehicle (dH\(_2\)O), an ICAM-1 neutralizing antibody (clone YN1/1.7.4, eBioscience; 100 µg/ml) or an isotype control antibody (clone eB149/10H5, eBioscience; 100 µg/ml) for the entire duration (2 h) of the cell suspension adhesion assay described above.
In addition, to examine the adhesive function of the extracellular domain of ICAM-1 in regulating initial myotube formation following homotypic myoblast adhesion, ICAM-1+ cells were treated with differentiation medium supplemented with the respective antibodies/vehicle at 1 d of differentiation for 2 or 24 h.

**Serum-free media treatment of ICAM-1:** To explore the possibility that components of horse serum contributed to the phenotype of ICAM-1+ cells, we also cultured ICAM-1+ cells in DMEM supplemented with insulin (5 µg/ml), transferrin (5 µg/ml) and selenium (5 ng/ml) (ITS medium; Sigma-Aldrich).

**Immunofluorescence:** Cells were prepared in the same manner described above (70% methanol/30% acetone fixative), and incubated for 2 h with an antibody that recognizes anti-sarcomeric MHC (clone MF20, Developmental Studies Hybridoma Bank; 1:20), anti-CD11a (1:50; clone M17/4; eBiosciences), or anti-CD11b (1:100; clone M1/70; BD Pharmingen). Cells were then washed and incubated with a fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and mounted with DAPI (Southern Biotech). Image capture was performed using exact protocols described above.
3.3.2 Specific Aim 2 Hypothesis 2: Results

ICAM-1 Expression Augments Homotypic Adhesion of Myoblasts

The influence of ICAM-1 expression on homotypic myoblast adhesion was assessed through 2 h cell suspension assays. The average number of cells within aggregates and the percent aggregation were ~2 fold higher for ICAM-1+ cells compared to CT and EV cells (Figures 20A–C). These data demonstrate that ICAM-1 expression augments homotypic adhesion of fusion competent myoblasts.

Figure 20: ICAM-1 expression facilitates myoblast-myoblast adhesion. Homotypic adhesion of myoblasts was quantified in a cell suspension assay using myoblasts collected after 1 d of treatment with differentiation medium. A) Representative images of cytospin prepared slides of control (CT), empty vector (EV), and ICAM-1+ cells at 120 min of incubation (scale bar = 100 µm). Glutaraldehyde-induced fluorescence (green) was used to count the number of cells in individual aggregates. B) Average number of cells within aggregates of CT, EV, and ICAM-1+ cells at 15, 60, and 120 min of incubation. C) Percent aggregation of CT, EV, and ICAM-1+ cells at selected intervals throughout 120 min of incubation. # = higher for ICAM-1+ compared to CT and EV cells (main effect for cell line). n = 4 or more replicates/group for each dependent measure. Mean ± SEM.
Extracellular Domain of ICAM-1 Augments Myoblast Adhesion, Myotube Formation, and Myonuclear Accretion

The involvement of the extracellular domain of ICAM-1 expression in mediating early events of myogenesis was assessed through antibody treatment of ICAM-1+ cells. Neutralization of ICAM-1+ fusion competent myoblasts at 1 d of differentiation with an ICAM-1 antibody (ICAM-1 Ab) for 2 h reduced the number of cells within aggregates by ~1.5 – 3 fold compared to Vehicle and Isotype Control (Isotype Ab) treatments (Figure 21A), but did not influence percent aggregation (Figure 21B).

Furthermore, treatment of ICAM-1+ cells at 1 d of differentiation with an ICAM-1 neutralizing antibody for 2 and 24 h reduced myotube formation, myonuclear number, and fusion index by 1.1 – 3.6 fold (Figures 22A–D). Taken together, we interpret our
findings to indicate that the extracellular domain of ICAM-1 augments the preceding homotypic adhesion of fusion competent myoblasts, which serves as an important mechanism for their ensuing fusion to generate nascent myotubes. Furthermore, the extracellular domain facilitates the ongoing addition of nuclei through myoblast adhesion and subsequent fusion with nascent myotubes.

**Figure 22:** ICAM-1 expression augments nascent myotube formation and myonuclear accretion through mechanisms involving its extracellular domain. ICAM-1+ cells were treated at 1 d of differentiation with vehicle (dH₂O), isotype control antibody (Isotype-Ab; 100 µg/ml) or ICAM-1 antibody (ICAM-1 Ab; 100 µg/ml) for 2 or 24 h. **A)** Representative images of myosin heavy chain (green) and nuclei (blue) in ICAM-1+ cells after 2 and 24 h treatment with vehicle, Isotype Ab, or ICAM-1 Ab (scale bar = 100 µm). Quantitative analysis of myoblast fusion indices: **B)** total number of myotubes, **C)** average number of nuclei within myotubes, and **D)** fusion index. # = lower for ICAM-1 Ab compared to Isotype-Ab and vehicle (main effect for treatment), * = lower for ICAM-1 Ab compared to Isotype-Ab and vehicle at indicated duration of treatment (interaction effect). n = 3 replicates/group for each dependent measure. Mean ± SEM.
**Skeletal Muscle Cells do not Express CD11a or CD11b**

The extent by which the interaction of ICAM-1 to known primary ligands (i.e. β2 integrins) initiate the phenotypic alterations observed in ICAM-1+ cells was assessed by software analysis of CD11a/CD11b and nuclei staining (Figure 23). Although CD11a and CD11b was expressed in peritoneal leukocytes (positive control; Figure 23A), we did not detect their expression in skeletal muscle cells (control, EV and ICAM-1+) throughout 6 d of differentiation (Figures 23B–D). Western blotting and immunoprecipitation for CD11a and CD11b were not performed on lysates of skeletal muscle cells, as both techniques failed to detect a band at their anticipated molecular weight (160-180 kDa) in peritoneal leukocytes using several modifications of standard protocols and established antibodies. Nevertheless, our immunofluorescence findings are in agreement with prior work that demonstrated that cultured human myoblasts and myotubes do not express β2 integrins.\textsuperscript{8,28,31,85}
Influence of Serum on the Phenotype of ICAM-1+ Cells

The extent by which the interaction of ICAM-1 with serum components, specifically fibrinogen (a known ligand for ICAM-1),71,82 initiates the phenotypic alterations observed in ICAM-1+ cells was assessed by software analysis of MHC and nuclei staining of ICAM-1+ cells cultured in serum free ITS medium. We reported that myoblast fusion indices, as well as several measures of myotube size, of ICAM-1+ cells under serum free ITS medium conditions were similar in values to those observed during treatment of differentiation medium with serum (Figures 24A–F). These findings indicate that phenotypic alterations resulting from the expression of ICAM-1 were not dependent on the presence of serum in differentiation medium.

Figure 24: Influence of serum on myotube indices. ICAM-1+ cells were treated with DMEM containing 2% horse serum (serum medium) or insulin, transferrin, and selenium (serum-free medium) for up to 6 d. Quantitative analysis of: A) myotube number, B) number of nuclei within myotubes, C) fusion index, D) myotube diameter, E) width, and F) area. # = significantly higher for serum-free medium compared to serum medium throughout 6 d of differentiation (main effect for medium). n = 2 – 3 replicates/group for each dependent measure. Mean ± SEM.
3.3.3 **Specific Aims 2 Hypothesis 2: Interpretation**

Our collective results demonstrate that mechanisms through which ICAM-1 expression augmented initial events of myogenesis manifested with the homotypic adhesion and fusion of fusion competent myoblasts to generate nascent myotubes, as well as ongoing myoblast adhesion and fusion with existing myotubes to add nuclei. These findings are consistent with prior reports on the contributions of other members of the immunoglobulin superfamily of adhesion molecules, such as neural cell adhesion molecule (NCAM; CD56) and vascular cell adhesion molecule-1 (VCAM-1; CD106)\(^{69,70}\), during early events of myogenesis.

Specifically, antibody neutralization of NCAM and VCAM-1 \textit{in vitro} has been reported to reduce myoblast-myoblast adhesion and/or the percentage of nuclei within myotubes (fusion index)\(^{85,86}\). In our present study, antibody neutralization of ICAM-1 failed to reduce the percent aggregation of fusion competent myoblasts. This finding is most likely attributable to an insufficient amount of time for the ICAM-1 antibody to block the extracellular domain of ICAM-1 prior to the formation of aggregates, as 45% of the vehicle treated cells formed aggregates within 15 min of incubation (Figure 21B). Despite this, antibody neutralization elicited a decrease in the number of fusion competent myoblasts within aggregates, total number of myotubes, as well as number and percent of nuclei within myotubes. Hence, we interpret the findings from our antibody neutralization study to indicate that ICAM-1 augmented homotypic adhesion of myoblasts, nascent myotube formation, and myonuclear accretion through mechanisms involving the adhesive function of its extracellular domain.
Underlying mechanisms by which the extracellular domain of ICAM-1 initiates early events of myogenesis are not well understood. In comparison, the expression of VCAM-1 by myoblasts has been suggested to facilitate their fusion via ligand binding to VLA-4 (α4 integrin; CD49d) expressed by myoblasts and myotubes\(^{86}\). Although ligand binding of the α-subunits of β2 integrins (CD11a or CD11b) to ICAM-1 promotes cell-to-cell adhesion in vascular endothelial cells\(^{11,12}\), we and others have found that cultured skeletal muscle cells do not express CD11a or CD11b\(^{8,28,31,84}\). A potential ligand for ICAM-1 is fibrinogen, which has been shown to facilitate leukocyte adhesion to the endothelium and transendothelial monocyte migration by acting as a bridging molecule between the two cell types\(^{67,68}\). In our present study, we observed that fibrinogen, as well as other serum components, did not influence events of myogenesis associated with skeletal muscle cell expression of ICAM-1. Hence, our findings indicate that enhanced myoblast adhesion and fusion are not initiated by ICAM-1 binding to established ligands, or other unidentified ligands expressed in serum. Interestingly, myoblast and myotubes appear to express hyaluronan\(^{87}\), an acidic polysaccharide of the extracellular matrix that has been reported to bind to ICAM-1\(^{88}\).

In contrast, NCAM is thought to facilitate cell-to-cell adhesion through homophilic interactions on opposing membranes\(^{89}\), and to regulate myoblast fusion through glycosylation of the extracellular domain\(^{90}\). As ICAM-1 shares sequence homology with NCAM\(^{91}\), has been documented to form dimers or multimers on plasma membranes\(^{92,93}\), and contains multiple glycosylation sites\(^{94}\), homophilic binding and glycosylation could serve as mechanisms for ICAM-1 mediated myoblast adhesion and subsequent fusion.
Taken together, ICAM-1 expression could enhance adhesion and subsequent fusion of myoblasts through multiple mechanisms involving its extracellular domain. Additional work is required to gain greater insight on mechanisms for initiating and controlling ICAM-1 signaling in skeletal muscle cells. Due to the inherent limitations of antibody neutralization, future studies undertaking such objectives should incorporate more sophisticated tools, such as genetically engineered ICAM-1 mutant constructs.
3.4 **Specific Aim 2 Hypothesis 3:** ICAM-1 Expression by Cultured Skeletal Muscle Cells Augments Myoblast Fusion through Mechanisms Involving the Signal Transducing Function of its Cytoplasmic Domain.

Prior studies which characterized the function of ICAM-1 have identified its extracellular domain to possess adhesive properties, whereas its intracellular/cytoplasmic domain is capable of generating multiple intracellular signaling pathways\(^{25,73}\). We have successfully demonstrated the involvement of the extracellular domain in augmenting myoblast adhesion and fusion. As prior studies in endothelial cells have demonstrated that ligation-induced activation of the extracellular domain is capable of transducing an intracellular signal, we investigate the involvement of the cytoplasmic domain in mediating the early events of myogenesis following myoblast adhesion.

In addition to its role as an attachment site for leukocyte adhesion, ICAM-1 also functions as a potent signal transducer primarily through its cytoplasmic domain. While the cytoplasmic domain itself does not possess any intrinsic catalytic activity, prior studies have shown that the association of the ICAM-1 cytoplasmic domain with downstream cytoskeletal proteins such as α-actinin\(^{95}\), or adaptor proteins such as mosein and erzin\(^{63,96,97}\), has been demonstrated to transduce multiple intracellular signaling pathways. Significantly, the ligation-induced activation of ICAM-1 has been demonstrated to mediate downstream signaling within the MAPK\(^{98}\) and Rho GTPase\(^{99,100}\) pathways, both which are critical signaling pathways that regulate early events of myogenesis.
A key objective of our cell culture model is to establish the involvement of relevant intracellular signaling pathways in ICAM-1 mediated myogenesis. In particular, p38 MAPK signaling increases expression and transcriptional activity of myogenin, and is therefore a major regulator of skeletal muscle cell differentiation\textsuperscript{101,102}. In accordance to our earlier hypothesis, we do not expect to see any differences in p38 MAPK expression, as ICAM-1 expression is not believed to influence differentiation in skeletal muscle cells. Rather, we speculate that ICAM-1 expression serves to augment subsequent events of cellular fusion occur following the successful differentiation of myoblasts.

To test our hypothesis, we utilize a cell penetrating peptide that inhibits the function of the cytoplasmic domain of ICAM-1 to assess its function in augmenting nascent myotube formation and subsequent myonuclear accretion. Additionally, we assess the involvement of p38 MAPK signaling in regulating early events of myogenesis across all cell lines.
3.4.1 Specific Aim 2 Hypothesis 3: Methods

*Cell Penetrating Peptides:* To manipulate ICAM-1 signaling in skeletal muscle, we targeted the ICAM-1 cytoplasmic domain with a novel cell permeable peptide. Briefly, a “blocking peptide” was used to inhibit binding of the cytoplasmic domain of ICAM-1 to downstream adaptor and signaling proteins (ICAM-1-P; Ohio Peptides)\(^{103,104,105,106}\). This peptide contained an antennapedia cell-permeative sequence/penetratin (RQIKIWFQNRRMKWKK), and a truncated sequence – 13 out of 27 amino acids of the cytoplasmic tail of murine ICAM-1 (QRKIRIYKLQKAQ, **Figure 25**). The control peptide contained the amino acid sequence for antennapedia and rat rhodopsin (CKPMSNFRFGENH), an irrelevant peptide (CT-P; Ohio Peptides).

![Figure 25: Amino acid sequences for murine ICAM-1, and ICAM-1 blocking peptide.](image)

To determine the signal transducing function of the ICAM-1 cytoplasmic domain in augmenting the initial stages of myogenesis (myotube formation and myonuclear accretion), ICAM-1+ cells were treated with 100 µg/ml of the cell penetrating peptide for ICAM-1 or its control peptide, or vehicle (dH\(_2\)O) at 1 d of differentiation for 2 and 24h.
**Immunofluorescence:** Cells were prepared in the same manner described above (70% methanol/30% acetone fixative), and incubated for 2 h with an antibody that recognizes anti-sarcomeric MHC (clone MF20, Developmental Studies Hybridoma Bank; 1:20). Cells were then washed with PBS and incubated with a fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and mounted with DAPI (Southern Biotech). Image capture was performed using exact protocols described above.

**Western Blotting:** p38 MAPK expression was quantified as a readout of the extent to which ICAM-1 expression influences cell signaling for myoblast differentiation. Cultured skeletal muscle cells were sonicated, boiled and separated (25 µg/lane), and transferred to PDVF-FL membranes (Millipore) using the same protocols described above. Membranes were subsequently blocked in 5% non-fat dry milk, and incubated overnight at 4ºC with an antibody that recognizes total-p38α (Santa Cruz Cat, # SC-535; 1:2500), or phospho-p38 (Thr180/Tyr182) (Cell Signaling, # 9211; 1:1000). Detection of antibody binding was achieved by using an Alexa Fluor® 680 anti-rabbit secondary antibody (Invitrogen; 1:5000 – 1:7500), and the Odyssey® infrared detection system (LI-COR; Biosciences). Data is presented in raw scanning units.
3.4.2 Specific Aim 2 Hypothesis 3: Results

**Cytoplasmic Domain of ICAM-1 Augments Nascent Myotube Formation and Subsequent Myonuclear Accretion**

The involvement of the cytoplasmic domain of ICAM-1 expression in mediating early events of myogenesis was assessed via peptide treatment of ICAM-1+ cells. Peptide inhibition of ICAM-1+ cells at 1 d of differentiation for 2 and 24 h reduced myotube formation, number of myonuclei, and fusion index by 1.2 – 4.8 fold compared to CT-P and vehicle treated cells (Figures 26A–D). We interpret our findings to indicate that the cytoplasmic domain of ICAM-1 augments early events of myogenesis fusion through enhanced myoblast fusion, leading to increased nascent myotube formation and greater myonuclear accretion.

**Figure 26:** ICAM-1 expression augments nascent myotube formation and subsequent myonuclear accretion through mechanisms involving its cytoplasmic domain. ICAM-1+ cells were treated with a cell penetrating peptide that inhibits its cytoplasmic domain (ICAM-1-P; 100 µg/ml), an irrelevant control peptide, (CT-P; 100 µg/ml), or vehicle (dH2O) at 1 d of differentiation for 2 or 24 h. **A**) Representative images of myosin heavy chain (green) and nuclei (blue) in ICAM-1+ cells after 2 and 24 h treatment with vehicle, CT-P, or ICAM-1-P (scale bar = 100 µm). Quantitative analysis of myoblast fusion indices: **B**) total number of myotubes, **C**) average number of nuclei within myotubes, and **D**) fusion index. # = lower for ICAM-1-P compared to CT-P and vehicle (main effect for treatment), * = lower for ICAM-1-P compared to CT-P and vehicle at indicated duration of treatment (interaction effect). n = 4 or more replicates/ group for each dependent measure. Mean ± SEM.
ICAM-1 expression on skeletal muscle cells does not influence p38 MAPK signaling

To assess the involvement of the ICAM-1 cytoplasmic domain on the ability of myoblasts to differentiate, p38 MAPK expression was quantified as a readout of the extent to which ICAM-1 expression influences cell signaling for myoblast differentiation. Phosphorylated and total levels of p38 MAPK were similar between the cell lines throughout 3 d of differentiation (Figures 27A–B). In addition, treatment of ICAM-1+ cells with the ICAM-1-P at 1 d of differentiation for 2 and 24 h failed to influence phosphorylated levels of p38 MAPK (Figures 27C and E). Lastly, as p38 MAPK
facilitates gene transcription of myogenin, we assessed the role of the cytoplasmic domain in regulating myogenin expression. Similarly, we observed that treatment of ICAM-1+ cells with the ICAM-1-P at 1 d of differentiation for 2 and 24 h failed to influence myogenin expression (Figures 27D and F). Taken together, these data firmly demonstrate that the cytoplasmic domain of ICAM-1 does not influence p38 MAPK signaling or myogenin expression in skeletal muscle cells, which further supports our earlier findings that ICAM-1 expression does not alter myoblast differentiation.
3.4.3 Specific Aims 2 Hypothesis 3: Interpretation

Our collective results indicate that the signal transducing cytoplasmic domain of ICAM-1 serves a critical role in augmenting initial events of myogenesis whereby myoblasts are fusing together to generate nascent myotubes, as well as fusing with existing myotubes to facilitate myonuclear accretion. In conjunction with our work on myoblast adhesion, our findings establish a potential novel mechanism by which ICAM-1 expression augments early events of myogenesis, whereby the extracellular domain initially facilitates greater adhesion of fusion competent myoblasts, and the cytoplasmic domain subsequently augments myoblast fusion to myoblasts and myotubes through increased activation of pertinent myogenic signaling pathways.

We have begun exploring relevant signaling pathways pertaining to ICAM-1 mediated myoblast fusion. As prior studies have reported that the cytoplasmic domain of ICAM-1 is capable of activating downstream signaling with the MAPK pathway, we assessed p38 MAPK signaling to better distinguish the function of ICAM-1 expression on skeletal muscle differentiation from events of myoblast fusion. While ICAM-1 ligation with fibrinogen has been documented to prevent apoptosis in endothelial cells through MAPK signaling\(^21\), our present study demonstrated p38 MAPK levels were not elevated with ICAM-1 expression in skeletal muscle cells. Furthermore, peptide inhibition did not inhibit p38 MAPK signaling, or myogenin expression. These findings correspond with our earlier reports that ICAM-1 expression does not influence myoblast differentiation. Importantly, our findings clearly establish that ICAM-1 mediated myogenesis, in particular the early events of myoblast adhesion and fusion, occurred through signaling mechanisms independent of myoblast differentiation.
Mammalian myoblast fusion occurs in two phases, which commences with homotypic fusion of myoblasts to generate nascent myotubes, and subsequently involves ongoing fusion of myoblasts with nascent myotubes to develop multinucleated myotubes. Despite the documented relevance of cell-to-cell contact and adhesion as requisite preceding events that facilitate myoblast fusion, these processes are quite far removed from the actual fusion process. Rather, findings from distinct model systems including Drosophila embryogenesis, mouse embryogenesis and regeneration, as well as rodent tissue culture have identified pertinent genes, signaling molecules, intracellular pathways, and membrane events that are intimately involved in the mechanics of myoblast fusion.

The roles of the actin cytoskeleton and their polymerizing agents have emerged as leading mechanisms that drive myoblast fusion. Remodeling of the actin cytoskeleton via the assembly of stress fibers and focal adhesion kinases, and its associated membrane projections, such as lamellipodia and filopodia, have been demonstrated to play a fundamental role in facilitating myoblast fusion. Specifically, lamellipodia and filopodia extended by skeletal muscle cell in differentiation have been postulated to contact neighboring myoblasts and bring them in close contact for subsequent fusion.

Prior studies have shown that members of the Rho GTPase subfamily mediate cytoskeletal rearrangement by augmenting the assembly of focal adhesions and actin stress fibers upon activation through cell-to-cell adhesion in different cell types. In particular, activation of the Rac1 GTPase functions as a “molecular switch” that regulates remodeling of the actin cytoskeleton, as well as lamellipodia and filopodia dynamics. Consequently, Rac1 plays an integral role in facilitating the initial
stages of myogenesis by promoting myoblast fusion, and thereby enhancing nascent myotube formation\textsuperscript{66,119,120,121,122}.

During myoblast fusion in \textit{Drosophila}, Rac1 is activated by the guanine nucleotide exchange factor (GEF), Myoblast city (Mbc)\textsuperscript{123,124}, and is highly enriched in fusion competent myoblasts\textsuperscript{125}. In this capacity, Rac1 serves to augment the local activation of SCAR prior to myoblast fusion, as Rac1 mediated signaling of the WAVE/SCAR complex promotes actin nucleation through the Arp2/3 complex\textsuperscript{126,127}. Besides regulating actin dynamics pertinent to myoblast fusion in flies, Rac1 appears to have similar conserved functions in mammalian myoblast fusion. In particular, the activation of Rac1 is dependent on M-Cadherin adhesion via the Rho GEF trio, forming a multiprotein complex containing M-cadherin, Rac1, and Trio at the onset of mouse myoblast fusion\textsuperscript{119}. This complex is mediated by ADP ribosylation factor 6 (Arf6), which regulates fusion through phospholipase D and phosphatidylinositol 4,5-biphosphate signaling pathways\textsuperscript{128}. These studies elucidate the fundamental role of Rac1 in mediating actin dynamics, as well as its dynamic interplay with key signaling mechanisms that are associated with the mechanics of myoblast fusion.

Prior investigators have reported that the cytoplasmic domain of ICAM-1 activates Rho GTPases in other cell types\textsuperscript{99,100}. In this capacity, ICAM-1 signaling has been demonstrated to initiate actin polymerization, lamellipodia and filopodia dynamics, and the formation of cup-like docking structures for leukocytes in endothelial cells through its cytoplasmic domain. As Rac1 is activated by ICAM-1 in endothelial cells, we propose that enhanced myoblast fusion associated with skeletal muscle cell expression of ICAM-1 is mediated by elevated Rac1 activity, through ICAM-1’s cytoplasmic domain.
Future work should attempt to establish Rac1 as a primary signaling mechanism by which ICAM-1 augments myotube formation and myonuclear accretion. Furthermore, as ICAM-1 signaling has been reported to activate other members of the Rho GTPase family, such as Cdc42 and RhoA\textsuperscript{97,129,130}, future work should attempt to elucidate their potential roles in events of ICAM-1 mediated myogenesis. Lastly, future studies should emphasize on the function of the ICAM-1 cytoplasmic domain in remodeling the actin cytoskeleton of skeletal muscle cells, by investigating the role of ICAM-1 expression in facilitating events that lead to myoblast fusion, such as the migration of myoblasts.
3.5 **Specific Aim 2 Hypothesis 4:** ICAM-1 Expression by Cultured Skeletal Muscle Cells Augments Myotube Size through Mechanisms Involving Fusion of Well-Aligned Myotubes, and Enhanced Rates of Protein Synthesis via Increased Akt/p70s6k Signaling.

Findings from our cell culture model revealed that skeletal muscle cell expression of ICAM-1 augmented the initial stages of myogenesis, as demonstrated by increased homotypic adhesion of myoblasts, nascent myotube formation, and myonuclear accretion to nascent myotubes, through mechanisms involving the adhesive and signal transducing functions of the extracellular and cytoplasmic domains of ICAM-1, respectively. Additionally, our findings revealed that ICAM-1 expression augmented events of myogenesis subsequent to the initial formation of nascent myotubes, as indicated by the ongoing fusion of myoblasts to multinucleated myotubes, as well as the fusion of myotubes with one another at later stages of myogenesis (5 – 6 d of differentiation), leading to increased myonuclei number within an individual myotube (Figures 17–18). These myogenic events are critical to the maturation of myotubes, as nascent myotubes undergo hypertrophy by adding nuclei through the fusion of myoblasts with multi-nucleated myotubes, and the fusion of multi-nucleated myotubes to one another. Consequently, increased myonuclei number in myotubes associated with skeletal muscle cell expression of ICAM-1 was accompanied by greater myotube size (Figure 19), which mirrored our in vivo findings of larger ICAM-1+ myofibers after muscle overload.

As ICAM-1 mediated myotube fusion corresponded to robust increases in myotube size measurements at 5 and especially 6 d of differentiation, we speculate that enhanced fusion of myotubes is a mechanism by which ICAM-1 expression augments
myotube hypertrophy. Events of myotube fusion, as well as ongoing myoblast-myotube fusion, are dependent on cellular alignment, which occurs through the parallel apposition of the membranes of skeletal muscle cells with one another, and serves to facilitate cellular communication/crosstalk. Visual assessment of morphological characteristics revealed that ICAM-1+ myotubes were more closely aligned through 6 d of differentiation compared to C2C12 and empty vector cells (Figure 17). Hence, the parallel arrangement of myotubes with one another may serve as a mechanism by which ICAM-1 expression augments myotube fusion to facilitate substantial increases in myonuclear accretion and promote their subsequent hypertrophy.

Observations from our cell culture model reported corresponding increases in myonuclei number and various measurements of myotube size. An increase in myonuclei number within a myotube has been proposed to serve as a potential mechanism for its hypertrophy by increasing its capacity to synthesize proteins. To this end, findings from our mouse knockout model demonstrated that ICAM-1 expression augmented whole muscle protein synthesis after mechanical overload (Figure 12). Of significance, the cytoplasmic domain of ICAM-1 has been documented to increase protein synthesis of cytokines in endothelial cells. Hence, we hypothesize that ICAM-1 expression augments myotube hypertrophy through enhanced levels of protein synthesis mediated by its cytoplasmic domain.

In addition to MAPK and RhoGTPases, the cytoplasmic domain of ICAM-1 has also been reported to elicit the Akt signaling pathway. The Akt/mTOR pathway is upregulated during hypertrophy, and is a crucial regulator of skeletal muscle hypertrophy in vivo, as well as protein synthesis in skeletal muscle cells.
Increased mTOR activity results in p70s6k phosphorylation, which is thought to promote increases in skeletal muscle mass with resistance exercise\textsuperscript{134}. Furthermore, increased mTOR activity phosphorylates 4EBP1, leading to its disassociation from eIF4E, and subsequent initiation of mRNA translation\textsuperscript{134}. Hence, we speculate that the cytoplasmic domain of ICAM-1 augments myotube protein synthesis through increased signaling along the Akt/mTOR pathway.

To corroborate our earlier findings of enhanced myotube fusion and size in ICAM-1+ cells, we investigate the parallel alignment of myotubes as a mechanism by which further myonuclear accretion and myotube fusion occurs, as well as assess rates of protein synthesis, as an underlying mechanism for increased myotube hypertrophy. The involvement of relevant signaling pathways is established by determining the total and phosphorylated levels of Akt and p70s6k. The ICAM-1 peptide is utilized to further assess the role of the cytoplasmic domain of ICAM-1 in mediating events of myogenesis subsequent to the initial formation of myotubes leading to eventual myotube hypertrophy.
3.5.1 Specific Aim 2 Hypothesis 4 Methods

Cell Penetrating Peptides: To determine the signal transducing function of the ICAM-1 cytoplasmic domain in augmenting events of myogenesis leading to myotube hypertrophy, ICAM-1+ cells were treated with 50 µg/ml of the cell penetrating peptide for ICAM-1 or its control peptide, or vehicle (dH20) at 5 d of differentiation for 2 and 24 h. The peptide concentrations were reduced from 100 µg/ml at 1 d of differentiation to 50 µg/ml, as preliminary studies revealed that 100 µg/ml of ICAM-1-P resulted in substantial cellular detachment at 5 d of differentiation.

Myotube Alignment: To reveal underlying mechanisms leading to ICAM-1 mediated fusion of myotubes, the parallel alignment of myotubes throughout 6 d of differentiation were quantified through two dimensional fast Fourier transform (FFT) analysis, using a macro function created by Media Cybernetics that adopted published procedures\textsuperscript{135,136}. Briefly, the software decomposed a luminance image of MHC expression into its sine and cosine components and then converted the spatial information into a mathematically defined frequency domain. The resulting plot showed low frequency pixels at the origin, or center; whereas, high frequency pixels are found away from the origin. Pixel intensities were summed for each angle (0–360°), normalized to the lowest intensity value, and plotted against angle to produce a FFT alignment plot\textsuperscript{135}. The peak sum intensity and the overall shape of the FFT alignment plot reflect the degree of alignment\textsuperscript{135}. The shape of the alignment plot was quantified by calculating the area under the curve using the trapezoid rule method.
Protein Synthesis: Rates of protein synthesis in cultured cells were determined throughout 6 d of differentiation via the SUnSET method described above\textsuperscript{60,61}, as an outcome measure of myotube hypertrophy. Cells were treated with 1 uM puromycin for 30 min at 37°C in a 5% CO\textsubscript{2} humidified environment, and subsequently collected and analyzed for puromycin incorporation into nascent proteins through western blot analysis.

Akt/p70s6k: Signaling proteins involved in translational control of protein synthesis were quantified by western blot analysis of cell lysates at 3 – 6 d of differentiation for total and phosphorylated levels of relevant kinases along the Akt/mTOR pathway: Akt (Ser473) and p70s6k (Thr389).

Immunofluorescence: Cells were prepared in the same manner described above (70% methanol/30% acetone fixative), and incubated for 2 h with an antibody that recognizes anti-sarcomeric MHC (clone MF20, Developmental Studies Hybridoma Bank; 1:20). Cells were then washed with PBS and incubated with a fluorochrome-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) and mounted with DAPI (Southern Biotech). Image capture was performed using exact protocols described above.

Western Blotting: Cultured skeletal muscle cells were sonicated, boiled and separated on 10% SDS-PAGE gels (25 µg of protein per lane), and transferred for 1 h to PVDF-FL membranes (Millipore) in Towbin’s transfer buffer containing 10% methanol using a semi-dry (20 volts) or a wet transfer (200 mA) protocol described above. Membranes were subsequently blocked in 5% non-fat dry milk, and incubated overnight at 4°C with an antibody that recognizes mouse anti-puromycin (clone 12D10; 1:5,000; kindly provided by Dr. Philippe Pierre, Centre d'Immunologie de Marseille-Luminy), total Akt
(Cell Signaling Cat # 9272; 1:1000), phospho-Akt (Ser473) (Cell Signaling Cat # 9271; 1:500), total p70s6k (Cell Signaling Cat# 2708; 1:1000), or phospho-p70s6k (Thr389) (Santa Cruz Cat # SC-11759; 1:500). Detection of antibody binding was achieved by using an Alexa Fluor® 680 anti-rabbit (Akt/p70s6k) or isotype specific IgG2A anti-mouse (puromycin) secondary antibody (Invitrogen; 1:5000 – 1:7500), and the Odyssey® infrared detection system (LI-COR; Biosciences). Data is presented in raw scanning units. Coomassie blue stained gels served as loading controls for determination of protein synthesis in puromycin treated cells.
3.5.2 Specific Aim 2 Hypothesis 4: Results

**ICAM-1 Expression Augments Parallel Alignment of Myotubes**

The influence of ICAM-1 expression on myotube alignment was quantified using FFT analysis of MHC and nuclei staining (Figures 28A–C). The peak sum intensities and areas under the curve were 1.3 – 2 fold higher in ICAM-1+ cells compared to CT and EV cells at 2 – 6 d of differentiation (Figures 28D–E). This resulted in a high degree of alignment in ICAM-1+ myotubes, as opposed to CT and EV myotubes, which showed more than one axis of alignment as indicated by low peak sum intensities and a broad

![Figure 28: ICAM-1 expression augments parallel alignment of myotubes. A) Representative fluorescent images of myosin heavy chain (green) and nuclei (blue) labeling in control (CT), empty vector (EV), and ICAM-1+ (IC+) cells at 3 d of treatment with differentiation medium (scale bar = 100 µm). B) Representative Fast Fourier Transform (FFT) images derived from the analysis of corresponding images of myosin heavy chain and nuclei staining. C) Corresponding FFT alignment plots showing normalized sum intensity on the y-axis and degrees (0 – 360) on the x-axis. Quantitative analysis of FFT alignment plots: D) peak normalized sum intensity, E) area under the curve in CT, EV, and ICAM-1+ cells at 1 – 6 d of differentiation. # = significantly higher for ICAM-1+ compared to CT and EV cells throughout 6 d of differentiation (main effect for cell line), * = significantly higher for ICAM-1+ compared to CT and EV cells at indicated day of differentiation (interaction effect). n = 6 replicates/group for each dependent measure. Mean ± SEM.](image)
pattern in FFT alignment plots. Hence, our data demonstrate that ICAM-1 expression augments the parallel alignment of myotubes, which could serve as a mechanism for ICAM-1 mediated fusion of myotubes at later stages of myogenesis.

**Cytoplasmic Domain of ICAM-1 Augments Parallel Alignment of Myotubes, Fusion of Multinucleated Myotubes and Overall Myotube Size**

The involvement of the cytoplasmic domain of ICAM-1 expression in mediating later stages of myogenesis was assessed via peptide treatment of ICAM-1+ cells. Treatment of ICAM-1+ cells with ICAM-1-P at 5 d of differentiation for 2 or 24 h reduced indices of myotube alignment by 2.6 – 3.3 fold compared to CT-P and vehicle treated cells (Figures 29A–B). Accordingly, peptide inhibition corresponded with substantial decreases in the number of nuclei within a myotube and fusion index, and a concomitant 1.6 – 2.7 fold increase in the number of myotubes (Figures 30A–D). These findings demonstrated that diminished myotube alignment, associated with peptide inhibition of ICAM-1, compromised the fusion of myotubes in ICAM-1+ cells, indicating a crucial

![Figure 29: ICAM-1 expression augments parallel alignment of myotubes through mechanisms involving its cytoplasmic domain. ICAM-1+ cells were treated with the ICAM-1 peptide (ICAM-1-P; 50 µg/ml), control peptide (CT-P; 50 µg/ml), or vehicle (dH2O) at 5 d of differentiation for 2 or 24 h. Quantitative analysis of FFT alignment plots: A) peak normalized sum intensity, B) area under the curve in ICAM-1+ cells after treatment with vehicle, CT-P, or ICAM-1-P. # = lower for ICAM-1-P compared to CT-P and vehicle (main effect for treatment), * = lower for ICAM-1-P compared to CT-P and vehicle at indicated duration of treatment (interaction effect). n = 4 replicates/group for each dependent measure. Mean ± SEM.]
Figure 30: ICAM-1 expression augments fusion of myotubes through mechanisms involving its cytoplasmic domain. ICAM-1+ cells were treated with ICAM-1 peptide (ICAM-1-P; 50 µg/ml), control peptide (CT-P; 50 µg/ml), or vehicle (dH$_2$O) at 5 d of differentiation for 2 or 24 h. A) Representative fluorescent images of myosin heavy chain (green) and nuclei (blue) labeling in ICAM-1+ cells (scale bar 100 µm). Quantitative analysis of myotube fusion indices: B) total number of myotubes, C) average number of nuclei within myotubes, and D) fusion index in ICAM-1+ cells after treatment with vehicle, CT-P, or ICAM-1-P. # = lower for ICAM-1-P compared to vehicle and CT-P treated cells (main effect for treatment), * = lower for ICAM-1-P compared to vehicle and CT-P treated cells at indicated duration of treatment (interaction effect). n = 4 replicates/groups for each dependent measure. Mean ± SEM.

role of the cytoplasmic domain of ICAM-1 in augmenting myotube fusion through the enhanced parallel alignment of myotubes. Lastly, the compromised fusion of myotubes and associated reductions in myonuclei number in ICAM-1-P treated cells resulted in substantial decreases in measurements of myotube size (Figures 31A–B).
Figure 31: ICAM-1 expression augments myotube size through mechanisms involving its cytoplasmic domain. ICAM-1+ cells were treated with ICAM-1 peptide (ICAM-1-P; 50 µg/ml), control peptide (CT-P; 50 µg/ml), or vehicle (dH2O) at 5 d of differentiation for 2 or 24 h. Quantitative analysis of myotube size indices: A) total area, and B) mean diameter in ICAM-1+ cells after treatment with vehicle, CT-P, or ICAM-1-P. # = lower for ICAM-1-P compared to CT-P and vehicle (main effect for treatment). * = lower for ICAM-1-P compared to CT-P and vehicle at indicated duration of treatment (interaction effect). n = 4 replicates/group for each dependent measure. Mean ± SEM.

Therefore, our results illustrate the critical function of the cytoplasmic domain of ICAM-1 in augmenting later events of myogenesis through increased myotube fusion, which results in the formation of large myotubes with a robust abundance of nuclei.

ICAM-1 Expression Augments Protein Synthesis

The extent to which ICAM-1 expression influences protein synthesis was quantified using a nonradioactive western blotting technique60,61. Protein synthesis in myotubes was 3 fold higher in ICAM-1+ cells compared to CT and EV cells throughout 6 d of differentiation (Figures 32A–B). In addition, treatment of all cell lines with the ICAM-1-P at 5 d of differentiation for 2 h reduced protein synthesis in ICAM-1+ cells to levels observed in CT and EV cells, which were not influenced by peptide treatment (Figures 33A–B). These findings demonstrate that ICAM-1 expression augments levels
of myotube protein synthesis through a mechanism involving its cytoplasmic domain, which serves to facilitate myotube hypertrophy.

**Figure 32:** ICAM-1 expression augments *in vitro* levels of protein synthesis. Protein synthesis is measured via the incorporation of puromycin into nascent peptides chains. A) Representative western blot of puromycin in control (CT), empty vector (EV), and ICAM-1+ (IC) cells (25 µg/lane) at 1–6 d of treatment with differentiation medium. S = standards (250–15 kDa). B) Quantitative analysis of western blot detection of puromycin incorporation in CT, EV and ICAM-1+ cells through 6 of differentiation. # = significantly higher for ICAM-1+ compared to CT and EV cells throughout 6 d of differentiation (main effect for cell line). n = 4 replicates/group. Mean ± SEM.

**Figure 33:** ICAM-1 expression augments *in vitro* levels of protein synthesis through mechanisms involving its cytoplasmic domain. Cells were treated with the ICAM-1 peptide (ICAM-1-P; 50 µg/ml), control peptide (CT-P; 50 µg/ml), or vehicle (dH₂O) at 5 d of differentiation for 2 h prior to measuring protein synthesis (via puromycin incorporation). A) Representative western blot of puromycin in control (CT), empty vector (EV), and ICAM-1+ cells treated with vehicle (V), control peptide (C), or ICAM-1 peptide (I). S = standards (250–15 kDa). B) Quantitative analysis of puromycin incorporation in CT, EV and ICAM-1+ cells treated with vehicle, control peptide (CT-P), or ICAM-1 peptide (ICAM-1-P). * = ICAM-1 peptide reduced protein synthesis in ICAM-1+ cells to levels detected in CT and EV cells (significant interaction). n = 4 replicates/group for each dependent measure. Mean ± SEM.
**ICAM-1 Expression Augments Akt/p70s6k Signaling**

The extent to which ICAM-1 expression by skeletal muscle cells influences Akt/p70s6k signaling was quantified through western blotting analysis. Phosphorylated, but not total, levels of Akt (Ser473) and p70s6k (Thr389) were 2 fold higher in ICAM-1+ cells compared to CT and EV cells at 3 – 6 d of differentiation (Figures 34C–D). There were no differences across cell lines in total levels of Akt and p70s6k. In addition, treatment of ICAM-1+ cells with the ICAM-1-P at 5 d of differentiation for 2 h reduced phosphorylated, but not total, levels of Akt and p70s6k by ~2 – 3 fold respectively (Figures 35C–D). These findings indicate the cytoplasmic domain of ICAM-1 augmented myotube size and protein synthesis via a mechanism involving Akt and p70s6k signaling.
**Figure 35:** ICAM-1 expression augments Akt/p70s6k signaling through mechanisms involving its cytoplasmic domain. Cells were treated with the ICAM-1 peptide (ICAM-1-P; 50 µg/ml), control peptide (CT-P; 50 µg/ml), or vehicle (dH₂O) at 5 d of differentiation for 2 h. Representative western blots of: A) phosphorylated Akt (Ser473; P-Akt) and total Akt, as well as B) phosphorylated p70s6k (Thr389; P-p70) and total p70 in ICAM-1+ cells treated with vehicle, control peptide (CT-P), or ICAM-1-peptide (ICAM-1-P). Quantitative analysis of phosphorylated levels of: C) Akt (Ser473), and D) p70s6k (Thr389) in ICAM-1+ cells treated with vehicle, control peptide (CT-P), or ICAM-1-peptide (ICAM-1-P). * = lower for ICAM-1-P compared to vehicle and CT-P. n = 4 replicates/group for each dependent measure. Mean ± SEM.
3.5.3 Specific Aim 2 Hypothesis 4: Interpretations

A novel aspect of our cell culture model is the quantification of myotube alignment. Mammalian skeletal muscle is composed of parallel bundles of muscle fibers, which arise from the assembly of multinucleated myotubes into well-aligned myofibers\textsuperscript{70,71,72}. However, nascent myotubes adopt a random pattern of alignment \textit{in vitro}\textsuperscript{109,137,138}. Recent studies focusing on skeletal muscle tissue engineering have attempted to control for cellular orientation through the use of micropatterned substrates to create well-aligned skeletal muscle cells as a model for studying \textit{in vitro} muscle tissue regeneration\textsuperscript{139,140,141,142}. These studies underscored the clinical significance of myotube alignment during events of muscle regeneration and dysfunction. Current findings from our cell culture model indicate that the increased uniformity of myotube alignment associated with ICAM-1 expression served a crucial role in directing the parallel fusion of myotubes, as well as facilitating large additions in myonuclei within a myotube, thereby resulting in the formation of robust myotubes. Significantly, peptide inhibition of the signal transducing cytoplasmic domain of ICAM-1 diminished the alignment of ICAM-1+ cells, resulting in a random pattern of orientation in myotubes, which compromised their subsequent ability to fuse with one another. This finding indicates that mechanisms of myotube alignment and myotube fusion are mediated by intracellular signaling events.

Of significance, studies have identified possible cues that direct the alignment of myotubes to include spatial, structural, chemical, and mechanical factors that affect cell adhesion, motility, orientation, and polarization, such as Wnt11 signaling, the localization of focal adhesions, and accumulation of postsynaptic acetylcholine receptors\textsuperscript{140,143,144}. 
Collectively, these mechanisms strongly indicate the potential involvement of intracellular events that initiate actin cytoskeleton remodeling. Based on prior studies that reported enhanced actin polymerization and membrane projection dynamics associated with ICAM-1 signaling in endothelial cells \(^99,100\), we propose that the cytoplasmic domain of ICAM-1 expression augments events of myotube alignment through increased Rho GTPase activity. Further work is necessary to discern the role of ICAM-1 in augmenting the various cues that affect myotube alignment and orientation.

ICAM-1 mediated events of increased myoblast-myotube fusion, as well as myotube-myotube fusion, resulted in substantial myonuclear accretion. An increase in myonuclear number has been postulated to increase myotube size by enhancing the myonuclear domain \(^54\), which facilitates an increased capacity for myotubes to synthesize proteins. In particular, sustained elevations in protein synthesis serve to induce skeletal muscle cell hypertrophy \(^61,133\). To this end, our findings demonstrate that increased levels of protein synthesis associated with ICAM-1 expression correspond to increases in myonuclear accretion, which ultimately contributes to the generation of larger myotubes. Significantly, phosphorylated levels of Akt and p70s6k were elevated with ICAM-1 expression, which indicates that ICAM-1 expression augments protein synthesis through increased signaling along the Akt/mTOR pathway, which is an established cell signaling pathway for skeletal muscle hypertrophy \(^3,4,5,6,145\). Furthermore, as peptide inhibition reduced rates of protein synthesis and phosphorylated levels of Akt/p70s6k in ICAM-1+ cells, we propose that the cytoplasmic domain of ICAM-1 contributed to enhanced rates of protein synthesis via increased Akt/p70s6k signaling.
The hypertrophy phenotype of ICAM-1+ cells is consistent with our in vivo findings after muscle overload\textsuperscript{34}. Specifically, ICAM-1+ myofibers in overloaded muscles were larger than myofibers that did not express ICAM-1, and protein synthesis and myofiber size were attenuated in overloaded ICAM-1\textsuperscript{−/−} compared to wild type mice\textsuperscript{34}. Hence, our in vivo and in vitro findings demonstrate that the expression of ICAM-1 by differentiated skeletal muscle cells facilitates their hypertrophy.

Taken together, findings from our cell culture model reveal a novel paradigm for skeletal muscle cell expression of ICAM-1 in augmenting myotube hypertrophy in which the cytoplasmic domain of ICAM-1 augments the parallel alignment of myotubes and their subsequent fusion to facilitate substantial myonuclear accretion, as well as robust levels of protein synthesis associated with Akt/p70s6k signaling.
3.6 Specific Aim 2: Summary

Findings from our cell culture model firmly establish the critical role of ICAM-1 in augmenting crucial events of myogenesis following skeletal muscle cell proliferation and differentiation. Specifically, we observe skeletal muscle cell expression of ICAM-1 expression to augment myogenic processes pertaining to myoblast fusion and myotube hypertrophy. In this capacity, we report that ICAM-1 expression enhanced events of homotypic myoblast adhesion, myotube formation, myonuclear accretion, parallel myotube alignment, myotube fusion, and myotube size. These findings corroborate with the results from our in vivo overload model, and serve to further demonstrate that ICAM-1 expression facilitates vital myogenic processes at multiple stages of skeletal muscle development and growth.

Furthermore, through the use of pharmacological inhibitors, we identify distinct mechanisms involved in ICAM-1 mediated myogenesis. Antibody neutralization revealed the adhesive function of the extracellular domain of ICAM-1 to mediate early events of myogenesis leading to the formation of nascent myotubes. In contrast, peptide inhibition revealed that subsequent events of myotube hypertrophy occurred through increased myonuclear number, enhanced myotube-myotube fusion, and elevated rates of protein synthesis associated with increased Akt/p70s6k activity, all of which were mediated by the signal transducing function of the ICAM-1 cytoplasmic domain.

Future studies should attempt to investigate the function of ICAM-1 in cellular events prior to homotypic myoblast adhesion, such as the migration of myoblasts, as well as mechanisms whereby ICAM-1 signaling is activated in skeletal muscle cells. In
addition, the incorporation of ICAM-1 mutant constructs would allow for greater mechanistic distinction between the function(s) of the extracellular domain to its cytoplasmic tail, and vice versa. Lastly, the relevant signaling pathways associated with ICAM-1 mediated cellular fusion, such as Rac1/Cdc42, should be assessed.
Chapter 4

Conclusion

Taken together, findings from our in vivo overload model, along with results from our cell culture model, establish a novel role for adhesion molecules of the inflammatory response in regulating skeletal muscle growth processes. In particular, we have demonstrated that ICAM-1 expression is critical in augmenting a myriad of critical myogenic processes that pertain to the formation, development, and maturation of skeletal muscle tissue. Consequently, we have identified potential working models whereby ICAM-1 expression by skeletal muscle cells augment skeletal muscle growth processes. Our in vivo model suggest that the dynamics/interactions between β2 integrins expressed by myeloid cells and ICAM-1 expressed by skeletal muscle cells serve to promote the release of cytokines, which augment regenerating myofiber formation, protein synthesis, as well as whole muscle and myofiber hypertrophy after mechanical overload (Figure 36).

Figure 36: Schematic of the proposed mechanism by which interactions between β2 integrins expressed on inflammatory cells and ICAM-1 on skeletal muscle cells promote mechanical load-induced skeletal muscle regeneration and hypertrophy.
Conversely, our cell culture model suggests that ICAM-1 mediated events of \textit{in vitro} myogenesis is regulated through alternative mechanisms that is devoid of β2 integrin interaction, and instead, involve dynamic interactions between skeletal muscle cells (Figure 37). In this proposed model, ICAM-1 signaling in skeletal muscle cells could be activated via an extrinsic or intrinsic mechanism. In the cell extrinsic pathway, ICAM-1 signaling is triggered upon ligand binding between ICAM-1 and novel ligands expressed by opposing skeletal muscle cells, such as hyaluronan, or through homophilic interactions between ICAM-1 on opposing membranes. Conversely, the close proximity between skeletal muscle cells may act to initiate ICAM-1 activity in a cell intrinsic manner, and generate a downstream signal in response to a variety of spatial, structural, chemical, and mechanical cues, such as glycosylation of the ICAM-1 extracellular domain.

Future studies should closely scrutinize underlying mechanisms for the activation of ICAM-1 signaling to facilitate the development of new/alternative approaches to rehabilitate musculoskeletal injuries and promote the maintenance and/or growth of skeletal muscle, as well as to identify potential molecular targets for translational strategies.
References


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116) Yoon S, Molloy MJ, Wu MP, Cowan DB, Gussoni E. C6ORF32 is upregulated during muscle cell differentiation and induces the formation of cellular filopodia. *Dev Biol.* 2007;(301):70-81


# Appendix A

## Institutional Animal Care and Use Committee Form

![Image of the form]

**Information**

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<th>Renewal - Please do not activate until old protocol expires</th>
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**Title of Protocol**

Immunobiology of Skeletal Muscle – MDEG Final

**PI Name**

Francis X. Pizza

**Date**

Jun 21, 2012

**Protocol Type**

Research

**Special Concerns**

Mouse

**Department**

Kinesiology

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**Lab/Room**

1416A and 1417

**Campus**

Main Campus

**Health Science Campus**

**Off-Label Use**

francis.pizza@utoledo.edu

**Assurances - Principal Investigator**

I certify that the information presented in this application is complete and accurate.

1. I understand that the principal investigator is responsible for the protection of the welfare and ethical treatment of animals and for strict adherence to institutional policies and procedures, federal, state, and local laws and regulations governing the protection of animals in research.

2. I agree to the following:

   1. To obtain prior approval from the IACUC before amending or altering this research protocol, including (but not limited to) changes in animal number, surgical procedures, anesthesia, or euthanasia methods.

   2. To provide for the humane care and use of all animals used in research, and to ensure that all procedures and experimental techniques are humane and ethical.

   3. To provide proper training and oversight to all study personnel in the proper conduct of animal research, as well as proper administration with the consent of the animal.

   4. To satisfactorily comply with all reporting and continuing review requirements of the IACUC.

**Signature**

Francis X. Pizza

**Date**

09-24-2012

**Approval Information (IACUC USE ONLY)**

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**IACUC Chair**

Amy C. Slocum

**Dates**

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**Protocol Number**

First Annual Review

07-22-2013

Second Annual Review

08-23-2014

3 Year Expiration

09-29-2015

3 Year Renewal Meeting Agenda

July, 2015