

SYSTEMIC ENVIRONMENTS ON SKELETAL MUSCLE DEVELOPMENT

**EFFECTS OF THE SYSTEMIC ENVIRONMENT IN HEALTH AND DISEASE ON
SKELETAL MUSCLE DEVELOPMENT**

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ABSTRACT

Children with cystic fibrosis (CF) show signs of systemic inflammation. Studies suggest that systemic inflammation is related to increased protein breakdown, which, in turn, is related to lower fat-free mass in CF patients. However, the direct implications of systemic inflammation on skeletal muscle tissue development are unknown. The focus of this thesis was to elucidate the effect of systemic factors from children with CF and healthy children on C2C12 myoblast myogenesis *in vitro*, and to assess whether systemic factors altered by exercise can modulate myogenesis, or provide an anti-inflammatory protection from an inflammatory mediator.

Our first study demonstrated that myoblasts treated with pooled CF serum had higher levels of proliferation, as measured by the total number of nuclei, compared to control serum. There was no exercise effect on proliferation in CF, while post-exercise serum from healthy controls showed an increase in proliferation compared to resting serum.

In our next study, we confirmed that myoblasts treated with individual samples of CF serum had greater proliferation than control serum. Differentiation, as measured by the myonuclei fusion index, was decreased in myoblasts treated with CF serum. No exercise effect was observed for proliferation or differentiation.

Our final study illustrated that an inflammatory mediator, lipopolysaccharide (LPS), decreased differentiation, as measured by myonuclei fusion index, in myoblasts treated with CF resting serum but increased differentiation in myoblasts

treated with control resting serum. Post-exercise recovery from children with CF reversed the effects of LPS, while exercise and recovery serum from healthy controls blunted the effects of LPS.

Collectively, our data suggest that systemic factors can have an effect on myogenesis, with differences observed between CF and control serum. Although post-exercise serum did not consistently affect myogenesis, anti-inflammatory effects are evidenced by the protection these factors provided from the effects of an inflammatory mediator.

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DEDICATION

To my mother, Em Thi Bui. I am grateful to have you in my life; grateful that you are here as I write these words and to see me cross this finish line. I love you, forever and always.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
bHLH	basic-helix-loop-helix transcription factors
BMI	Body mass index
BSA	Bovine serum albumin
CF	Cystic fibrosis
CFTR	Cystic Fibrosis Conductance Regulator
CRP	C-reactive protein
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-linked immunosorbent assay
ETDA	Ethylenediaminetetraacetic acid
EX	Serum or plasma sample collected immediately following exercise
eMHC	Embryonic myosin heavy chain
FBS	Fetal bovine serum
FEV ₁	Forced expiratory volume in 1 sec
FFM	Fat-free mass
FGF2	Fibroblast growth factor 2
FLT-3	Fms-like tyrosine kinase-3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony stimulating factor
GH	Growth hormone
GM	Growth media
HGF	Hepatocyte growth factor
IGF-1	Insulin-like growth factor
IL-1	Interleukin 1
IL-1 β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-6R α	IL-6 receptor alpha
IL-8	Interleukin-8
IL-10	Interleukin 10
IL-13	Interleukin 13
IP-10	interferon- γ -inducible protein 10
JAK	Janus Kinase
LBM	Lean body mass
LPS	Lipopolysaccharide
MFI	Myonuclei fusion index
MLP	Muscle LIM protein
MRI	Magnetic resonance imaging

mRNA	Messenger ribonucleic acid
MHC	Myosin heavy chain
MLP	Muscle LIM protein
MPC	Myogenic precursor cells
MRF4	Myogenic regulatory factor 4
MRFs	myogenic regulatory factors
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation antigen
NSAIDs	Nonsteroidal anti-inflammatory drugs
NK	Natural killer cell
³¹ P-MRS	³¹ Phosphorous-magnetic resonance spectroscopy
Pax7	Paired box 7
PBMC	Peripheral blood mononuclear cells
PCr	Phosphocreatine
PFA	Paraformaldehyde
PHV	Peak height velocity
PMP	Peak mechanical power
PMNs	Polymorphonuclear leukocyte
pSTAT3	Phosphorylated STAT3
RANTES	Regulated upon activation normal T-cell expressed and secreted
REST	Serum or plasma sample collected before exercise
REC	Serum or plasma sample collected 1 hour after exercise
RPM	Revolution per min
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDF-1	stromal cell-derived factor-1
SOCS3	suppressor of cytokine signaling 3
STAT3	Signal transducer and activator of transcription 3
TARC	Thymus and activation-regulated chemokine
TNF- α	Tumor necrosis factor alpha

PREFACE

This thesis is prepared in the “sandwich” format as outlined in the “Guide for the preparation of Master’s and Doctoral Theses” from McMaster University. Chapter 1 serves as a rational and general literature review of this thesis. Chapter 2-4 each represent an independent study manuscript, one of which has been published, one submitted for publication, and one in preparation to be submitted at the time of this thesis submission. All published, submitted, and prepared manuscripts were written by the author of this thesis, who is also the first author on all manuscripts. The preamble section preceding each chapter describes the contributions of other authors to the multi-authored work. Finally, Chapter 5 serves as the discussion section used to summarize overall finding and discuss implications.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Cystic fibrosis (CF) is an inherited genetic disease that affects nearly 4,000 Canadians (Cystic Fibrosis Canada, 2015). The disease is commonly known for its progressive pulmonary impairment, but CF encompasses much more than a lung disease (Amin & Ratjen, 2008). Indeed, CF is a multi-organ disease (Amin & Ratjen, 2008; Cystic Fibrosis Canada, 2015), and a common outcome of the condition is systemic inflammation (Nguyen et al., 2012; Tirakitsoontorn et al., 2001). Systemic inflammation in CF is thought to originate from the chronic pulmonary infections and local inflammation that spill over into the circulation (van Heeckeren et al., 2000). An association between systemic inflammation and protein degradation in CF patients has been observed, suggesting poor growth in these patients may be partly related to an inflamed systemic environment (Ionescu et al., 2004). As such, one of the aims of this thesis was to understand whether systemic factors from children with CF would directly affect tissue growth, specifically skeletal muscle tissue, *in vitro*.

This thesis also aims to understand whether exercise-induced systemic factors would have any effect on tissue development. Childhood is a time of rapid growth (Rauch et al., 2004), and exercise is an important contributor to healthy growth and development (Bar-Or & Rowland, 2004). However, the mechanisms translating exercise into healthy growth and development during childhood are not well understood. The main reason for this lack of knowledge stems from the ethical

limitation of obtaining pediatric tissue samples. This limitation has confined pediatric exercise research and allowed it to fall behind the vast body of literature in the adult population. This thesis will take steps towards determining the cellular consequences of exercise pertaining to skeletal muscle development in the pediatric population. A better understanding of the cellular responses to exercise factors would significantly advance our knowledge in the field of pediatric exercise science.

Exercise can also have anti-inflammatory properties (Petersen & Pedersen, 2005), which may benefit those with chronic systemic inflammation (Abd El-Kader et al., 2013; Wang et al., 2014; Lamina et al., 2014; Ho et al., 2013). It can also protect skeletal muscle development from the negative effects of an inflammatory mediator (Strle et al., 2007; Strle et al., 2008). Thus, another aim of this thesis was to examine whether exercise-induced systemic factors from children with CF and healthy controls would blunt the effects of an inflammatory mediator on myogenesis. Examining the anti-inflammatory effects of exercise on tissue development in the pediatric population is a novel approach, and would contribute to our understanding of the impact of exercise on growth. The following literature review was written to explain the rationale for the aims of this thesis and to discuss current gaps in the literature.

1.2 Cystic Fibrosis

1.2.1. Overview

Nearly ~4000 Canadians live with CF (Cystic Fibrosis Canada, 2015). The disease stems from a dysfunctional chloride channel expressed in many organs, including the lungs, digestive tract, sweat glands, pancreas, and sex organs (Bar-Or & Rowland, 2004; Gardner, 2007), resulting in a wide range of pathologies (Jacquot et al., 2008). Due to a number of scientific discoveries and the development of effective treatments, the median age of survival for an individual with CF has increased to more than 50 years (Cystic Fibrosis Canada, 2015). Unfortunately, half the mortality cases in 2013 occurred in patients less than ~35 years of age (Cystic Fibrosis Canada, 2015). Coupled with the incurable nature of CF, patients and caregivers feel heavily burdened by this disease (Götz & Götz, 2000) and report poor quality of life (Ribeiro Moço et al., 2015; Chevreul et al., 2015; Jamieson et al., 2014). Continued research is required to provide optimal and more effective treatment to further extend life expectancy and improve quality of life in the individuals born into this life-long, multi-organ disease.

1.2.2. Failure to thrive

Impaired growth is evident in pediatric patients with CF. Approximately 23% of children with CF are below the 10th percentile for height, ~20% are below the 10th percentile for weight (Cystic Fibrosis Canada, 2013), and ~29% are below the 25th percentile for BMI (Cystic Fibrosis Canada, 2015). Failure to thrive is also

prominent in adults as ~25% are considered underweight based on BMI (Cystic Fibrosis Canada, 2015). Thus, impaired growth begins in childhood and persist into adulthood, which suggests that therapeutic intervention strategies should begin during early childhood development.

There are many explanations for impaired growth. The primary reason is pancreatic insufficiency, which manifests as an inability to secrete the necessary bicarbonate and enzymes to digest food and absorb nutrients (Matel & Milla, 2009). Pancreatic insufficiency affects ~85% of patients with CF during the earlier years of life (Amin & Ratjen, 2008; Coste et al., 2007; Cystic Fibrosis Canada, 2015), the other ~15% of patients eventually become insufficient later in life (Matel et al., 2009). Even with pancreatic enzyme replacement therapy, children with CF weigh less and are shorter than their healthy peers, even when they present with greater caloric intake (Stark et al., 1997). Moreover, intestinal malabsorption (Coste et al., 2007) and higher resting energy cost (~120-150% of healthy children) (Selvadurai et al., 2003, Matel et al., 2009) can contribute to negative energy balance, which further exacerbates the observed growth impairments.

While there has been a focus on the nutritional deficiency contributing to patients' failure to thrive, the present thesis set out to explore another possible mechanism of impaired growth in patients with CF. Specifically, the effects of chronic systemic inflammation on skeletal muscle development. In recent years, the negative effects of chronic systemic inflammation on health and disease have garnered considerable attention from the scientific community, with a number of

studies supporting the central role of systemic inflammation in the development a number of diseases (Petersen & Pedersen, 2005).

1.2.3. Systemic inflammation in cystic fibrosis and possible consequences

Cystic Fibrosis stems from a mutation of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, and is inherited in an autosomal recessive manner (Riordan et al., 1989; Amin & Ratjen, 2008). The CFTR gene codes for a transmembrane chloride channel (Anthony et al., 1999; Welsh & Smith, 1993) that is expressed in many areas of the body including the respiratory tract (Amin & Ratjen, 2008). Indeed, pulmonary disease is the main cause of death for patients with CF, accounting for nearly ~90% of mortality and morbidity (Jacquot et al., 2008). In the respiratory tract, CFTR chloride channels are found on epithelial cells, as well as the serosal cells of the submucosal glands. Mutations to these channels result in chloride retention and subsequent sodium and water retention in the cells. This manifests as thick mucus secretion that build up in the alveolar sacs and airways of the lungs, impairing the action of the cilia lining the airways and inhibiting the clearance of inhaled microorganisms (Amin & Ratjen, 2008). Chronic bacterial infections are common consequences of CF. Bacterial strains often observed in CF patients include, but are not limited to, *Haemophilus influenza*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans*, *Pseudomonas aeruginosa*, *Aspergillus fumigatus*, *Methicillin-Resistant Staphylococcus aureus*, *Alcaligenes*, and *Burkholderia cepacia* (Amin & Ratjen, 2008; Cystic Fibrosis Canada, 2015; Strausbaugh &

Davis, 2007). *Pseudomonas aeruginosa* is the most common infection and contributes to the deterioration of lung function and clinical outcomes (Corey & Farewell, 1996; Henry, et al., 1992). The consequence of these chronic infections is chronic pulmonary inflammation, which leads to a vicious cycle of obstruction, infection, and inflammation (Moss, 2009; Amin & Ratjen, 2008).

Inflammation in the lungs is thought to spill over into systemic circulation in CF (van Heeckeren et al., 2000; Valletta et al., 1992). Indeed, van Heeckeren *et al.*, (2000) observed elevated pulmonary cytokine concentrations of tumor necrosis factor alpha (TNF- α), interleukin 1 (IL-1), and interleukin 6 (IL-6) immediately following and for up to 4 days after an induced pulmonary infection via *Pseudomonas aeruginosa*-laden agarose beads instilled into lungs of male C57BL/6 mice. This was followed by elevated systemic concentrations of serum IL-6 and decrease in body weight (van Heeckeren et al., 2000), highlighting the potential negative effects of systemic inflammation on body mass. The concept that systemic inflammation may be associated with reduced body mass is supported by research in patients with CF. Systemic inflammation is present in patients with CF, as evidenced by elevated circulating levels of TNF- α , IL-6, and C-reactive protein (CRP) (Ionescu et al., 2002; Ionescu et al., 2004; Dufresne et al., 2009). Within the CF population, patients who have increased inflammation tend to have increased protein breakdown, as measured by urinary pseudouridine. Moreover, increased protein breakdown is also associated with lower fat-free mass (FFM) (Ionescu et al., 2002; Ionescu et al., 2004). Indeed, patients with CF have been reported to

suffer from lower FFM than their healthy counterparts (King et al., 2010; Bianchi et al., 2006; Ionescu et al., 2000).

Researchers have yet to elucidate the direct effects of systemic factors on skeletal muscle or its development. Understanding the effects of systemic inflammation on tissues such as skeletal muscle may provide researchers and clinicians with more insight into the clinical symptoms of CF. We hope that this would lead to future solutions for combatting poor growth in CF patients; a very clinically relevant endeavour given the presence of failure to thrive in patients with CF (Cystic Fibrosis Canada, 2013; Cystic Fibrosis Canada, 2015). As such, a closer examination of skeletal muscle development and systemic inflammation in CF is warranted. The subsequent sections will discuss the importance of examining skeletal muscle development in children, the role of systemic immunology on skeletal muscle, and the pediatric exercise literature related to skeletal muscle and systemic immunology.

1.3 Skeletal muscle, immunology, and exercise in children

1.3.1. Skeletal muscle in health and development and the role of exercise

Skeletal muscle is the largest tissue by mass and is required to produce movement (McArdle et al., 2007). The rate of muscle growth is greatest around the age of peak height velocity – the age corresponding to the greatest rate of height acquisition. In healthy children, peak lean body mass (LBM) velocity occurs at around 12 years of age for girls and 14 years of age for boys, gaining nearly ~5,050

g/yr and 8,850g/yr, respectively (Rauch et al., 2004). Therefore, optimal growth in childhood is critical, and interventions during this period may be more effective than at any other time in life.

Muscle can affect the growth of other tissues in the body and play a role in metabolism. Indeed, muscle contraction provides the largest physiological loads on bone (Burr, 1997) and peak LBM velocity precedes peak bone mineral content velocity, suggesting that muscle development and associated contractile forces facilitate optimal bone deposition (Rauch et al., 2004). Skeletal muscle is responsible for the majority of glucose uptake and storage through either insulin- or contraction-mediated mechanisms (Stump et al., 2006). Skeletal muscle is also involved in lipid metabolism by carrying out lipolysis (Wolsk et al., 2010) and fat oxidation (Pedersen, 2011). These observations emphasize the vital role of skeletal muscle and its contractile activity in inducing physiological adaptations that lead to optimal growth.

Exercise is a powerful stimulus for muscle. More specifically, exercise training in children can enhance muscle hypertrophy (Eliakim et al., 1997; Eliakim et al., 1998; Eliakim et al., 2001) and muscle strength (Falk & Tenenbaum, 1996). Despite low circulating testosterone (Vingren et al., 2010), gains in muscle size provide strong evidence that acute changes in other systemic and local factors must play an essential role in skeletal muscle adaptations to exercise in children, but the mechanisms of skeletal muscle adaptation during growth are not well understood. Because childhood is characterized by periods of rapid growth, it

serves as a unique model to elucidate – in a relatively short time frame – the impact of systemic factors released due to contractile activity on mechanisms of skeletal muscle adaptation that would not be gleaned from adult investigations.

1.3.2. The immune system and its role in muscle growth

Exercise is able to activate and release systemic immune cells and factors with significant anabolic potential. For example, exercise induces the migration of circulating progenitor cells, which are incorporated into skeletal muscle tissue thereby contributing to adaptation (i.e., hypertrophy) (Palermo et al., 2005). Mobilized progenitor cells (e.g., CD34⁺) may indirectly contribute to skeletal muscle adaptation by secreting growth-potentiating factors *in situ* (Majka et al., 2001). Exercise further contributes to adaptive processes in skeletal muscle by increasing local expression of anabolic factors. Instrumental in skeletal muscle development is insulin-like growth factor 1 (IGF-1) (Glass, 2003), as it has been observed to induce muscle protein synthesis (Frost et al., 1997) and enhance the potential for incorporation of progenitor cells into skeletal muscle (Sacco et al., 2005). Cytokines, such as interleukin-8 (IL-8), are also produced locally with muscle contraction (Chan et al., 2004) and possess chemoattractant properties (Koch et al., 1992), including mobilization of stem cells from bone marrow (Fibbe et al., 1999). Taken together, these observations hint at a potential mechanism whereby contractile activity induces the production of local factors and recruits systemic factors, which may directly or indirectly enhance skeletal muscle adaptation in healthy individuals.

In contrast, over-activation of the immune system can induce muscle atrophy or inhibit growth. This effect is in part driven by cytokines in skeletal muscle, for example, IL-6 is capable of inducing muscle degradation by activating the proteasomes and cathepsin proteolytic pathways (Ebisui et al., 1995). Furthermore, TNF- α inhibits skeletal muscle growth, regeneration, and repair by causing resistance to growth hormone (GH) (Frost et al., 2003; Hong-Brown et al., 2003). Thus, increases in these cytokines will drive muscle wasting, a condition commonly seen in individuals in pro-inflammatory states (i.e., cancer) (DeJong et al., 2005). Exercise is known to increase pro-inflammatory cytokines, including IL-6 and TNF- α (Tirakitsoontorn et al., 2001), suggesting that it may also play a role in the impairment of muscle growth. Taken together, the systemic factors activated by exercise can act to enhance or reduce and even inhibit muscle growth; this highlights the importance of balancing the anabolic and catabolic nature of exercise immune responses.

1.3.3. Pediatric exercise and skeletal muscle

Muscle biopsies have been performed on healthy children in the past. In the 1970's, results of a muscle biopsy study in healthy children between the ages of 11 and 16 years were published by a Swedish group (Eriksson & Saltin, 1974). Tissue samples were used to compare muscle metabolism during exercise in children and adults. Researchers reported that children possessed smaller stores of muscle glycogen and were also less anaerobic than adults (Eriksson et al., 1974). The same research group reported that lower anaerobic capacity in children

may be attributable to lower concentrations of phosphofructokinase, an enzyme required for glycolysis (Eriksson et al., 1974; Eriksson et al., 1973; Eriksson & Saltin, 1974). Muscle biopsies are no longer performed in healthy children today. In relation to exercise, limited studies have included muscle biopsies in children with a known disease (Mantz et al., 1993) or suspected muscle abnormalities due to clinical signs of exercise intolerance (Grafakou et al., 2003; Kollberg et al., 2007; Mayr et al., 2006; van Ekeren et al., 1991). The majority of these studies were primarily interested in the contents of the muscle tissue to confirm muscle abnormalities or dysfunction. Only one study explored the effects of exercise on skeletal muscle remodeling in a child with carnitine-palmitoyl-transferase deficiency (Mantz et al., 1993). In this study, muscle tissue was collected 11 days after “casual exercise”, as described by the authors, and muscle regeneration was examined using light and electronic microscopy. The results were descriptive in nature, exploring the presence of immune cells and damaged muscle cells with no comparison to a control sample. These studies collectively illustrate that the molecular consequences of exercise on muscle tissue have been scarcely explored.

Researchers have devised a number of tools and techniques to study muscle in healthy children in the absence of tissue samples. Among these is ³¹phosphorous-magnetic resonance spectroscopy (³¹P-MRS), which has been used to examine muscle metabolism in children during and after exercise (Barker & Armstrong, 2010; Wells et al., 2011; Fleischman et al., 2010; Kappenstein et al.,

2013). The technology relies on the unique properties of substances such as adenosine diphosphate (ADP) and phosphocreatine (PCr), which have specific energy signatures. When the molecules are exposed to a magnetic field and an external energy pulse, the amount of energy released is quantified and concentrations of the substances are determined. Studies utilizing ^{31}P -MRS suggest that muscle mitochondrial capacity is fully developed by the age of 9-10 years, that children experience less perturbation of pH than adults at a given workload suggesting better oxidative adenosine triphosphate (ATP) flux (Barker et al., 2010), and that children have a greater rate of energy recovery (Fleischman et al., 2010).

The major advantage of ^{31}P -MRS is its non-invasive nature. More specifically, since children are performing exercise in a magnetic resonance imaging (MRI) machine, blood samples and tissue collection are not required. However, the disadvantage of using ^{31}P -MRS is the fact that the calculations used to quantify concentrations are based on assumptions. For example, ATP at rest, which is used to calculate the concentrations of PCr and ADP, is assumed to be 8.2 mmol/L and not to differ between individuals, or between muscle fibre types. This assumption, along with many others, appear to be true for adults (Barker et al., 2010), but have not, and cannot, be validated in children because of the inability to perform muscle biopsies. Indeed, this method is applying findings from adults to children, which appears to be a common trend in the pediatric field.

Other scientists have turned to animal models to study the effects of exercise on muscle. To this author's knowledge, only two studies have evaluated muscle remodeling in juvenile animal models following exercise (Chen et al., 2002; Velders et al., 2008). In juvenile rats (29-day-olds), voluntary exercise (i.e., wheel running) for 7 days resulted in an 18% increase in muscle mass and 23% increase in fibre size. Gene expression using real-time reverse transcription polymerase chain reaction (RT-PCR) showed increased gene expression of muscle LIM protein (MLP), which is a regulator of myogenic differentiation (MyoD) 1 gene (Velders et al., 2008). MyoD protein is responsible for early muscle stem cell differentiation (Legerlotz & Smith, 2008). Thus, an increase in MLP may be indicative of increased muscle development. These studies provide some insight into the mechanisms linking exercise and developing muscle; whether these findings translate to the human pediatric population remains to be explored.

Collectively, the study of muscle development and the effects of exercise in children are sparse. Indeed, there have been few attempts to determine the cellular consequences of exercise on muscle adaptation in the pediatric population, particularly as it relates to growth. The present thesis will aim to bridge an *in vitro* cell culture model with pediatric exercise samples to determine how muscle cells respond to exercise.

1.3.4. Pediatric exercise immunology

There has been a considerable increase in the number of studies examining the effects of exercise on immune responses of children (Timmons, 2006).

Examining the immune response to exercise in children has been limited to non-invasive measures. Several different types of tissue, the majority of which have used blood, to quantify cell types, determine function, and now recently, examine gene expression in immune cells have been done. Quantifying immune cells and mediators has been performed using a host of methods. Various immune cells such as lymphocytes, monocytes, and neutrophils, have been quantified using the automated Coulter counter (Timmons et al., 2004). Progenitor/stem cells (Arnold et al., 2010; Zaldivar et al., 2007), natural killer cell (NK) subsets (cytotoxic vs. regulatory) (Timmons & Bar-Or, 2007b), and T and B cells expressing the apoptosis receptor CD95 (Timmons & Bar-Or, 2007a) have been enumerated by flow cytometry. Many cytokines and chemokines, such as IL-6, IL-8, TNF- α (Timmons et al., 2006), granulocyte colony stimulating factor (G-CSF), Fms-like tyrosine kinase-3 (FLT-3), stromal cell-derived factor-1 (SDF-1) (Zaldivar et al., 2007), eotaxin, regulated upon activation normal T-cell expressed and secreted (RANTES), thymus and activation-regulated chemokine (TARC), and interferon- γ -inducible protein 10 (IP-10) (Tahan et al., 2006) have been quantified using enzyme-linked immunosorbent assay (ELISA). The quantification of activated immune cells, using the CD62L receptor as a marker of activation, has been examined via flow cytometry (Nemet et al., 2002). Components of the complement system were determined using radial immunodiffusion (Wolach et al., 1998). The majority of these studies have shown that various components of the immune

system are mobilized by muscle contraction, suggesting that these immune cells and mediators play a role in exercise adaptation.

Although most of the studies examining the immune response to exercise were geared towards quantifying immune components, relatively few have also examined the effects of exercise on immune cell function. Polymorphonuclear leukocyte (PMNs) cells from blood samples collected following exercise have been isolated and used to assess the effect of exercise on migration capacity. Migration of PMNs was examined using a chemotactic microchamber with N-formylmethionyl-leucyl-phenylalanine (Wolach et al., 1998). Migration of PMNs was also determined indirectly via flow cytometry by examining the up-regulation of the CD54 receptor that aids in adhesion and migration (Perez et al., 2001). Moreover, PMNs' capacity to fight bacteria has also been examined by incubating these cells with cultured *Staphylococcus aureus* (Wolach et al., 1998). While exercise had the capacity to alter adhesion molecules, it had no effect on the PMNs' ability to kill bacteria. This suggests that exercise may activate immune cells for reasons that are not entirely related to fighting pathogens.

Finally, gene expression of peripheral blood mononuclear cells (PBMC) has been studied using microarray and RT-PCR. Genes associated with NK cell-mediated cytotoxicity, antigen processing and presentation, cytokine-cytokine receptor interaction, apoptosis, cytokine and chemokines - all of which have relevance for health - are altered by an acute bout of exercise (Radom-Aizik et al., 2009a; Radom-Aizik et al., 2009b; Tahan et al., 2006). Results suggest that

immune cells are sensitive to the physiological changes induced by exercise resulting in intracellular modifications that can up- and down-regulate a number of genes. Alternatively, changes in gene expression of immune cells may reflect an exercise-induced redistribution of cells from sequestered sites into the circulation.

Other types of samples have been used to examine the immune system and its components including saliva (Scheett et al., 1999; Thomas et al., 2009; Filaire et al., 2004; Tharp, 1991), pulmonary sputum (Yoshikawa et al., 1998), and urine (Tirakitsoontorn et al., 2001; Scheett et al., 1999; Lee et al., 2006). These studies mainly identified and quantified immune cells, cytokines, or immunoglobulin presence and exercise-induced alterations in their concentrations. Despite the non-invasiveness of obtaining these biological samples, studies utilizing these techniques are fewer in number compared with those using blood samples. This may be linked to the fact that components of the immune system measured in blood samples are more indicative of systemic effects, while alternative techniques may be related to the local environment (i.e., pulmonary sputum reflects the lung environment).

From the studies that have investigated immune responses to exercise in the pediatric population, the link between mobilization of immune mediators and tissue adaptation (i.e., in muscle), has not yet been explored. A goal of this thesis is to explore how exercise-induced systemic immune mediators affect skeletal muscle cells.

1.4 Myogenesis

1.4.1. An overview of muscle development and regeneration

The largest contributor to our body mass is skeletal muscle (Yusuf & Brand-Saberi, 2012), which is composed of highly differentiated and specialized cells (Pagel et al., 2014). Given its important functions, it is not altogether surprising that skeletal muscle demonstrates an incredible ability to regenerate following injury (Pagel et al., 2014). The events that lead to the embryonic development of skeletal muscle and its regeneration are, within the scope of this thesis, similar (Pagel et al., 2014; Yin et al., 2013; Chargé & Rudnicki, 2004) and will be discussed in detail. To the author's knowledge, there is no literature to suggest that the mechanisms regulating the growth and development of skeletal muscle in children are vastly different from those observed during embryonic development or adult tissue regeneration. However, similarities and differences in juvenile muscle growth and adult skeletal muscle regeneration will be discussed.

Skeletal muscle originates from the mesoderm layer during embryonic development, and specifically from the somites. Mononucleated and mitotically active myoblasts fuse together to form myotubes that serve as an important structural component of skeletal muscle development by behaving as scaffoldings. Myoblasts fuse onto these scaffoldings that grow in size and form a multinucleated contractile muscle fibre (Yusuf & Brand-Saberi, 2012; Simionescu & Pavlath, 2011)

In adult tissue, satellite cells are precursor myoblasts responsible for the myogenic regenerative properties of skeletal muscle (Yusuf et al., 2012). While

other sources of cells may also behave as myogenic precursors, such as vessel-derived stem cell mesoangioblasts (Sampaolesi et al., 2006), multipotent myogenic cells related to the endothelial cell lineage (Zheng et al., 2007), and pericytes that are present in the microvasculature (Dellavalle et al., 2007), satellite cells contribute to the vast majority of the myogenic precursor pool. Satellite cells were first identified in the tibialis anticus of a frog in 1961 (Mauro, 1961). Alexander Mauro used electron microscopy to describe cells with a large nucleus with a thin cytoplasmic body lying between the sarcolemma and basal lamina of their associated muscle fibres. These cells were named “satellite cells” due to their location (Mauro, 1961). Years later, researchers definitively recognized satellite cells as the precursors to myoblasts that play a key role in muscle regeneration (Scharner & Zammit, 2011)

In a healthy human adults, satellite cells make up 2-7% of the muscle nuclei (Yin et al., 2013). However, their numbers vary between species, as well as by muscle fibre type, with oxidative muscles fibres displaying higher numbers of satellite cells compared to glycolytic muscles fibres (Schultz, 1989). In addition, lower numbers of satellite cells were observed with sarcopenia, which is the age-related loss of muscle mass (Shefer et al., 2006; Collins et al., 2007), and in patients with diseases such as muscular dystrophies (Blau et al., 1983). In contrast, satellite cell number can increase following exercise training (Macaluso & Myburgh, 2012; Mackey et al., 2011; Verney et al., 2008).

Satellite cells are mitotically quiescent cells that have limited gene expression and protein synthesis. They become activated by physical trauma, disease, or exercise (Chargé & Rudnicki, 2004). When activated, satellite cells become known as myoblasts that undergo multiple rounds of division and increase in number, a process known as proliferation. Most of these myoblasts will then differentiate to fuse and form myotubes, and eventually multinucleated myofibres; a small fraction of these myoblasts will revert back to satellite cells and replenish the myogenic precursor pool (Le Grand & Rudnicki, 2007).

1.4.2. The regulation of myoblast proliferation and differentiation

There are 3 overlapping stages in muscle regeneration: i) inflammatory response, ii) activation, proliferation, differentiation, and fusion of satellite cells, iii) maturation and remodeling of newly formed myofibres (Yin et al., 2013). The first stage of muscle regeneration starts with the dissolution of the myofibre sarcolemma, which can be caused by injury, trauma, or exercise. This will in turn activate the complement cascade and induce the inflammatory response. This is followed by recruitment of circulating leukocytes to the site of damage by a chemoattractive stimulus (Tidball, 1995; Orimo et al., 1991). The first leukocytes to infiltrate are the neutrophils (Fielding et al., 1993) followed by 2 distinct subpopulations of macrophages. The first of these subpopulations of macrophages is identified as CD68⁺/CD163⁻, and peaks at about 24 hours following injury. These macrophages tend to secrete pro-inflammatory cytokines such as TNF- α and IL-1, in addition to clearing debris by phagocytosis. The second subpopulation of

macrophages is present 2-4 days after injury and is characterized as CD68⁺/CD163⁺. These macrophages tend to secrete anti-inflammatory cytokines such as interleukin 10 (IL-10) and are present until the termination of the inflammatory response (Yin et al., 2013).

The next stage consists of the activation, proliferation, differentiation, and fusion of satellite cells; together these phases are termed the myogenic programme or myogenesis. This highly orchestrated process is not thoroughly understood. Quiescent satellite cells and proliferating myoblasts both express paired box 7 (Pax7) protein (Olguín & Pisconti, 2012), making it sustainable as an early marker of myogenesis. Interestingly, Pax7 is required for the renewal of satellite cells as an absence of satellite cells is observed in transgenic models devoid of Pax7 (Seale et al., 2000). The events that regulate proliferation and differentiation are orchestrated by 4 muscle regulatory basic-helix-loop-helix transcription factors (also known as bHLH or MRFs), which are myogenic factor 5 (Myf5), MyoD, myogenic regulatory factor 4 (MRF4), and myogenin that play a role in the regeneration process (Perry & Rudnick, 2000; Sambasivan & Tajbakhsh, 2015).

While the mechanisms that trigger satellite cell activation are not completely known, factors such as sphingosine-1-phosphate, extrinsic stretching that lead to hepatocyte growth factor (HGF) release, and fibroblast growth factor are thought to be involved (Le Grand & Rudnicki, 2007). Signals from the damaged environment activate satellite cells, allowing the cells to exit the quiescent state

and enter the myogenic programme. At this point, activated satellite cells and their progeny are referred to as myogenic precursor cells (MPC) or adult myoblasts. One of the earliest transcription factors involved in proliferation is Myf5. Similar to Pax7, Myf5 is expressed in ~90% of quiescent satellite cells (Beauchamp et al., 2000) and in activated myoblasts, and is required for myoblast proliferation and maintaining the satellite cell pool (Sambasivan et al., 2015). Proliferation is thought to be regulated by Myf5, as evidenced by the observation that Myf5⁻ myoblasts have a tendency towards early differentiation (Montarras et al., 2000). The expansion of the myoblast pool is essential in muscle regeneration process so as to provide enough cells for regeneration and renew the quiescent satellite cell pool. The second muscle regulatory transcription factor that makes an appearance in this process is MyoD. MyoD is required for initiation of differentiation. In fact, MyoD⁻ myoblasts exhibit enhanced proliferation and delayed differentiation (Sabourin et al., 1999). The current hypothesis is that myoblasts will proliferate or differentiate depending on whether Myf5 or MyoD expression predominates (Rudnicki et al., 2008; Yin et al., 2013). Moreover, co-expression of Myf5 and MyoD leads to an intermediate stage of proliferation and differentiation (Yin et al., 2013). The next stage of myogenic differentiation is terminal differentiation, which is irreversible. The muscle regulatory transcription factors involved in this process are MRF4 and myogenin (Cornelison et al., 2000; Smith et al., 1994; Yablonka-Reuveni & Rivera, 1994). At this point structural and contractile muscle proteins, such as actin, myosin, and troponin are expressed (Yin et al., 2013).

After proliferation, wherein myoblasts exit the cell cycle, myoblasts committed to differentiate undergo cell-to-cell fusion with existing and damaged myofibres, or they participate in *de novo* formation of myofibres that is similar to the 2-stage process seen during embryonic myogenesis. The first stage involves individual myoblasts fusing together to form nascent myotubes with only a few nuclei. The second stage occurs when additional myoblasts fuse with nascent myotubes, gradually forming a mature myofibre with contractile proteins. Newly formed myotubes by default go on to express embryonic, neonatal, and fast myosin heavy chain (MHC), thus, providing markers for last stages of muscle differentiation (Yin et al., 2013).

The third and final stage is the maturation and remodeling of newly formed myofibres. The process is dependent on the development of blood vessels, and the formation of myotendinous connections and neuromuscular junctions. The absence of these innervations results in atrophic fibres, making these innervations essential to the maturation and maintenance of muscle fibres (Zanou & Gailly, 2013).

1.4.3. Skeletal muscle growth and development in youth

Increases in skeletal muscle mass during postnatal growth are attributed to two processes. One of these is the enlargement of muscle fibres, known as hypertrophy, which occurs when an individual is in a positive net protein balance, wherein the rate of protein synthesis exceeds the rate of protein breakdown. The second process involves an increase in the number of muscle fibres, known as

hyperplasia, and occurs in the earlier stages of postnatal development via the proliferation and fusion of satellite cells (Schiaffino et al., 2013; White et al., 2010). The process of hyperplasia differs from adult muscle growth, which is mainly attributed to hypertrophy. However, recent evidence has resulted in some debate suggests that satellite cells may play a key role in adult muscle mass hypertrophy through myoblast fusion (Blaauw & Reggiani, 2014; Adams, 2006).

There are other apparent differences observed in juvenile versus adult skeletal muscle. Satellite cells make up ~30% of neonatal muscle in mice (Allbrook et al., 1971; Hawke & Garry, 2001), which is significantly higher than the 2-7% observed in adults (Yin et al., 2013). During the course of postnatal growth the number of satellite cells decreases while the number of myonuclei increases (Enesco & Puddy, 1964). It is thought that as the nuclear addition to the growing muscle ceases in juveniles, satellite cells enter a mitotically quiescent G_0 state (White et al., 2010). Interestingly, the proliferation rate of satellite cells is elevated in juvenile muscle and likely reflects the growth stage during this period of life (Yin et al., 2013) or the demand of wear and tear (Schultz, 1989).

Despite these differences, there are some similarities between juvenile and adult satellite cells. Much like adults, the juvenile satellite cell population is heterogeneous, consisting of fast-cycling and slow-cycling cells. The fast-cycling cells make up 80% of satellite cells, and the majority of cells experience a limited number of mitotic divisions prior to myogenic differentiation and fusion. On the other hand, slow-cycling satellite cells are thought to maintain the pool of fast-

cycling satellite cells through the process of asymmetrical division (Schultz, 1996). Similar observations in the function between these two subpopulations of satellite cells in both juvenile and adult muscle may reflect common processes and events in myogenesis in postnatal muscle growth and adult muscle regeneration (Yin et al., 2013).

1.4.4. The role of systemic factors on myogenesis

The number of satellite cells declines with age (Shefer et al., 2006; Collins et al., 2007), and aging muscle does not regenerate as well as younger muscle following injury (Sadeh, 1988). The impaired regenerative process typically observed in aging muscle is thought to be due to a reduced number of satellite cells, and not due to intrinsic impairments within the satellite cells themselves (Dedkov et al., 2003). This is supported by various studies, one of which is by Shefer *et al.*, (2006) where satellite cells from aged mice had diminished proliferation potential but similar differentiation potential compared to satellite cells from younger mice in an *in vitro* model (Shefer et al., 2006). Importantly, the impaired proliferative capacity in aged satellite cells was due to a reduction in the initial pool of satellite cells in aged muscle, which consequently delayed the amplification of satellite cells required for differentiation. Moreover, both aged and young satellite cells had similar proliferative capacity when treated with fibroblast growth factor 2 (FGF2), a factor known to promote myoblast proliferation (Shefer et al., 2006). Other researchers have indirectly alluded to systemic factors playing a role in muscle regeneration capacity using grafting methods. Specifically, muscle

from old rats regenerated successfully when grafted into muscle of a young host, but muscle from a young rat displayed impaired regeneration when grafted into an old host (Carlson & Faulkner, 1989; Carlson et al., 2001). Another study showed that aged muscle differentiated into fibroblasts, thus, reducing its regenerative capacity. This impairment was a result of elevated canonical Wnt signaling induced by components from the systemic factors in old mice (Brack et al., 2007).

The powerful effects of systemic factors on skeletal muscle regeneration are illustrated in an eloquently designed study by Conboy *et al.* (2005)(Conboy et al., 2005). These researchers examined the effect of systemic factors from young mice on the proliferation and regenerative capacity of satellite cells from old mice using both an *in vivo* and *in vitro* model. In the *in vivo* model, researchers established parabiotic pairings in which mice developed vascular anastomoses that resulted in a shared circulatory system. Parabiotic pairings were established between young (2-3 months) and old mice (19-26 months), termed 'heterochronic parabioses'. This allowed old mice to be exposed to systemic factors from young mice. Parabiotic pairings were also established between two young mice and between two old mice, termed isochronic pairing, to serve as controls. Following 5 weeks of established parabiosis, the hindlimb muscles of each mouse was given 5-bromodeoxyuridine injections to induce injury. Five days after injury, the young mice in heterochronic and isochronic pairing had similar number of activated satellite cells and regeneration index, indicating similar proliferative and differentiation capacity. In the old mice, isochronic pairs displayed reduced activated satellite cell numbers

and regeneration index, while the heterochronic pairs had significantly higher proliferative and regenerative capacity. It is important to note that circulating cells in young mice were labeled with a green fluorescent protein. Interestingly, less than 0.1% of the regenerated myotubes in the old mice in heterochronic pairs were of young mouse origin, suggesting that systemic factors from young mice can promote improved proliferation and differentiation capacity in aged satellite cells. Conboy *et al.* (2005) were keen to reproduce their findings in an *in vitro* model by which they cultured the primary satellite cells from young and old mice, with and without association with myofibre explants, and treated them with young and old serum. Results were similar to their *in vivo* model (Conboy et al., 2005).

Collectively, this body of literature indicates that the environment satellite cells are exposed to can have astounding effects on their capacity for proliferation, and consequently their ability to differentiate. This highlights the relevance of the objectives of this thesis, particularly as they relate to determining the effects of systemic factors from children with CF and healthy controls on proliferation and differentiation of myoblasts.

1.5 The effect of cytokines on myogenesis

1.5.1. Interleukin 6: The good and the bad

IL-6 is of particular interest based on the author's earlier finding of elevated systemic levels of IL-6 in the participants involved in this thesis (Nguyen et al., 2012). Known traditionally as a pro-inflammatory cytokine, IL-6 is secreted by many

cell types and tissues including monocytes, macrophages, neutrophils, B and T lymphocytes (Biffi et al., 1996), adipose tissue (Bruun et al., 2007), and muscle (Steensberg et al., 2002; Helge et al., 2003). The role of IL-6 in inflammation is to stimulate acute-phase protein production (Heinrich et al., 1990) in the liver that go on to initiate systemic inflammation via the complement system (Pickup, 2004). Other roles of IL-6 include the stimulation of B cell maturation and differentiation, T cell activation, and the release of hormones such as GH (Heinrich et al., 1990), illustrating that IL-6 can function to aid in an organism's immunity.

On the other hand, IL-6 can also have a negative effect on skeletal muscle by promoting protein degradation through lysosomal and ubiquitin-proteasome activity by increasing levels of ubiquitin messenger ribonucleic acid (mRNA) (Williams et al., 1998), mRNA encoding lysosomal proteins, and transcription of proteasome proteins (Ebisui et al., 1995). Indeed, IL-6 infusion results in muscle atrophy and -17% reduction on myofibrillar protein content in rats (Haddad et al., 2005). Further, studies have reported that IL-6 is associated with reduced growth rates and skeletal muscle development (De et al., 1997; De et al., 2006; Bodell et al., 2009), while chronically elevated systemic IL-6 in aging and disease are seen with muscle atrophy (Baltgalvis et al., 2008; Ersler & Keller, 2000).

However, IL-6 is also involved in skeletal regeneration/development. IL-6 induces myoblast proliferation (Serrano et al., 2008; Zhang et al., 2013) and differentiation (Baeza-Raja & Munoz-Canoves, 2004; Hoene et al., 2013). The paradoxical role of IL-6 in promoting both muscle development and catabolism may

be attributed to a dose effect, in which low or “healthy” levels of IL-6 can be beneficial, while chronically elevated levels can be detrimental to skeletal muscle. Difference between acute exposures versus chronic exposure may be another reason. Moreover, the origin of IL-6 may play a role as it has been postulated that IL-6 originating from skeletal muscle and released post-exercise may have anti-inflammatory characteristics (de Lemos et al., 2012; Petersen & Pedersen, 2005; Gleeson et al., 2011), while pro-inflammatory IL-6 related to catabolism has been hypothesized to originate from adipose tissue, but has yet to be confirmed (Pedersen et al., 2003).

1.5.2. Interleukin 6: Signalling pathway

IL-6 induces myoblast proliferation (Serrano et al., 2008) via the Janus Kinase/Signal transducer and activator of transcription (JAK/STAT) signalling pathway (Rawlings et al., 2004), specifically the JAK/STAT3 pathway (Yin et al., 2013). The IL-6 receptor alpha (IL-6R α) is expressed in satellite cells (McKay et al., 2013), and binding of IL-6 to IL-6R α results in receptor dimerization (Rawlings et al., 2004; Yin et al., 2013). Tyrosine kinase activity is present on one of the dimerized receptors and the close proximity of the two receptors lead to the phosphorylation, thus the activation of JAK2. JAK2 goes on to phosphorylate a transcription factor that resides latently in the cytoplasm known as STAT3. The activation of STAT3 results in dimerization and translocation into the nucleus where it binds to specific regulatory sequences to promote or repress the transcription

target genes that induce myoblast proliferation (Rawlings et al., 2004; Yin et al., 2013).

There are multiple ways in which the JAK/STAT3 pathway can be regulated, one of which involves suppressor of cytokine signaling 3 (SOCS3). SOCS3 is one of the genes targeted by STAT3, and illustrates the simple characteristic negative feedback loop of the JAK/STAT3 pathway. The production of SOCS3 turns off the JAK/STAT3 pathway in three ways. The first is via the binding of SOCS3 to the phosphotyrosine on the receptor to physically block the recruitment of STAT3 to the receptor, thus, preventing STAT3 phosphorylation. SOCS3 can also bind to JAK or to the receptor to inhibit JAK kinase activity. Lastly it can induce the ubiquitination of JAK or the receptors and targeting them for proteasomal degradation (Rawlings et al., 2004; Yin et al., 2013). The suppression of IL-6 signalling by SOCS3 was associated with a blunted satellite cell response (McKay et al., 2013), and IL-6⁻ mice had attenuated STAT3 signalling and impaired satellite cells *in vivo* and *in vitro* (Serrano et al., 2008). Given the effects of IL-6 on myoblasts and elevated resting levels of IL-6 in children with CF, examining the effects of systemic factors from children with CF on myoblasts JAK/STAT3 pathway is of particular interest.

1.5.3. Anti-inflammatory effects of exercise on myogenesis

In healthy individuals, an acute bout of exercise will trigger a short-term and transient systemic inflammatory response. This is seen with increases in systemic leukocytes and pro-inflammatory cytokines such as C-reactive protein (CPR), TNF-

α and IL-6 (Kasapis & Thompson, 2005). The inflammatory response is followed by a long-term anti-inflammatory effect (Kasapis & Thompson, 2005) thought to be an adaptive mechanism against inflammation (de Lemos et al., 2012). One proposed mechanism of anti-inflammatory effects of exercise is the increase production of anti-inflammatory cytokines, some of which originate from skeletal muscle (Petersen & Pedersen, 2005; Pedersen & Febbraio, 2008; Gleeson et al., 2011). IL-6 is one of the first cytokines to make a systemic appearance during exercise (Pedersen et al., 2008), with increases correlating with muscle mass in the exercise activity, as well as, with mode, duration, and intensity (Golbidi et al., 2012). Although traditionally known as a pro-inflammatory cytokine, the ability of IL-6 to i) inhibit or down regulate the production of pro-inflammatory cytokines TNF- α and IL-1 (Schindler et al., 1990; Xing et al., 1998), ii) inhibit lipopolysaccharide (LPS)-induced production of TNF- α from monocytes in cell culture (Schindler et al., 1990) and in humans *in vivo* (Starkie et al., 2003), iii) inhibit neutrophil accumulation during inflammation (Xing et al., 1998), and iv) stimulate the production of anti-inflammatory cytokine IL-10 (Steensberg et al., 2003) highlights its anti-inflammatory properties.

IL-10 is a potent anti-inflammatory mediator that acts to down regulate or prevent the production of an array of pro-inflammatory cytokines such as IL-1 and TNF- α resulting in the incapacitation of T cells to sustain an inflammatory response (Moore et al., 2001; Hong-Brown et al., 2003; Hong et al., 2009). At the level of the muscle, IL-10 promotes muscle growth and regeneration by activating

macrophages, which is concomitantly associated with an increase in myoblast proliferation without affecting differentiation (Deng et al., 2012). IL-10 also acts to restore myogenesis from TNF- α and interleukin 1 beta (IL-1 β) inhibitory effects of myogenesis (Strle et al., 2007; Strle et al., 2008). These findings would suggest that the systemic anti-inflammatory response to exercise might have positive effects on skeletal muscle development or at least provide protection from inflammatory insults. As such, examining whether systemic factors can protect myoblasts from the negative effects of an inflammatory mediator was of particular interest in this thesis.

1.6 The use of C2C12 in investigating skeletal muscle development

There are various methods used to study skeletal muscle growth and development with exercise. Among these are *in vivo* methods, in which organisms are studied as a whole. *In vivo* studies includes hypertrophy analyses as measured by MRI (Mitchell et al., 2014) or taking muscle biopsies followed by immunofluorescent staining analyses to determine satellite cell expansion (Bellamy et al., 2014) or fibre type (Joanisse et al., 2013). There are also *in vitro* studies that refer to the use of cell culture. Cells used for cell culture studies can be primary cells or a cell line. Primary cells are isolated from muscle biopsies, and are then cultured. Researchers can either obtained the muscle biopsies and isolate the primary cells independently (Conboy et al., 2005; Johnston et al., 2010), or purchase these primary cells from various companies (Lonza, 2015).

Given the aim of this thesis is to investigate the effects of systemic factors on skeletal muscle development, an *in vitro* method was more appropriate as it would allow for the direct exposure of systemic factors to skeletal muscle cells. The benefit of using a cell line instead of primary cells is apparent due to the difficulties in obtaining pediatric muscle biopsies. Purchasing pediatric primary cells was also not a viable option due to the cost and difficulties with growing and maintaining primary human cells.

Ideally, the use of a human skeletal muscle cells line would be more applicable given the use of pediatric human systemic factors for this thesis. However, human cell line are known to be more difficult to work with than a rat or mice cell line, as such, it was more feasible to use a murine line for our experiments. The findings of this dissertation can provide information for future work that involves human cells.

C2C12 cells are myoblasts derived from satellite cells that have the capacity to proliferate and differentiate, making them ideal for studying various stages of skeletal muscle development. C2C12 myoblasts are the subclone of the C2 myoblasts that originate from the skeletal muscle of C3H mice; these myoblasts have been immortalized and can proliferate indefinitely (Yaffe & Saxel, 1977; Burattini et al., 2004). The benefits of using the C2C12 cell line include the fact that they are relatively easy to maintain and grow, and they are less costly than other cell lines. In addition, the C2C12 cell line is well characterized (Madison et al., 2014), and behaves much like their progenitor lineage (Burattini et al., 2004), which

may reflect the type of myogenesis observed during normal growth and development in children. In addition, C2C12 myoblasts are responsive to human growth factors (Frost et al., 2002; Sadowski et al., 2001), which suggest that C2C12 myoblasts would be responsive to human systemic factors. A potential alternative model also used in skeletal muscle growth and regeneration studies is the cell line L6 (rat myoblasts) (Chromiak et al., 1991). The C2C12 myoblasts were selected for this thesis based on knowledge and significant expertise amassed using this cell line by our collaborators (Dr. Gianni Parise's laboratory: Johnston et al., 2010; Johnston, et al., 2011).

Although there are a number of strengths associated with using the C2C12 myoblasts, the limitations of using this cell line to examine the effects of human systemic environments on skeletal muscle development must also be acknowledged. First, it is important to consider differences in the homology of systemic protein from humans and the corresponding mouse receptors. Second, while skeletal muscle cell lines are composed of what were once primary cells taken from skeletal muscle, they have since been altered to allow for indefinite growth and proliferation (e.g., immortalized). This comes at the cost of cell lines being genetically abnormal, and maintaining these cell lines in an artificial environment for prolonged period of time may, in turn, alter their function (Owens, et al., 2013). This may limit the application of our *in vitro* results to an *in vivo* model. Given the primary ethical limitation of obtaining pediatric muscle samples, the use of the C2C12 cell line in this thesis represents an important first step towards

understanding how resting and exercise-induced systemic environments from children can affect skeletal muscle development.

1.7 Objectives and Hypotheses

To overcome the ethical limitations of muscle biopsies in children, our laboratory has developed an *in vitro* model to elucidate muscle responses to an exercise “environment”. Serum samples, which hold systemic factors released during exercise, will be used to investigate the effects of exercise on mouse myoblasts. Although it is acknowledged that this methodology does not directly assess the local effects of exercise on muscle, it can provide us with a clearer picture of the systemic effects of exercise that have been shown to improve tissue health in mice (Safdar et al., 2011). Furthermore, an exercise-induced systemic change in the form of a cytokine response has been reported independent of a local response, which may highlight the importance of systemic factors over local factors on exercise adaptation (Nemet et al., 2002).

1.7.1. General Objective

The general objective of this thesis was to examine the effects of resting and post-exercise systemic factors from children with CF and healthy controls on skeletal muscle development *in vitro*.

1.7.2. Specific Objectives

The specific objectives of the studies in this thesis were to:

- (1) Determine the effects of systemic factors from children with CF and healthy controls on myoblast proliferation, and determine the effect of post-exercise systemic factors on proliferation *in vitro*.
- (2) Determine the effects of systemic factors from children with CF and healthy controls on myoblast differentiation, and determine the effect of post-exercise systemic factors on differentiation *in vitro*.
- (3) Determine the effect of post-exercise systemic factors from children with CF and healthy controls on differentiation of myoblasts exposed to an inflammatory mediator *in vitro*.

1.7.3. Specific Hypotheses

The specific hypotheses of the studies in this thesis were that:

- (1) Myoblasts exposed to systemic factors from children with CF will display greater degree of proliferation in comparison to those exposed to systemic factors from healthy controls. Post-exercise systemic factors will cause greater myoblasts proliferation compared with systemic factors at rest (Chapter 2);
- (2) Myoblasts exposed to systemic factors from children with CF will have lower differentiation compared with myoblasts exposed to systemic factors from healthy controls. Post-exercise systemic factors will reduce differentiation in myoblasts in comparison to systemic factors at rest (Chapter 3);
- (3) Myoblasts exposed to post-exercise systemic factors from children with CF will not blunt the effects of an inflammatory mediator on myoblasts

differentiation; conversely, the effects of an inflammatory mediator on myoblasts differentiation will be blunted with exposure to post-exercise systemic factors from healthy controls (Chapter 4).

CHAPTER 2

The effects of resting and exercise serum from children with cystic fibrosis on C2C12 myoblast proliferation *in vitro*

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Dr. Brian Timmons, Dr. Sandeep Raha, Jeff Baker, and I contributed to the design of the study. I was responsible for participant recruitment and blood sample collection, with assistance from Joyce Obeid. Dr. Linda Pedder provided support for recruitment of patients with cystic fibrosis. Dr. Gianni Parise provided the laboratory, equipment, and cells. I completed data collection, with assistance from Jeff Baker. I was responsible for the analysis of the data presented. Joyce Obeid assisted with statistical analyses. I drafted the manuscript, with support from Dr. Brian Timmons, Jeff Baker, Joyce Obeid, Dr. Sandeep Raha, Dr. Gianni Parise, and Dr. Linda Pedder.

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The effects of resting and exercise serum from children with cystic fibrosis on C2C12 myoblast proliferation in vitro

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Abstract

Chronic systemic inflammation is a clinical symptom in children with cystic fibrosis (CF), but the effects on skeletal muscle development are unknown. The aims of this study were to determine (1) the effects of systemic factors from children with CF and healthy controls on myoblast proliferation, and (2) whether exercise serum can have an effect on proliferation in vitro. Eleven children with CF and 11 biological age-matched controls completed two 30-min bouts of cycling at an intensity set at 50% peak mechanical power. Serum samples were collected before exercise (REST), immediately following exercise (EX), and after 60 min of recovery (REC). Serum samples prepared in group-specific pools were used for cell culture experiments. C2C12 myoblasts were incubated in 5% serum and media for 1 h and then immediately harvested for protein and mRNA analysis, or incubated in growth media for 2 days to examine proliferation. C2C12 myoblasts treated with CF serum displayed greater proliferation phenotype than myoblasts treated with control serum. Proliferation did not change with EX or REC serum from children with CF compared to CF REST serum, while proliferation was increased with EX and REC serum from control compared to control REST serum. These findings suggest that systemic factors from children with CF at rest and after exercise can alter myoblast proliferation responses when compared to systemic factors from healthy children, which may have implications on skeletal muscle development.

Introduction

Children with cystic fibrosis (CF) suffer from chronic systemic inflammation (Tirakitsoontorn et al. 2001; Nguyen et al. 2012) that may negatively impact whole body skeletal muscle mass via increased protein catabolism (van Heeckeren et al. 2000; Ionescu et al. 2002, 2004). In patients with CF, those who have increased protein breakdown as measured by urinary pseudouridine, tend

to have increased IL-6 and TNF- α (Ionescu et al. 2002), and lower fat-free mass (Ionescu et al. 2002, 2004). This systemic inflammation observed in patients with CF may stem from chronic pulmonary infection (Valletta et al. 1992; van Heeckeren et al. 2000). Indeed, mice infected with *Pseudomonas aeruginosa* localized in the lungs have increased pulmonary inflammation in the days following infection. This was mirrored by an increase in the inflammatory cytokine, IL-6, whereby the concentration of IL-6

observed in the lungs correlated with the amount of weight loss observed in these infected mice, with evidence of reduced skeletal muscle leg mass (van Heeckeren *et al.* 2000). Collectively, the available evidence suggests that factors in the systemic circulation may negatively affect whole body skeletal muscle development. However, few studies have addressed this important issue in children with CF by studying the specific effects of the systemic environment on molecular and signaling pathways of skeletal muscle development.

Systemic inflammatory profiles can be transiently altered with acute bouts of exercise, even in children with CF (Nguyen *et al.* 2012). Often an increase in inflammatory cytokines is observed with exercise; however, an exercise-induced increase in IL-6, for example, is viewed by many as being anti-inflammatory (Petersen and Pedersen 2005). Given that chronic levels of systemic inflammation may have a negative impact on whole body skeletal muscle, but that specific episodes of exercise may create an anti-inflammatory systemic environment, we wanted to examine the effects of these different systemic environments on indices of skeletal muscle development. Specifically, we wanted to compare the effects of serum from healthy children with serum from children with CF, in rest, exercise, and recovery conditions, on markers of skeletal muscle development.

Since skeletal muscle from children is difficult to obtain for ethical reasons, we used the well-established C2C12 skeletal muscle murine cell line as the target cells to examine the effects of systemic environments on indices of muscle development. C2C12 cells are a subclone of myoblasts from muscle in the leg of a C3H mouse (Yaffe and Saxel 1977; Burattini *et al.* 2004) and are responsive to human factors (Sadowski *et al.* 2001; Frost *et al.* 2002). Muscle development encompasses several stages, including proliferation, differentiation, and fusion. We examined aspects of myoblast proliferation as it represents the early stages of muscle development (Peault *et al.* 2007). Specifically, we determined how different systemic environments affected proliferation signaling pathways and gene expression, and whether these molecular changes translated into an altered phenotype of proliferation, as measured by an increased number of cells. To provide additional insight into the regulation of skeletal muscle development, we also examined markers of differentiation. Since inflammation can induce greater C2C12 myoblasts proliferation at the cost of reduced differentiation into myotubes (Dogra *et al.* 2006), we hypothesized that compared with healthy controls, serum from children with CF would induce greater signaling of proliferation pathways due to their chronic systemic inflammation (Nguyen *et al.* 2012), resulting in a greater proliferation phenotype. Moreover, given that IL-6 is associated with

increasing myoblast proliferation (Toth *et al.* 2011) and exercise is known to increase circulating IL-6 (Nguyen *et al.* 2012), we hypothesized that changes in the systemic environment induced by a specific episode of exercise would enhance proliferation.

Methods

Participants

Participants' characteristics are shown in Table 1. Eleven children with CF (two females) with complete blood samples were included in this study. Patients were recruited from the Cystic Fibrosis Clinic at the McMaster Children's Hospital (Hamilton, Ontario, Canada). Children with CF who could not perform reproducible pulmonary function tests were excluded from the study. Six patients with CF were taking nonsteroidal anti-inflammatory drugs (NSAIDs) and/or inhaled or nasal spray corticosteroids. One participant was diagnosed with cirrhosis of the liver. Eleven sex- and biological age-matched (Mirwald *et al.* 2002) healthy control children were recruited from the surrounding community. Healthy control children were included in the study only if they had no known physical, mental, metabolic, and inflammatory diseases. All parents/guardians and children provided written informed consent and assent, respectively, prior to enrollment in this study, which was approved by the Hamilton Health Science/Faculty of Health Science Research Ethics Board, with consent and assent forms signed by parents/guardian and children, respectively.

Table 1. Participants' characteristics.

	CF (n = 11)	Matched controls (n = 11)	P value
Age (years)	14.4 ± 2.2	13.9 ± 2.2	0.59
Estimated years from predicted age of PHV	0.56 ± 1.71	0.72 ± 1.57	0.83
FEV ₁ (% predicted)	90.3 ± 22.0	93.4 ± 7.4	0.73
Height (m)	1.62 ± 0.13	1.68 ± 0.13	0.33
Height (% ₁₀₀)	45.5 ± 22.3 ¹	73.7 ± 29.6	0.02
Weight (kg)	49.5 ± 11.9	54.6 ± 9.4	0.27
Weight (% ₁₀₀)	37.2 ± 18.1 ¹	62.2 ± 25.2	0.02
BMI (% ₁₀₀)	35.6 ± 19.4	48.9 ± 23.2	0.16
% Body fat	20.0 ± 4.8	19.2 ± 9.6 ²	0.79
FFM (kg)	39.8 ± 10.2	44.3 ± 9.7 ²	0.31

Values are expressed in mean ± SD. PHV, peak height velocity; FEV₁, forced expiratory volume in 1 sec; %₁₀₀, percentile; BMI, body mass index; FFM, fat-free mass.

¹Significant difference between groups.

²n = 10 for age-matched controls.

Exercise and blood sampling

Participants completed two visits. During the first visit, anthropometric variables (height, weight, body composition using bioelectrical impedance analysis) and FEV_1 were measured along with peak mechanical power (PMP), assessed using the *McMaster All-Out Progressive Continuous Cycling Test* on a cycle ergometer (Fleisch-Metabo, Geneva, Switzerland). Height, weight, and body mass index (BMI) percentiles were calculated using reference values of weight-for-age and stature-for-age from the Centers for Disease Control and Prevention (2009). Fat-free mass (FFM) was calculated using an age-specific BIA equation from Schaefer et al. (1994). Percent body fat was calculated as $([Body\ weight - FFM]/body\ weight) \times 100$. Reference data for FEV_1 were obtained from Wang et al. (1993) and were used to calculate percent predicted.

The second visit was scheduled a minimum of 2–3 days after the first. Participants were asked to refrain from consuming any food or liquid, with the exception of water, 3 h prior to the visit. They also refrained from participating in any strenuous physical activity for at least 24 h before the visit. The second visit consisted of 2 \times 30-min bouts of cycling at a constant pace of ~60 rpm and an intensity equivalent to 50% PMP. We chose to study the effects of 60 min of moderate-intensity exercise as this reflects the internationally accepted recommendation for daily physical activity for children (Tremblay et al. 2011; World Health Organization 2013). Blood samples were collected using an in-dwelling catheter placed in the antecubital region of the arm. Blood samples were collected before exercise (REST), at the end of the 2 \times 30-min bouts of cycling (EX) and after 60 min of recovery (REC). Blood was collected into vacutainers that were either placed on ice (plasma) or allowed to clot for 30 min at room temperature (serum). Samples were then centrifuged for 20 min at 2000g and 4°C. All plasma and serum samples were aliquoted and stored at –80°C for future analysis. Plasma samples were analyzed for the inflammatory cytokine IL-6 and serum samples were used for cell culture experiments.

Cell culture experiments

To provide a sufficient volume of serum to execute the proposed cell culture experiments, pooling of serum was necessary (i.e., the amount of blood required from one child surpassed what we considered to be ethically justified). Hence, we prepared group-specific pools of serum (i.e., CF and healthy controls), representing equal volume from each individual within a group, for each of the REST, EX, and REC time points.

C2C12 myoblasts

C2C12 myoblast cell line was purchased from American Type Culture Collection (Rockville, MD). Myoblasts were grown on 100-mm cell culture dishes in growth media (GM) consisting of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin and incubated at 37°C in 5% CO₂.

Protein and gene expression

Cells at passage 8 were grown until 70% confluence was reached at which point the cells were used in the experimental setup. For treatment, GM was removed and the cells were washed with PBS. Cells were then given 7 mL of treatment consisting of DMEM supplemented with 5% serum from children with CF or from healthy controls, with each time point represented (i.e., REST, EX and REC) and 1% penicillin–streptomycin. Cells were incubated at 37°C in 5% CO₂ for 1 h, after which treatment media was removed, cells were washed with PBS, and then harvested with 1 mL of TRIzol® Reagent (Life Technologies, Burlington, Ontario, Canada). Plates were placed on ice and samples were collected and aliquoted into 2-mL tubes. Samples were vortexed and stored at –80°C for further analyses.

Protein isolation

Samples in TRIzol reagent were thawed and treated with 0.2 mL of chloroform, shaken for 15 sec, incubated at room temperature for 5 min, and centrifuged at 12,000g and 4°C for 15 min. The upper aqueous phase was placed in 2.0-mL tubes and stored at –80°C for RNA isolation. The interphase was discarded and the phenol–chloroform phase was placed in a 2.0-mL tube for protein isolation. Protein isolation was completed using the protein precipitation method as per manufacturer's instruction (TRIzol reagent, Life Technologies). Protein pellets were resuspended using 200 μ L of 1% SDS with protease inhibitors (Complete mini; Protease inhibitor cocktail tablets; Roche Diagnostics, Laval, Quebec, Canada) and phosphatase inhibitors (PhosSTOP, Roche Diagnostics, Laval, Quebec, Canada). Pellets were incubated at room temperature in resuspension reagents for 20–40 min and then heated at 50°C in a heat block until completely resuspended. Samples were centrifuged at 10,000g for 10 min at 4°C and transferred to a new tube. Protein concentration was assessed using Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL), and samples stored at –20°C until further analyses.

Western blotting

We chose to study proteins involved in the JAK/STAT3 pathway, as it has been identified to result in increased C2C12 myoblast proliferation when activated (Spangenburg and Booth 2002). Specifically, STAT3 and its activated form (p-STAT3) were measured. Suppressor of cytokine signaling (SOCS3) is a key regulator of inflammation and its upregulation inhibits the JAK/STAT3 pathway (Crocker *et al.* 2008; Diao *et al.* 2009). STAT3, pSTAT3, and SOCS3 protein expressions were analyzed with actin used as a loading control. Equal amounts of protein (5 mg for STAT3; 25 mg for p-STAT3 and SOCS3) and Laemmli buffer were boiled at 95°C for 5 min. Samples were loaded on in the wells of a 12.5% gel and run at 120 V for approximately 2 h and transferred to polyvinylidene fluoride (PVDF; Millipore, Etobicoke, Canada) membranes at 120 V on ice for 1 h. Membranes were blocked with 5% nonfat powdered milk in 1x TBST at room temperature for 1 h, then incubated overnight in primary antibody (STAT3 rabbit antibody, dilution 1:2000, catalog number #4904. pSTAT3 Tyr705 rabbit antibody, dilution 1:500, catalog number #9145, Cell Signaling Technology, Boston, MA; SOCS3 rabbit antibody, dilution 1:500, catalog number #ab16030, Abcam Inc., Cambridge, MA; Actin rabbit antibody, dilution 1:1000, catalog number # A2066. Sigma-Aldrich Co., St. Louis, MO) in either 5% BSA (BSA, Santa Cruz Biotechnology, Santa Cruz, CA) in 1x TBST for STAT3, p-STAT3, SOCS3 or 5% nonfat powdered milk for actin at 4°C. Following multiple washes with 1x TBST, blots were incubated in goat anti-rabbit HRP (dilution 1:2000, catalog number #7074, Cell Signaling Technology) in 5% nonfat powdered milk in 1x TBST for 60 min at room temperature. Following multiple washes with 1x TBST, proteins were detected with ECL (SuperSignal West Dura, Thermo Fisher Scientific) using FluorChem SP (Alpha Innotech Corporation, San Leandro, CA). Protein bands corresponding to the predicted molecular weight of STAT3 and p-STAT3 (94 kDa), SOCS3 (27 kDa), and actin (42 kDa) were quantified using the FluorChem SP Software with background correction.

RNA isolation and reverse transcription

Ribonucleic acid was isolated with E.Z.N.A Total RNA Kit I (Omega Bio-Tek, Norcross, GA) using the previously isolated upper aqueous phase. Total RNA isolation was carried out using the Omega protocol. RNA was transcribed to 500 ng of cDNA using the Applied Biosciences High Capacity cDNA reverse Transcription Kit (Applied Bioscience, Foster City, CA) and the Eppendorf Mastercycler epigradient thermal cycler (Eppendorf, Mississauga, Ontario, Canada).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using SYBR Green PCR master mix (AP Applied Biosystems, Warrington, UK) and 25- μ L reactions. Primers were custom made and purchased from the MOBIX Lab (DNA sequencing and Oligo Synthesis Facility, McMaster University, Hamilton, Canada). Markers of proliferation measured were SOCS3 and Pax7. Pax7 was measured as it is expressed by proliferating myoblasts (Buckingham 2007). In addition to proliferation markers, a differentiation marker was measured to gain additional insight into aspects of muscle development. The differentiation marker measured was myogenin, which is an early marker of differentiation that signifies commitment to myotube development (Andres and Walsh 1996). The primer sequences used are shown in Table 2. GAPDH was used as the housekeeping gene. Primers were reconstituted using 1x TE buffer of pH 8.0 to make 100 μ mol/L and stored at -20°C until further analyses. In PCR 0.2-mL tubes (Axygen Inc., Union City, CA), 12.5 μ L of SYBR green, 2 μ L forward primer, 2 μ L reverse primer, 7.5 μ L of H_2O , and 1 μ L (25 ng) of cDNA were combined to give a total volume of 25 μ L for all mRNA markers except GAPDH. For GAPDH, 6.5 μ L of H_2O and 2 μ L (50 ng) of cDNA was used instead. qRT-PCR was performed using a Eppendorf Mastercycler ep real-plex² real-time PCR system (Eppendorf). GAPDH

Table 2. Primer sequences.

Gene name	Forward sequence	Reverse sequence
SOCS3	5'-TGCAGGAGAGCGGATTCTAC-3'	5'-TGACGCTCAACGTGAAGAAG-3'
Pax 7	5'-GCTACCACTACAGCCAGTATG-3'	5'-GTCACCTAAGCATGGGTAGATG-3'
myogenin	5'-CTACAGGCGCTTGCTCAGCTC	5'-AGATTGTGGCGCTCTGTAGG-3'
GAPDH	5'-TGCACCACTCACTGCTTAG	5'-GGATGACAGGATGATGTTTC-3'

expression was not different between conditions. Changes in gene expression over time were expressed as fold change from REST values, using the $\Delta\Delta CT$ method (Schmittgen and Livak 2008). ΔCT values were used for statistical analyses.

Proliferation phenotype experiment using human serum

To determine the effects of systemic factors on C2C12 myoblasts proliferation at a phenotypic level, passage 8 cells were seeded in cell-treated 96-well plates at a concentration of 1000 cells per well in 100 μ L GM. Plates were incubated at 37°C in 5% CO₂ environment for 24 h to allow myoblasts to adhere and grow. GM was removed and cells were treated with 200 μ L of treatment media consisting of DMEM supplemented with 5% serum from children with CF or from healthy controls, with each time point represented (i.e., REST, EX, and REC) and 1% penicillin–streptomycin. Treated plates were incubated at 37°C in 5% CO₂ for 1 h, after which serum was removed and replaced with GM. Plates were then incubated at 37°C in 5% CO₂ for 2 days. After 2 days of incubation, plates were washed with 200 μ L of PBS, then treated with 2% PFA at a volume of 100 μ L, and incubated at room temperature for 30 min. Plates were then washed with 200 μ L of PBS, treated with 50 μ L of DAPI, and incubated for 10 min in the dark. Plates were washed with 200 μ L of PBS, air dried, and stored at 4°C until analysis. The number of nuclei was assessed using a Nikon Eclipse Ti (Nikon Instrument Inc. Melville, NY) at 10 \times magnification. Five random fields of view were captured from each well and the number of nuclei was determined using the NIS-Element AR 3.2 64-bit (Nikon Instrument Inc.) program. The sum of the nuclei from all five photographs was used to represent the total number of nuclei for each well.

Plasma analysis

Given the possible implication of IL-6's involvement in muscle proliferation (Toth *et al.* 2011) plasma samples were analyzed for IL-6 using enzyme-linked immunosorbent assays (ELISA) from R&D systems (Human IL-6 Quantikine HS ELISA kits, Minneapolis, MN). All exercise concentrations were corrected for changes in plasma volume (Dill and Costill 1974).

Statistical analyses

Statistical analyses were completed using SPSS version 17.0 (PASW Statistics version 17.0, SPSS Inc., Chicago, IL), unless otherwise stated. Data were tested for normal-

ity using Shapiro–Wilk test. All variables were normally distributed except for height percentile and IL-6. To determine differences in participants' characteristics, an independent *T*-test was used for normally distributed data and the Mann–Whitney *U*-test was used for non-normally distributed data. To determine differences between groups in protein expression, gene expression and proliferation phenotype and signaling two-way repeated measure ANOVAs were performed. The two factors examined were group (CF, Control) \times time (REST, EX, REC). To determine the effects of exercise, one-way repeated measure ANOVA was performed separately for children with CF and controls. Tukey's HSD post hoc analyses were performed to test for differences between means. All ANOVA analyses were completed using STATISTICA version 5.0 (StatSoft Inc., Tulsa, OK). To determine difference between groups in plasma IL-6, the Mann–Whitney *U*-test was used for each time point (i.e., REST, EX, and REC). To determine the effects of exercise on plasma IL-6 concentration, Kruskal–Wallis *H* test with Wilcoxon post hoc was used. Values are expressed as mean \pm SD unless stated otherwise. Significance was set at $P \leq 0.05$.

Results

Protein expression

There was no significant difference in STAT3, p-STAT3, or SOCS3 between C2C12 myoblasts treated with serum from children with CF or healthy controls (Fig. 1). There was no exercise effect observed with any of the protein markers in C2C12 myoblasts treated with serum from children with CF, although a trend toward a decrease in STAT3 at REC compared to REST was observed ($P = 0.062$). An exercise effect was observed for STAT3 and p-STAT3 expression in C2C12 myoblasts treated with healthy serum; STAT3 decreased with EX ($P < 0.01$) and REC ($P < 0.05$) compared to REST. Expression of p-STAT3 decreased in C1C12 myoblasts treated with healthy serum at REC ($P < 0.05$) compared with REST, while EX had no effect of p-STAT3.

mRNA expression

Similar gene expression of SOCS3, Pax7, and myogenin were observed in C2C12 myoblasts treated with serum from children with CF or healthy controls (Fig. 2). In C2C12 myoblasts treated with serum from children with CF, a trend for an exercise effect for SOCS3 was observed (one-way ANOVA, time effect, $P = 0.065$). In addition, a reduction in gene expression of SOCS3 at REC from REST ($P = 0.058$) (Fig. 2A) was observed. No exercise effect was observed in C2C12 myoblasts treated with

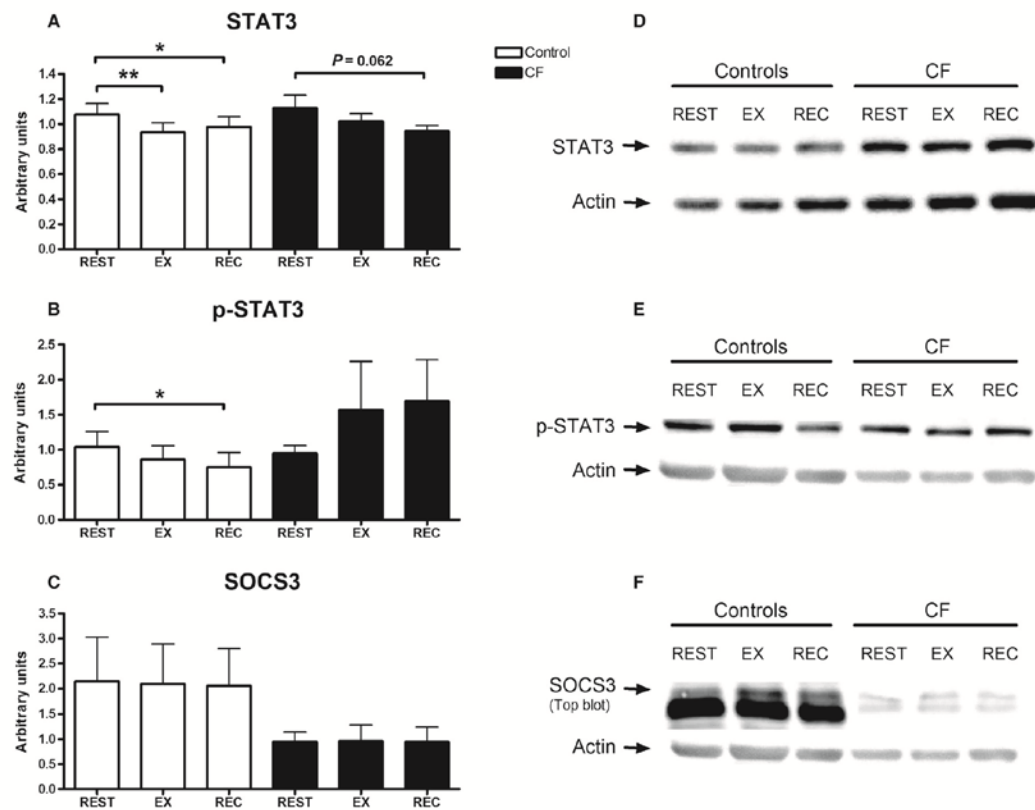


Figure 1. (A) STAT3, (B) STAT3 phosphorylation (p-STAT3), and (C) SOCS3 expression in myoblasts treated with CF or healthy serum. (D) STAT3, (E) p-STAT3, and (F) SOCS3 western blots. Myoblasts were treated for 1 h and harvested immediately after. Data are expressed in mean \pm SEM normalized to actin. STAT3: $n = 8$ for each condition; p-STAT3: control, $n = 7$, CF, $n = 7$; SOCS3: control, $n = 7$, CF, $n = 8$. There were no differences between groups. Significant difference from REST: * $P < 0.05$, ** $P < 0.001$. A trend was observed with STAT3 in C2C12 myoblasts treated with CF serum ($P = 0.062$).

serum from either children with CF or healthy controls for myogenin or Pax7.

Proliferation

Proliferation of C2C12 myoblasts treated with serum from children with CF was greater than with healthy controls (main effect for group, $P < 0.001$) (Fig. 3) 2 days after treatment. C2C12 myoblasts treated with serum from children with CF showed greater proliferation at REST ($P < 0.001$) and at EX ($P < 0.001$) compared to healthy controls, but proliferation was similar at REC (Figs. 4, 5). Exercise serum from children with CF had no effect on C2C12 proliferation. Exercise serum from healthy controls increased proliferation in C2C12 with values higher at EX ($P < 0.001$) and REC ($P < 0.01$) compared to REST.

Plasma IL-6

There was a significant difference in plasma IL-6 between groups at each time point (Fig. 6). Exercise tended to increase IL-6 in the children with CF, but this did not reach statistical significance ($P = 0.066$). Exercise increased IL-6 in the healthy controls ($P < 0.01$) and remained elevated at REC ($P < 0.01$).

Discussion

C2C12 myoblasts proliferated to a greater extent when treated with serum from children with CF compared to healthy control serum. One reason for this difference may be related to the higher concentration of IL-6 observed in our CF group, as IL-6 is known to induce myoblast proliferation via the JAK/STAT3 pathway (Toth et al. 2011).

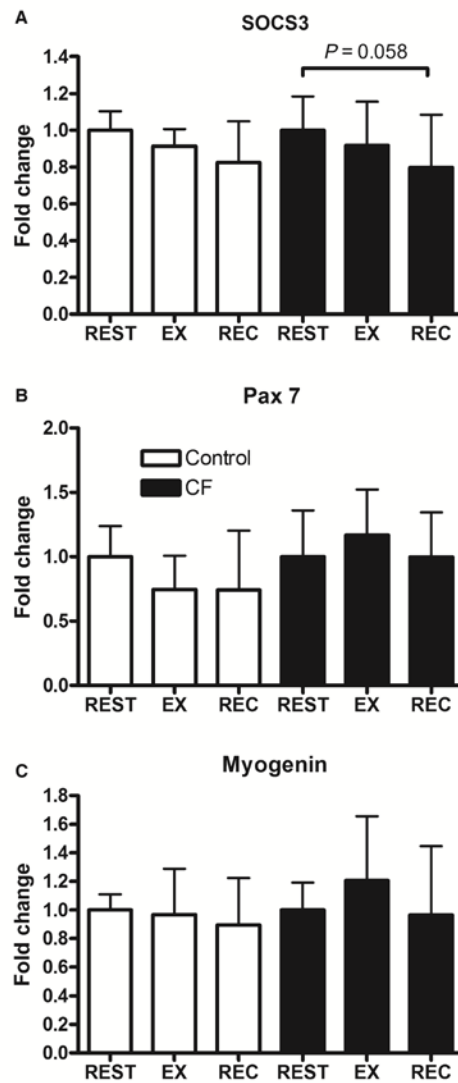
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Figure 2. Gene expression expressed as fold change \pm SD from REST for (A) SOCS3, (B) Pax7, and (C) myogenin in C2C12 myoblasts treated with CF serum or control serum. Myoblasts were treated for 1 h and harvested immediately after. C2C12 myoblasts were treated with serum from REST: before exercise, EX: after 60 min of cycling, REC: after 60 min of recovery. $n = 3$ for each condition. In C2C12 myoblasts treated with CF serum a trend ($P = 0.058$) toward reduced SOCS3 gene expression at REC from REST was observed.

According to the literature, other systemic factors may be playing a role in myoblast proliferation. IL-1 has the capacity to induce greater myoblast proliferation (Ots

CF Serum and Myoblast Proliferation

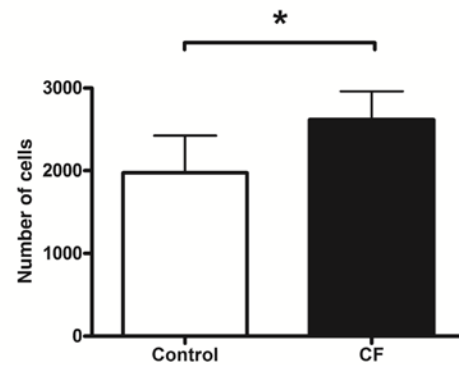


Figure 3. Myoblast proliferation. Average number of C2C12 cells treated with either CF or control serum. Data are expressed as mean \pm SD. Wells were seeded with 1000 myoblasts in 100- μ L growth media and allowed to adhere for 24 h. Myoblasts were then treated for 1 h with treatment media and allowed to proliferate in growth media for 2 days. *Significant difference between groups, $P < 0.001$.

et al. 2014) and while we did not measure IL-1 in our participants other studies have reported higher systemic levels in patients with CF (Greally et al. 1993). Therefore, although a higher concentration of IL-6 in our CF serum may be responsible for inducing greater proliferation, other inflammatory mediators may also be involved.

While some mediators can induce greater myoblast proliferation, others can inhibit proliferation. Reduced oxidative stress has been shown to increase myoblast proliferation (Zaccagnini et al. 2007), suggesting that high oxidative stress would result in lower myoblast proliferation. In addition, NSAIDs (Mikkelsen et al. 1985) can inhibit myoblast proliferation and corticosteroid (te Pas et al. 2000) can reduce the rate of proliferation during the early stages of proliferation. We did not measure oxidative stress in our CF patients, however, increased oxidative stress is observed in CF patients compared to healthy subjects (Reid et al. 2007). In addition, six of our patients with CF were on NSAIDs and/or inhaled or nasal spray corticosteroids, and the effects of NSAIDs were not accounted for in our study. Despite the known effects of oxidative stress, NSAIDs and corticosteroids on reducing proliferation, our myoblasts treated with CF had greater proliferation. Pooling our samples may have diluted the concentrations of NSAIDs and corticosteroids and reduced the inhibitory effects on proliferation. Another scenario may be that the systemic factors that stimulate myoblast proliferation were much more potent than the inhibitory effects of oxidative stress, NSAIDs, and corticosteroids.

The effects of exercise on circulating inflammatory cytokines in children with and without CF ($n = 12$ for

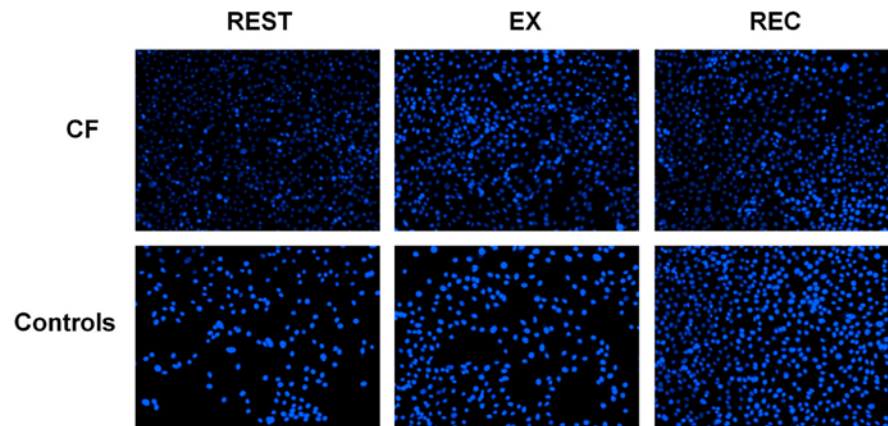


Figure 4. Photographs of myoblasts treated with serum from children with CF or healthy controls. Wells were seeded with 1000 myoblasts in 100- μ L growth media and allowed to adhere for 24 h. Myoblasts were then treated for 1 h with treatment media and allowed to proliferate in growth media for 2 days. Myoblasts nuclei were stained with DAPI. REST: before exercise, EX: after 60 min of cycling, REC: after 60 min of recovery. $n = 12$ for each conditions.

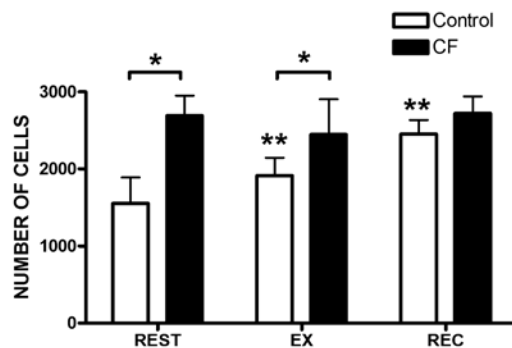


Figure 5. Effects of serum from children with CF and healthy controls on myoblast proliferation. Wells were seeded with 1000 myoblasts in 100- μ L growth media and allowed to adhere for 24 h. Myoblasts were then treated with treatment media for 1 h and allowed to proliferate in growth media for 2 days. Mean \pm SD number of nuclei. REST: before exercise, EX: after 60 min of cycling, REC: after 60 min of recovery. $n = 12$ for each conditions. *Significant difference between groups, $P < 0.001$. **Significant difference compared to REST, $P < 0.01$.

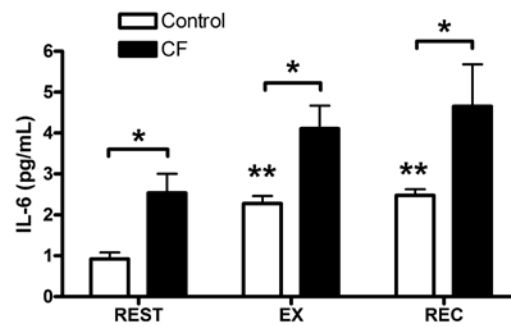


Figure 6. Effects of exercise on systemic IL-6 in children with CF and controls. Data are expressed in mean \pm SEM. REST: before exercise, EX: after 60 min of cycling, REC: after 60 min of recovery. $n = 11$ for each condition. *Significant difference between groups, $P < 0.05$. **Significant difference compared to REST, $P < 0.01$.

each group) from this study has been published elsewhere (Nguyen et al. 2012). While there were no exercise-related changes in proliferation for myoblast treated with CF serum, differences were observed with healthy control serum. More specifically, the EX and REC serum from controls caused greater proliferation compared to REST serum. Given that IL-6 concentrations were higher at these time points in healthy controls only, it is plausible

that the proliferative property of IL-6 may also explain increased C2C12 myoblasts proliferation in this group. Conversely, the lack of increase in myoblast proliferation with EX and REC serum from children with CF may be attributed to a ceiling effect. Systemic concentrations of IL-6 were already much higher in the CF group at REST, and did not substantially increase at EX or REC. Additional experiments are required to determine the true role of IL-6 in myoblast proliferation in this context. However, our data are novel as they highlight that serum from patients suffering from a systemic inflammatory disease can cause greater C2C12 myoblast proliferation. Furthermore, EX and REC serum from children with CF does

not alter this proliferative response, while EX and REC serum from healthy controls enhances it.

In this study, C2C12 myoblasts proliferated to a similar extent when exposed to serum from children with CF (either with REST, EX, or REC) and REC serum from healthy controls. This suggests that in healthy children, the systemic environment created during the immediate recovery period following exercise induces similar effects on C2C12 myoblast proliferation as CF serum. An increase in myoblast proliferation is required for differentiation (Thomas *et al.* 2000); however, an increase in proliferation in C2C12 myoblasts when treated with an inflammatory stimulus results in reduced capacity to differentiate (Dogra *et al.* 2006). In children with CF, given their chronic inflammatory state (Tirakitsoontorn *et al.* 2001; Nguyen *et al.* 2012), it is conceivable that the increased proliferation observed with CF serum would be conducive to impaired differentiation leading to impaired muscle development. Indeed, young rats given chronic IL-6 exposure experienced 13% reduced muscle growth (Bodell *et al.* 2009). However, it is difficult to speculate as to whether the observed increase in proliferation with recovery serum from healthy children is conducive to greater or impaired muscle development given that (1) the levels of IL-6 observed in healthy children were significantly lower than the CF group at all time points including at the recovery time point, (2) the elevated inflammatory state is acute, and (3) the inflammatory state was induced by exercise and not by a chronic infection or disease. Thus, to further investigate the effects of increased proliferation observed on muscle development, examining the effects of myoblast differentiation after serum exposure would be the next logical step. Although in our study, mRNA markers of proliferation and differentiation (Pax7, SOCS3, and myogenin) were unaffected by exposure to serum, further work should measure differentiation phenotype (e.g., myoblast fusion index) of C2C12 myoblasts exposed to different systemic environments. On the basis of our data, we would expect C2C12 differentiation to decrease upon exposure to serum from children with CF.

Systemic factors from healthy children following exercise decreased protein signaling involved in C2C12 myoblast proliferation. Specifically, STAT3 and p-STAT3 decreased in myoblasts treated with EX and REC serum from healthy controls, compared to REST serum. We expected these results to translate into less proliferation with myoblasts treated with EX and REC serum in our phenotype experiments. However, we observed the opposite effect with an increase in proliferation. These discordant findings may be due to timing, since protein samples were collected immediately following serum treatment, while the phenotype experiments were performed

on samples collected 2 days after serum treatment. Our results may suggest that either (1) systemic factors promoted an acute reduction in proliferation signaling in C2C12 myoblasts, with a later increase in proliferation phenotype; or (2) the JAK/STAT3 pathway is not responsible for the increased proliferation phenotype observed.

Our study focused on comparing the effects of systemic factors in children with CF and healthy controls by using a common target tissue (C2C12 myoblasts). We acknowledge that the use of human serum on a mouse cell line may limit the application of our results since we investigated the effects of systemic factors from one species on another species' tissue. Because the use of human serum on tissue is a novel approach, we sought to insure the use of serum on a tissue was feasible and that differences would be apparent. We used the C2C12 cell line because relative to human primary or human cell lines C2C12 myoblasts are more proficient in growth, proliferation, and relatively inexpensive. Human cells are known to be difficult to grow, slow to proliferate, and expensive. Additionally, it is common to grow and proliferate C2C12 myoblasts using fetal bovine serum, and to differentiate using horse serum. Thus, using serum from a species other than from a mouse on C2C12 myoblasts is common practice. Given the feasibility of our study, we plan to pursue the use of a human cell line or human primary cells in the future. Furthermore, since the CFTR protein is expressed in skeletal muscle (Lamhonwah *et al.* 2010), future work should also include skeletal muscle from a CF model to provide clearer insight into the relative roles of the systemic environment and local muscle factors in skeletal muscle development in children with CF. Finally, we pooled our serum samples in order to conserve sample volume and to insure the completion of all other analyses set forth, since pediatric blood samples are difficult to obtain and standard ethical procedure inhibits the collection of blood to a certain amount. Pooling our samples is a limitation of this study.

Conclusion

We took the novel approach of exposing C2C12 myoblasts to serum obtained from children with CF and healthy controls. We found that C2C12 myoblast proliferation was greater when treated with CF serum than control serum. In addition, proliferation did not differ between REST, EX, or REC CF serum, while an exercise effect was observed in healthy controls. Protein and mRNA markers of proliferation did not increase in C2C12 myoblasts treated with serum from children with CF or healthy controls. This work highlights the ability of systemic factors from patients with an inflammatory disease to alter aspects of skeletal muscle development.

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Conflict of Interest

None declared.

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CHAPTER 3

Serum from children with CF alters C2C12 Myoblast Proliferation and Differentiation *in vitro*

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Dr. Brian Timmons, Dr. Sandeep Raha, Jeff Baker, Sophie Joannis and I contributed to the design of the study. I was responsible for participant recruitment and blood sample collection, with assistance from Joyce Obeid. Dr. Linda Pedder provided support for recruitment of patients with cystic fibrosis. Dr. Gianni Parise provided the laboratory, equipment, and cells. I completed data collection, with assistance from Jeff Baker and Sophie Joannis. I was responsible for the analysis of the data presented. Joyce Obeid and Sophie Joannis assisted with statistical analyses. I drafted the manuscript, with support from Dr. Brian Timmons, Jeff Baker, Sophie Joannis, Joyce Obeid, Dr. Sandeep Raha, Dr. Gianni Parise, and Dr. Linda Pedder.

Serum from children with CF alters C2C12 Myoblast Proliferation and Differentiation

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To the Editor:

The growth of patients with cystic fibrosis (CF) is a clinical concern given the negative association between degree of relative underweight and survival [1]. In healthy children, skeletal muscle mass accounts for 30-40% of body weight [2] and the greatest rate of skeletal muscle acquisition occurs during puberty [3]. However, the systemic inflammatory state observed in patients with CF, often characterized by high levels of interleukin-6 (IL-6), may have negative effects on weight manifested as reduced fat free mass, skeletal muscle or body weight *in vivo* [4-6]. Unfortunately, our understanding of the direct effects of the systemic environment from patients with CF on skeletal muscle development is lacking as a result, in part, of the ethical concerns of obtaining skeletal muscle tissue from children. Given that specific episodes of exercise can also dramatically alter the

systemic environment, including IL-6, it is unclear whether these changes are beneficial or detrimental to processes of muscle development in cases where a chronic elevation is observed such as IL-6 in CF [7], or whether systemic factors such as IL-6 is related to muscle development *in vitro*. The wide ranging benefits of exercise in patients with CF is well known and advocated [8], but a better appreciation of whether these benefits extend to the effects of systemic environment on muscle development could help optimize exercise prescription. Here, we report on the effects of serum collected at rest and during exercise from children with CF on C2C12 myoblast proliferation and differentiation, and their relationship to systemic levels of IL-6.

This study was approved by our local Research Ethics Board. Twelve children with CF (FEV₁: 90.0 ± 21.6% predicted [9]) were recruited from the Cystic Fibrosis Clinic at the McMaster Children's Hospital (Ontario, Canada), and twelve sex- and biological age-matched [10] healthy controls were included. A detailed description of study protocol, participant characteristics, and systemic inflammatory response to exercise has been previously published [7]. Briefly, participants completed 2 × 30 min bouts of cycling set at 50% of their peak mechanical power. Blood was collected at rest (REST), immediately following exercise (EX), and 1 hour after exercise (REC). Serum was used for cell experiments and plasma was used to analyze interleukin 6 (IL-6) using ELISA.

Early skeletal muscle development was examined by myoblast proliferation while late development was examined by myoblast differentiation into myotubes.

C2C12 myoblasts were purchased from American Type Culture Collection (Rockville, MD). Passage 8 cells were seeded in cell treated 96-well plates at a concentration of either 1000 cells per well for proliferation experiments or 6000 cells per well for differentiation experiments in 100 µl growth media (DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin). Plates were incubated (37°C, 5% CO₂) for 8 hours (differentiation) or 24 hours (proliferation) to allow adherence. Growth media was removed and cells were treated with 100 µl (proliferation) or 200 µl (differentiation) of treatment media consisting of DMEM supplemented with 5% REST, EX or REC serum (based on preliminary experiments) from each child with CF or control, and 1% penicillin-streptomycin. Treated plates were incubated for 1 hour, after which serum was removed and replaced with either growth media to promote proliferation or differentiation media (DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin) to promote differentiation. Plates were then incubated for 3 days for proliferation or 6 days for differentiation, after which cells were fixed and stained for nuclei with DAPI. Differentiated cells were also stained for actin using Rhodamine Phalloidin (Life Technologies, Burlington, Canada). Nikon Eclipse Ti microscope and NIS-Element AR 3.2 64-bit program (Nikon Instrument Inc., Melville, NY) were used to assess number of nuclei and myonuclei for each well. Proliferation was assessed by the total nuclei count from the proliferation experiments and differentiation was assessed using the Myonuclei Fusion Index (MFI) from the differentiation experiments. Myonuclei were identified as nuclei

residing within a myotube as defined by longitudinal actin orientation and were counted if 3 or more nuclei were present in a myotube. MFI was defined as: $MFI = (\text{total number of myonuclei} / \text{total number of nuclei}) \times 100$. Two-way repeated measures ANOVAs (Statistica 5.0, StatSoft Inc., Tulsa, OK) were used to determine group, time, and interaction effects. Spearman's correlations (PASW 17.0, SPSS Inc., Chicago IL) were used to examine relationships between IL-6 and proliferation and differentiation.

Myoblasts treated with serum from children with CF had greater proliferation (Figure 1.A-B) but less differentiation (Figure 1.C-D) compared to myoblasts treated with serum from controls. Differentiation experiments showed myoblasts treated with serum from children with CF had a similar number of total nuclei as myoblasts treated with serum from controls (total nuclei treated with serum from CF, REST: 1717 ± 177 , EX: 1801 ± 143 , REC: 1658 ± 114 , average: 1726 ± 83 , total nuclei treated with serum from controls, REST: 1884 ± 83 , EX: 1936 ± 154 , REC: 2024 ± 127 , average: 1948 ± 71). However, a lower number of myonuclei was found in myoblasts treated with serum from children with CF compared to controls (main effect for group, CF: 198 ± 47 , controls: 321 ± 38 , $p = 0.01$). No effects of exercise were observed in any of the variables. Plasma IL-6 had a positive relationship with proliferation ($\rho = 0.31$, $p = 0.008$), but a negative relationship with differentiation ($\rho = -0.26$, $p = 0.028$).

Our findings suggest that both early and late stages of skeletal muscle development are altered due to systemic factors in children with CF. It appears that

serum from children with CF enhances myoblast proliferation at the cost of differentiation. Although we did not determine the specific factors responsible, our preliminary experiments suggest a potential role for IL-6. A positive relationship between IL-6 and proliferation mirrored by a negative relationship between IL-6 and differentiation is consistent with the higher circulating levels of IL-6 observed in our participants with CF compared to controls [7]. IL-6 is involved with induction of myoblast proliferation [11] and in reducing skeletal muscle growth [12]. However, to our knowledge the effects of IL-6 on myoblast differentiation have not been directly examined. Based on our data, further examination of the direct role of IL-6 in the impairment of skeletal muscle development in patients with CF is justified.

Prescribing exercise to patients with CF is a worthwhile clinical endeavor [8]. That exercise serum did not have an effect on myoblast proliferation or differentiation indicates that prescribing moderate-intensity exercise does not substantially alter how the systemic environment interacts with skeletal muscle development at least *in vitro*, despite known increases in systemic inflammation after a specific episode of exercise [7]. Our work indicates that systemic factors from children with CF in good clinical health resulted in impaired skeletal muscle development *in vitro*. Investigating the systemic factors involved warrants future work.

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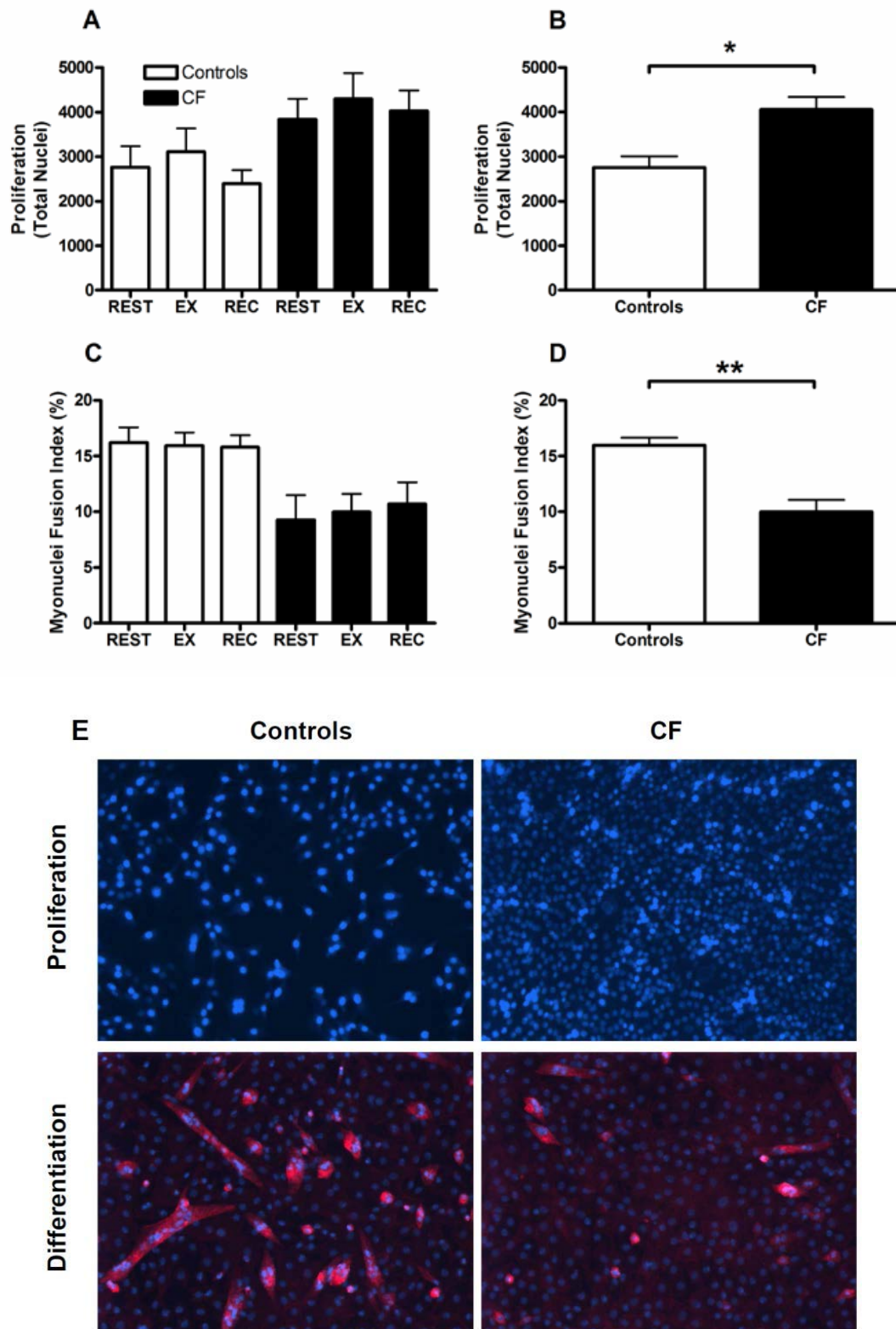


Figure 1. Effects of serum from children with CF and healthy controls on myoblast proliferation and differentiation. A) Proliferation: Total number of nuclei. B) Proliferation: Group differences in total nuclei. Average of REST, EX, and REC for CF and controls. C) Myotube fusion index. D) Group differences in myotube fusion index. Average of REST, EX, and REC for CF and controls. E) Photographs of myoblasts treated with rest serum from children with CF and healthy matched control. Values are expressed in mean \pm SEM. CF: cystic fibrosis. REST: myoblasts treated with serum collected before exercise, EX: myoblasts treated with serum collected after 1 hour of cycling. REC: myoblasts treated with serum collected 1 hour after exercise. n = 12 for each conditions. Myoblasts were treated for 1 hour and subsequently incubated in proliferation media for 3 days or incubated in differentiation media for 6 days. Nuclei were stained with DAPI (blue) and actin was stained using rhodamine phalloidin (red). Significant difference between groups, *p=0.03, **p=0.001.

CHAPTER 4

Exercise and Recovery Serum Rescues C2C12 Differentiation from the effects of Lipopolysaccharide *in vitro*

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Dr. Brian Timmons, Dr. Sandeep Raha, Jeff Baker, Sophie Joannis and I contributed to the design of the study. I was responsible for participant recruitment and blood sample collection, with assistance from Joyce Obeid. Dr. Linda Pedder provided support for recruitment of patients with cystic fibrosis. Dr. Gianni Parise provided the laboratory, equipment, and cells. I completed data collection, with assistance from Jeff Baker, Sophie Joannis, Ian Cooper, Joshua Nederveen. I was responsible for the analysis of the data presented. Joyce Obeid, Sophie Joannis, Joshua Nederveen assisted with statistical analyses. I drafted the manuscript, with support from Dr. Brian Timmons, Jeff Baker, Sophie Joannis, Joshua Nederveen, and Joyce Obeid. This manuscript will be sent to Ian Cooper Dr. Sandeep Raha, Dr. Gianni Parise, and Dr. Linda Pedder for revisions.

Exercise and Recovery Serum Rescues C2C12 Differentiation from the effects of Lipopolysaccharide *in vitro*

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ABSTRACT

Purpose: Determine whether exercise and recovery serum from children with cystic fibrosis (CF) and healthy controls can blunt the effects of lipopolysaccharide (LPS), a bacterial endotoxin and inflammatory mediator, on C2C12 myoblast differentiation. **Methods:** Twelve children with CF and 12 biological age- and sex-matched controls completed 2×30-min bouts of cycling. Serum samples were collected before exercise (REST), at the end of exercise (EX), and 60 min after exercise (REC). C2C12 myoblasts were treated with REST, EX, and REC serum with and without 10,000 ng/ml of LPS for 1 hour, then differentiation was induced for 6 days. Cells were stained and the myonuclei fusion index (MFI) was calculated. Data were analyzed using the difference (Δ) in MFI between serum incubated with and without LPS as a percentage of no LPS values (i.e., $(\text{REST with LPS} - \text{REST})/\text{REST} * 100 = \Delta\text{REST}$). **Results:** ΔREST and ΔEX were negative (mean \pm SEM: $-15.1 \pm 4.8\%$ and $-4.9 \pm 3.4\%$, respectively) while ΔREC was positive ($11.6 \pm 3.1\%$) in myoblasts treated with serum from children with CF. The positive ΔREC was significantly greater than ΔREST ($p=0.0003$) and ΔEX ($p=0.014$). With control serum, ΔREST , ΔEX and ΔREC were all positive ΔMFI ($37.1 \pm 7.9\%$, $11.9 \pm 8.6\%$, $12.3 \pm 6.0\%$, respectively) with ΔREST being greater than ΔEX and ΔREC ($p=0.039$ and $p=0.042$, respectively). **Conclusion:** EX serum from children with CF had no effect on LPS-induced differentiation, whereas EX serum from controls was able to blunt the effect of LPS. REC serum from both CF and controls was

able to reverse or blunt this effect. The anti-inflammatory capacity of exercise may be beneficial to muscle development, even under inflamed systemic environments.

INTRODUCTION

Children with cystic fibrosis tend to suffer from chronic systemic inflammation (1,2), which is largely attributed to the chronic pulmonary inflammation induced by chronic pulmonary infection seen in patients (3-5). This chronic inflammation can induce a catabolic state resulting in protein degradation (6, 7), and possibly impaired muscle development. Work from our laboratory has demonstrated that elevated levels of systemic inflammation, specifically IL-6, are related to increased proliferation and decreased differentiation in C2C12 myoblasts *in vitro* (Nguyen *et al.*, Submitted manuscript). Moreover, TNF- α , and tumor necrosis factor-like weak inducer of apoptosis (TWEAK) have also been shown to inhibit myoblast differentiation (8, 9).

The notion that exercise training can be an effective therapy for reducing resting systemic inflammation in patients with chronic inflammation is gaining traction, with evidence to support its effectiveness in patients with type 2 diabetes (10), COPD (11), hypertension (12), obesity (13), and even in sedentary but otherwise healthy adults (14). The anti-inflammatory effect of exercise is thought to be mediated, in part, by IL-10 (15) expression levels which are elevated following a bout of exercise (16). IL-10 mitigates inflammation by downregulating the production of pro-inflammatory cytokines TNF- α , IL-1, and IL-6 (17), mainly

through inhibition of the nuclear transcription factor (NF- κ B) pathway (17, 18). Another anti-inflammatory cytokine of interest in regards to muscle development is IL-13, which has recently been found to promote cell fusion (19).

The effects of an exercise-induced anti-inflammatory systemic response on muscle differentiation remain poorly understood. Thus, the primary aim of this study was to investigate the potential anti-inflammatory properties of systemic factors collected after exercise from children with CF and healthy children on myoblast differentiation. Specifically, to determine whether myoblast differentiation is altered when exposed to lipopolysaccharide (LPS), a bacteria endotoxin that induces an inflammatory response (20, 21) and are elevated in CF patients (22), and whether myoblast differentiation can be rescued when treated with LPS in combination with systemic factors collected after exercise. The second aim of this study was to determine whether circulating concentrations of anti-inflammatory cytokines IL-10 and IL-13, and pro-inflammatory cytokines TNF- α , IL-6, and TWEAK have a relationship with the recovery of myoblast differentiation capacity after exposure to LPS.

METHODS

Exercise and blood sampling

Twelve children with CF (FEV₁: 90.0 \pm 21.6% predicted (23)) were recruited from the Cystic Fibrosis Clinic at the McMaster Children's Hospital (Ontario, Canada), and 12 sex- and biological age-matched (24) healthy controls were included. This

study was approved by our local Research Ethics Board. A detailed description of recruitment and participant characteristics are published elsewhere (1). Briefly, participants completed two visits. During the first visit, peak mechanical power (PMP) was assessed using the *McMaster All-Out Progressive Continuous Cycling Test* on a cycle ergometer (Fleisch-Metabo, Geneva, Switzerland). The second visit was scheduled a minimum of 2-3 days after the first. This visit consisted of 2 × 30-min bouts of cycling at a constant pace of ~60 rpm and an intensity equivalent to 50% PMP. We chose to study the effects of 60-min of moderate-intensity exercise as this reflects the internationally-accepted recommendation for daily physical activity for children (25, 26). Blood samples were collected using an indwelling catheter placed in the antecubital region of the arm. Blood samples were collected before exercise (REST), at the end of the 2 × 30-min bouts of cycling (EX), and 60 min after exercise (REC). Blood was collected into 10-mL vacutainers and allowed to clot for 30 min at room temperature to obtain serum samples, a separate 10-mL EDTA vacutainer was collected to obtain plasma samples. Vacutainers were centrifuged for 20 min at 2000 × g and 4°C. Serum and plasma samples were aliquoted and stored at -80°C until use in the cell culture experiments and for quantifying circulating levels of pro-inflammatory and anti-inflammatory cytokines.

C2C12 myoblasts

C2C12 myoblast cell line was purchased from American Type Culture Collection (Rockville, MD, USA). Myoblasts were grown on 100-mm petri dishes in growth media (GM) consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin-streptomycin, and incubated at 37°C in 5% CO₂.

C2C12 differentiation

Passage 8 (P8) cells were seeded in cell-treated 96-well plates at a concentration of 5000 cells per well in 100 µl GM. Seeded plates were incubated at 37°C in a 5% CO₂ environment for 24 hours to allow myoblasts to adhere and grow. After 24 hours GM was removed and cells were treated with 100 µl of treatment media without and with LPS. Treatment media without LPS consisted of DMEM supplemented with 5% serum from children with CF or controls, with each time point represented (i.e., REST, EX, REC), and 1% penicillin-streptomycin. Treatment media with LPS included an additional 10,000 ng/ml of LPS (Sigma-Aldrich, catalog #L2654), with each time point represented (i.e., REST, EX, REC with LPS). Treated plates were incubated at 37°C in 5% CO₂ for 1 hour, after which treatment media was removed and replaced with 100 µl of differentiation media (DM) consisting of DMEM supplemented with 2% horse serum and 1% penicillin-streptomycin to induce differentiation. Plates were then incubated at 37°C in 5% CO₂ for 6 days. Cells were given fresh DM every 2 days. Myoblasts incubated in

either DMEM or GM and treated with and without LPS were used as a control (i.e., human serum was not included), to examine the effect of LPS on differentiation.

After 6 days of incubation, plates were fixed and stained for nuclei and embryonic myosin heavy chain eMHC (F1.652; embryonic isoform; DSHB; neat) to identify myotubes, and used as a marker of differentiation. More specifically, cells were washed with 100 μ l of PBS, fixed with 100 μ l of 2% PFA for 30 min, then washed again with 100 μ l PBS, incubated in 100 μ l of 0.25% Triton-X in PBS for 5 min, washed once more with 100 μ l PBS, and incubated with 100 μ l of 10% goat serum in PBS for 1 hour. Next, 60 μ l of primary antibody of embryonic myosin heavy chain (eMHC) was placed in each well and incubated for 1 hour. This was followed by 3 washes with 100 μ l PBS, which were each incubated for 5 min. Washes were followed by a 1 hour secondary antibody incubation at room temperature (Alexa fluor goat anti-mouse 594, 1:500, Invitrogen, Carlsbad, CA, USA). To stain for nuclei, wells were incubated in 100 μ l of DAPI for 10 min in the dark, washed 3 times with 100 μ l PBS, then air dried and stored at 4°C until further analysis.

Differentiation analyses

Markers of differentiation included the Myonuclei Fusion Index (MFI) and area of eMHC. Analyses were completed using five random fields of view captured from each well with a Nikon Eclipse Ti microscope (Nikon Instrument Inc. Melville, NY, U.S.A) at 10X magnification. The number of nuclei and myonuclei were

determined manually from the captured photographs in NIS-Element AR 3.2 64-bit software (Nikon Instrument Inc., Melville, NY, U.S.A). Myonuclei, defined as nuclei residing with a myotube identified by longitudinal eMHC orientation, were counted when 2 or more myonuclei were visible in a myotube. Nuclei or myonuclei were only included in the count if the entire nuclei/myonuclei was captured in the image. The sum of the nuclei and myonuclei from all 5 images were considered representative of the total number of nuclei and myonuclei for each well, respectively. MFI, as an indicator of differentiation capacity, was calculated as: $MFI = (\text{total number of myonuclei} / \text{total number of nuclei}) * 100$. The area of eMHC was obtained by defining the binary threshold and the area was calculated in the NIS-Element AR software. The sum of eMHC from all 5 images was used to represent the total area of eMHC from each well. All analysis was completed in a blinded manner.

Cytokine concentrations

Serum IL-10 concentrations were determined using high sensitivity human ELISA kits (R&D systems, HS100C, Minneapolis, MN, USA), while IL-13 concentrations were determined using human ELISA kits (Abcam, #ab46038, Toronto, Ontario, Canada).

Plasma IL-6 and TNF- α concentrations were determined with high sensitivity human ELISA kits (R&D systems, HS600B, HSTA00D, Minneapolis, MN, USA), while TWEAK concentrations were determined using human TWEAK

Instant ELISA kits (Bender MedSystems Inc., BMS2006INST, Burlingame, CA, USA). Plasma pro-inflammatory cytokines were used for correlation analyses. Group differences and the effects of exercise on these pro-inflammatory cytokines have been published previously (1).

Statistical Analyses

Data were tested for normality using the Shapiro-Wilk test (SPSS Statistics version 20.0, IBM, Armonk, New York, USA). All variables demonstrated a normal distribution, with the exception of markers of myoblasts differentiation treated with LPS in DMEM or GM, IL-10 concentrations, and IL-13 concentrations. Independent T-tests or Mann–Whitney U tests were used where appropriate to determine the effects of LPS with DMEM or GM on MFI, total nuclei, myonuclei, or eMHC. Two-way repeated measures ANOVA was used to determine whether myoblasts treated with serum from children with CF or controls spiked with LPS had an effect on MFI, total nuclei, myonuclei, or eMHC compared to myoblasts treated with serum without LPS. In addition, percent changes from serum with and without LPS (i.e., $((\text{REST with LPS} - \text{REST})/\text{REST}) * 100 = \Delta\text{REST}$) for each specific time point were calculated for MFI, total number of nuclei, number of myonuclei, and eMHC (i.e., ΔMFI , $\Delta\text{total number of nuclei}$, $\Delta\text{number of myonuclei}$, and ΔeMHC). Δ was compared within each group to determine an exercise effect using one-way repeated measure ANOVA. *Post hoc* Tukey's HSD were used where appropriate to examine specific mean differences.

Non-parametric analyses were performed on IL-10 and IL-13 concentrations. To determine the effects of exercise on cytokine concentrations within each group, Friedman's test was performed with Bonferroni correction for multiple hypothesis testing ($p \leq 0.025$ for significance). To determine differences between groups Mann–Whitney U test were performed between specific time points (i.e., CF REST and Control REST). Data from children with CF and controls were pooled to determine the relationship between pro-inflammatory or anti-inflammatory cytokines and Δ MFI, Δ total nuclei, Δ myonuclei, and Δ eMHC using Spearman rank order correlations. Unless otherwise specified, significance for all analyses was set at $p \leq 0.05$, and values are expressed as mean \pm SEM.

RESULTS

Effects of LPS on myoblast differentiation

Compared with myoblasts not treated with LPS, myoblasts treated with LPS in DMEM increased MFI (-LPS = $17.7 \pm 1.4\%$, +LPS = $22.0 \pm 1.8\%$, $p < 0.001$), total nuclei (-LPS = 2130 ± 69 , +LPS = 3001 ± 63 , $p < 0.001$), and myonuclei (-LPS = 346 ± 30 , +LPS = 624 ± 54 , $p < 0.001$), but not eMHC (-LPS = $3.19 \times 10^5 \pm 3.76 \times 10^4 \mu\text{m}^2$, +LPS = $3.32 \times 10^5 \pm 2.58 \times 10^4 \mu\text{m}^2$, $p = 0.52$). Myoblasts treated with LPS in GM increased in all differentiation variables (MFI: -LPS = $21.3 \pm 1.6\%$, +LPS = $28.1 \pm 2.3\%$, $p < 0.001$; total nuclei: -LPS = 2321 ± 64 , +LPS = 3083 ± 76 , $p < 0.001$; myonuclei: -LPS = 508 ± 46 , +LPS = 877 ± 82 , $p < 0.001$; eMHC: -LPS = $3.31 \times 10^5 \pm 2.36 \times 10^4 \mu\text{m}^2$, +LPS = $4.60 \times 10^5 \pm 3.72 \times 10^4 \mu\text{m}^2$, $p < 0.001$).

Myonuclei fusion index

Neither exercise nor LPS had an effect on MFI in myoblasts treated with CF serum (Figure 1A). This is in contrast to myoblasts treated with control serum where REST + LPS resulted in a greater MFI compared to REST alone and REC + LPS (Figure 1B). When examining the Δ MFI from CF serum, Δ REST and Δ EX were observed to be negative changes, while Δ REC was positive (Figure 1C). The positive Δ REC was significantly greater than the Δ REST and Δ EX. The Δ MFI from control serum was positive for each time point, with Δ REST being greater than Δ EX and Δ REC (Figure 1D).

Similar responses were observed for myoblasts treated with CF and control serum in total number of nuclei (Table 1) and Δ (Table 2). Conversely, myonuclei development differed between myoblasts treated with CF and control serum. Specifically, there was a main effect of LPS for control serum, with greater number of myonuclei observed with control serum with LPS compared to control serum without LPS. This main effect of LPS was not observed in myoblasts treated with CF serum. Much like the Δ MFI, the Δ REC in Δ myonuclei was significantly greater than the Δ REST and Δ EX in myoblasts treated with CF serum. The opposite was observed with control serum, where the Δ REST in Δ myonuclei was greater than both Δ EX and Δ REC.

Embryonic Myosin Heavy Chain

Myoblasts treated with CF serum demonstrated a significant effect of exercise and a significant effect for LPS in eMHC (Figure 2A). In addition, the Δ REST and Δ EX for Δ eMHC were negative, while Δ REC was positive (Figure 2C). The positive Δ REC was significantly greater than Δ REST and Δ EX. Neither exercise nor LPS had any effects on eMHC in myoblasts treated with control serum (Figure 2B & C).

Anti-inflammatory Cytokines

Serum concentrations of IL-10 from children with CF did not change with exercise, but increased at REC from REST in controls ($p = 0.008$, Figure 3). There were no differences between groups in IL-10 concentrations at any of the measured time points. Exercise had no effect on serum concentration of IL-13 in either children with CF or controls. However, children with CF had lower concentration of IL-13 at all time points (REST: $p = 0.008$, EX: $p = 0.024$, REC: $p = 0.024$).

Correlations

IL-6 was negatively correlated with Δ MFI, Δ total nuclei, and Δ myonuclei (Figure 4). IL-13 was positively correlated with Δ total nuclei ($p = 0.27$, $p = 0.024$) and myonuclei ($p = 0.28$, $p = 0.018$). TNF- α was positively correlated with Δ eMCH

($p = 0.29$, $p = 0.013$). There were no correlations between TWEAK or IL-10 with any of the Δ differentiation markers.

DISCUSSION

The effects of LPS on Δ MFI were reversed when myoblasts were treated with REC serum from children with CF, and blunted when treated with EX and REC serum from controls. This effect was also seen for Δ eMHC in myoblasts treated with CF serum, but not with control serum. Interestingly, Δ MFI at Δ REST was negative in myoblasts treated with CF serum, but positive in myoblasts treated with control serum. This difference may be related to the concentration of IL-13, an anti-inflammatory marker, which was higher in control compared with CF serum. Finally, higher IL-6 was associated with more negative Δ MFI.

Myoblast differentiation was rescued from the effects of LPS when exposed to a systemic post-exercise environment. In myoblasts treated with CF serum, the negative Δ MFI and Δ eMHC at Δ REST increased with REC serum, while in healthy controls, the positive Δ MFI at Δ REST decreased with EX and REC serum. This rescuing effect may be related to previous reports of an exercise-induced anti-inflammatory systemic environment (15, 16). Given that IL-10 can inhibit the pro-inflammatory stimulation of LPS (17, 18), it seems plausible that the observed increase in IL-10 from REST to REC in controls is partly responsible for the rescue effects of REC serum on myoblasts. Moreover, in order for skeletal muscle differentiation to occur under normal conditions, an initial pro-inflammatory

environment must be followed by a switch to an anti-inflammatory environment (27, 28). The observed increase in IL-10 at REC may be representative of this switch. However, this does not explain the fact that EX serum also induced a similar rescue response since there was no change in IL-10 at EX in controls. In addition, it is possible that other anti-inflammatory cytokines may be at play; for example, IL-4 is known to have anti-inflammatory properties (29) and promote myoblasts fusion and differentiation (30). It is unclear whether IL-4 is affected by acute exercise, but expression of IL-4 protein is increased *in vivo* in muscle following strength training (31). In our sample of participants, only 1 participant with CF and 5 controls had detectable IL-4 values when measured by ELISA (unpublished observation). Further investigation of IL-4 using more sensitive measures may provide additional insight into our findings.

An interesting finding from this study is the difference in response to LPS in myoblasts treated with serum from CF compared to controls. More specifically, LPS produced a negative Δ MFI with REST serum from children with CF, but a positive Δ MFI with REST serum from controls. Based on our observations from treating myoblasts with LPS in DMEM or LPS in GM, we expected that Δ MFI would be positive at Δ REST. This was confirmed in myoblasts treated with control serum but not those treated with CF serum. These findings suggest that the systemic environment in CF at REST altered the normal effect of LPS by impairing myoblasts differentiation. Although the exact mechanisms underlying the differential effects of CF and control serum on myoblasts are unclear, it may be

related to concentrations of IL-13. We found that the control group displayed 2- to 3-fold higher circulating levels of IL-13 than the CF group at every time point. The anti-inflammatory properties of IL-13 were found to inhibit LPS-induced TNF- α (32), and promote the recruitment of myoblasts as well as promote myoblast fusion (33). While other cytokines like IL-4 and IL-15 have also been linked to myoblasts fusion, Jacquemin *et al.* (2007) reported that only the neutralization of IL-13, but not IL-4 or IL-15, following IGF-1 treatment resulted in a reduction of fusion index (34), thus, highlighting the importance of IL-13. The anti-inflammatory effects of IL-13 include downregulation of a host of pro-inflammatory cytokines including IL-6, TNF- α , IL-1 α and IL-1 β , which are known to inhibit myoblasts fusion. Moreover, administration of IL-13 can rescue mice from LPS induced lethal endotoxemia (35). Given the functions of IL-13 as a promoter of myoblasts fusion and a potent anti-inflammatory mediator, it may help explain the different pattern in Δ MFI and Δ eMCH observed between myoblasts treated with CF and control serum.

Interestingly, the negative Δ MFI and Δ eMHC at Δ REST in myoblasts treated with CF serum was no longer negative, but became positive at Δ REC. This positive change at Δ REC is similar to what is observed in myoblasts treated with control serum (statistic not shown). Despite the CF group presenting with a higher resting systemic pro-inflammatory profile (1), our results suggest that exercise can be a potent stimulus to induce an anti-inflammatory effect similar to that of controls.

We reported a negative correlation between IL-6 and Δ MFI, as well as Δ total nuclei and Δ myonuclei. In regards to Δ MFI, different levels of IL-6 were seen

with different degrees of LPS's effect on myoblasts fusion. That is low levels of IL-6 was seen with greater myoblasts fusion with LPS, while moderates level of IL-6 was seen with little or no change in myoblasts fusion with LPS, and finally higher levels of IL-6 was seen with reduced levels of myoblasts fusion with LPS. Interestingly, IL-6 is known to promote proliferation (36) and differentiation (37) of myoblasts under normal conditions. However, higher levels of IL-6 are physiologically intolerable and alter the normal course of differentiation. Indeed, a study by Ionescu *et al.* (2002) that showed serum levels of IL-6 in CF patients with chronic inflammation were associated with higher levels of protein degradation (6). The authors also reported that IL-6 was elevated in those with lower fat-free mass, alluding to a catabolic state that is not conducive to muscle development (6). As such, it seems likely that the reduction in myoblast differentiation when treated with LPS and CF serum is linked to the higher circulating IL-6 observed in our participants with CF compared with healthy controls (1). In addition to the possible direct effects of systemic IL-6 on myoblasts fusion, LPS treatment may have increased myoblast secretion of IL-6 (38), which may have in turn exacerbated effects of IL-6 from the systemic environment. Although we cannot confirm this hypothesis since we did not collect cell supernatant during incubation, it may represent an important area for future investigation. Nevertheless, our study showed that IL-6 is related to the anti-inflammatory capacity of systemic environment on myoblasts differentiation from an inflammatory insult with LPS.

The mouse cell line used in the present study builds on our earlier research (39); however, an important limitation to consider is the use of human serum on a mouse myoblasts cell line. The rationale for the use of the C2C12 cell line is available in our previously published work (39). Future studies will seek to use human cell lines or human primary myoblasts, as well as investigate the effects of systemic factors on other cell types such as fibroblast, adipocytes, osteoblasts, and vascular endothelial cells. Moreover, future research examining the effect of neutralizing some inflammatory and anti-inflammatory cytokines, such as IL-6, IL10, and IL-13, may provide us with additional insight into the mediators involved in myoblasts differentiation and the anti-inflammatory effects of exercise.

To our knowledge, this is the first study to examine the anti-inflammatory effects of an exercise-induced systemic environment on C2C12 myoblasts differentiation *in vitro*. We observed that exercise and/or recovery serum from children with CF and healthy controls were able rescue myoblast differentiation from the effects of LPS. Between group differences were observed for the IL-10 response to exercise, as well as for IL-13 concentrations at rest and following exercise, which may be partly responsible for the between group differences in the effects of LPS on myoblasts differentiation. The negative relationship between IL-6 and the percent change in myonuclei fusion index suggests that IL-6 may play a role in myoblasts differentiation, and may also alter the effects of LPS on myoblasts differentiation, which warrants further investigation. Overall, our results indicate

that systemic environment characterized by a pro-inflammatory state can express anti-inflammatory properties following exercise.

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	<i>Total number of nuclei</i>		<i>Number of Myonuclei</i>	
	CF	Controls	CF	Controls
REST	2301 ± 168	2248 ± 102	596 ± 88	375 ± 56
EX	2599 ± 174*	2543 ± 129*	555 ± 79	525 ± 82
REC	2684 ± 156*	2555 ± 150*	531 ± 50	419 ± 61
REST + LPS	3108 ± 150**	3282 ± 115**	615 ± 77	821 ± 111**
EX + LPS	3052 ± 192**	3207 ± 128**	596 ± 80	647 ± 62‡
REC + LPS	3111 ± 137**	3023 ± 134**‡	785 ± 114**	614 ± 97**‡
Average without LPS	2528 ± 97	2449 ± 76	561 ± 42	440 ± 39
Average with LPS	3090 ± 91†	3171 ± 73†	665 ± 53	694 ± 54†

Table 1. Total number of nuclei and myonuclei in myoblasts treated with serum with and without lipopolysaccharide (LPS) from children with CF and controls. REST: Myoblasts treated with serum collected before exercise. EX: Myoblasts treated with serum collected after exercise. REC: Myoblasts treated with serum collected 60 min after exercise. + LPS: Serum was spiked with 10,000 ng/ml of LPS. *Significant difference from REST. **Significant difference from the same time point without LPS. ‡Significant difference between REST + LPS. †Significant main effect for LPS. Significance was set at $p \leq 0.05$. Values are expressed in mean ± SEM.

	ΔREST	ΔEX	ΔREC
ΔTotal number of nuclei (%)			
CF	34.2 \pm 3.7	20.2 \pm 2.4*	15.1 \pm 2.0*†
Controls	43.7 \pm 5.0	27.3 \pm 3.0*	20.9 \pm 2.8*
ΔNumber of Myonuclei (%)			
CF	10.9 \pm 5.0	12.8 \pm 5.2	32.5 \pm 4.6*†
Controls	99.6 \pm 14.3	49.3 \pm 12.4*	44.2 \pm 11.3*

Table 2. Percent change in the total number of nuclei and myonuclei in myoblasts treated with and without lipopolysaccharide (LPS) using serum from children with CF and controls. *Significant difference from Δ REST. †Significant difference from Δ EX. Δ REST: Change of REST + LPS from REST without LPS. Δ EX: Change of EX+ LPS from EX without LPS. Δ REC: Change of REC + LPS from REC without LPS. Significance was set at $p \leq 0.05$. Values are expressed as mean \pm SEM.

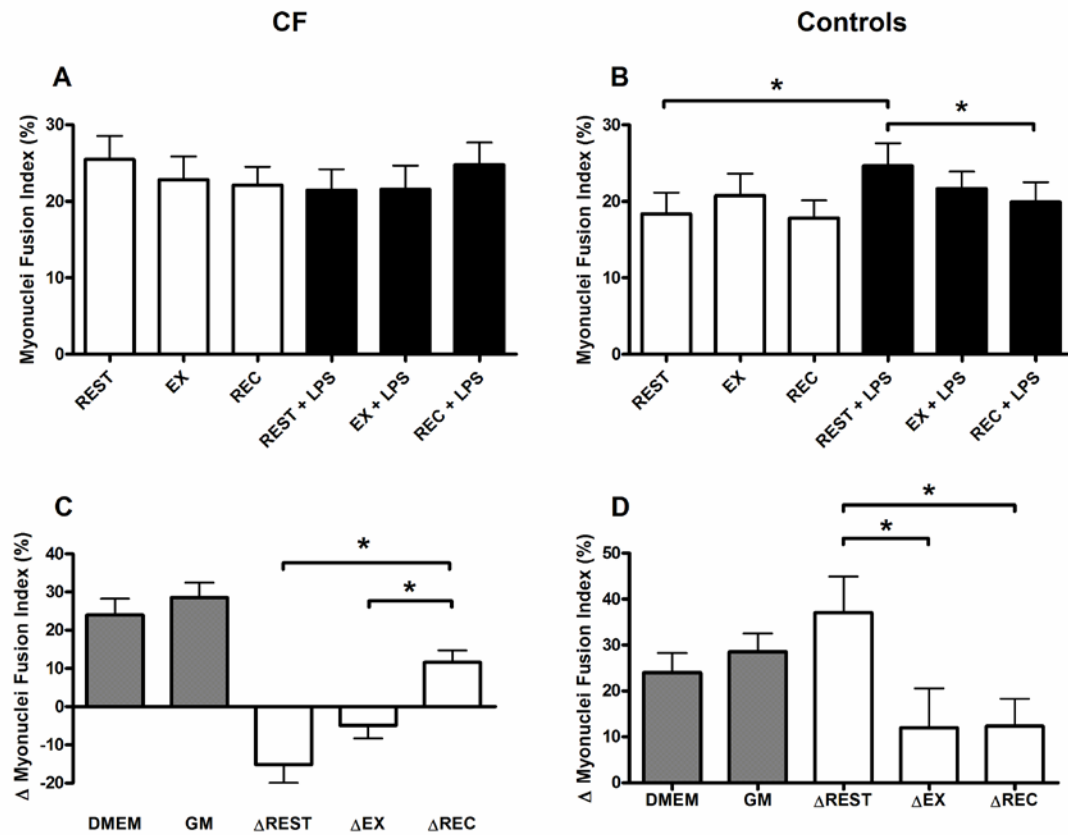


Figure 1. Myonuclei fusion index (MFI) in myoblasts treated with serum from (A) children with CF and (B) controls, with and without lipopolysaccharide (LPS). Data are also presented as Δ MFI in myoblasts treated with and without LPS using serum from (C) children with CF and (D) controls. Δ MFI in myoblasts treated with and without LPS in DMEM or GM are illustrated for visual comparisons to show the effect of LPS, and were not included in statistical analyses. REST: Myoblasts treated with serum collected before exercise. EX: Myoblasts treated with serum collected after exercise. REC: Myoblasts treated with serum collected 60 min after exercise. + LPS: Serum spiked with 10,000 ng/ml of LPS. Δ REST: Change of REST + LPS from REST without LPS. Δ EX: Change of EX + LPS from EX without LPS. Δ REC: Change of REC + LPS from REC without LPS. GM: growth media. Values are expressed as mean \pm SEM. *Significant at $p \leq 0.05$.

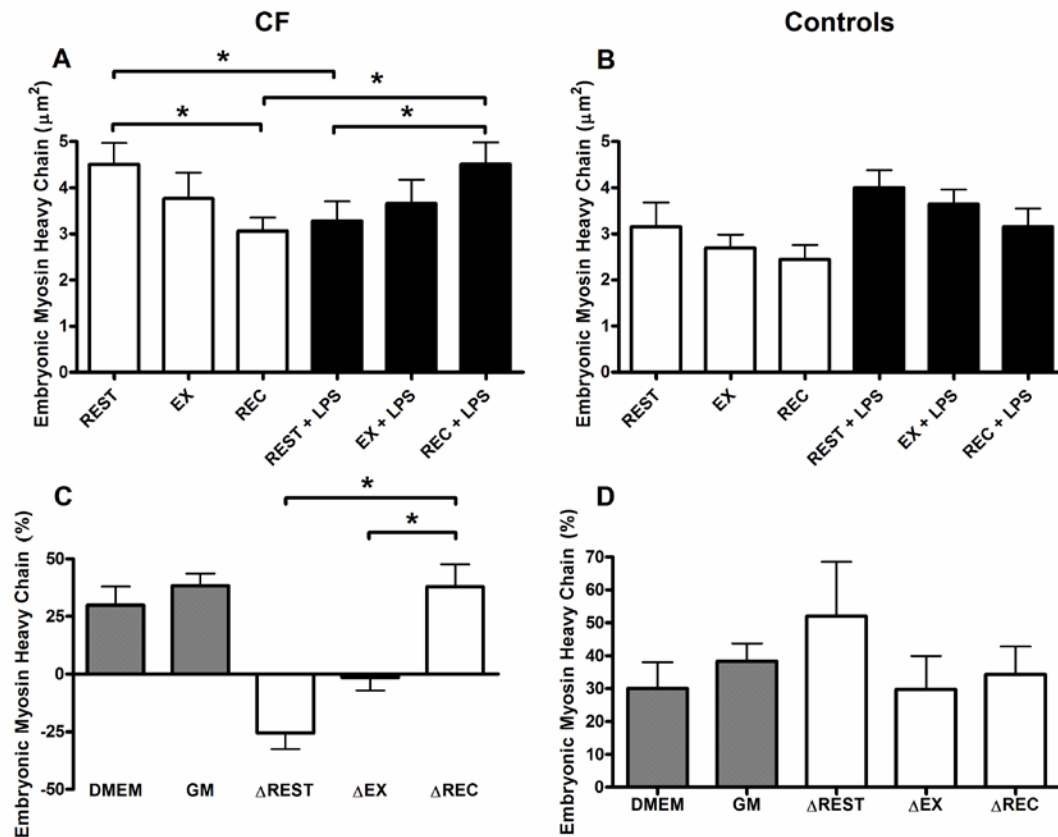


Figure 2. Area of embryonic myosin heavy chain (eMHC) in myoblasts treated with serum from (A) children with CF and (B) controls, without and with lipopolysaccharide (LPS). Data are also presented as Δ eMHC in myoblasts treated without and with LPS using serum from (C) children with CF and (D) controls. Δ eMHC in myoblasts treated with and without LPS in DMEM or GM are illustrated for visual comparisons to show the effect of LPS, and were not included in statistical analyses. REST: Myoblasts treated with serum collected before exercise. EX: Myoblasts treated with serum collected after exercise. REC: Myoblasts treated with serum collected 60 min after exercise. + LPS: Serum spiked with 10,000 ng/ml of LPS. Δ REST: Change of REST + LPS from REST without LPS. Δ EX: Change of EX + LPS from EX without LPS. Δ REC: Change of REC + LPS from REC without LPS. GM: growth media. Values are expressed as mean \pm SEM. *Significant at $p \leq 0.05$.

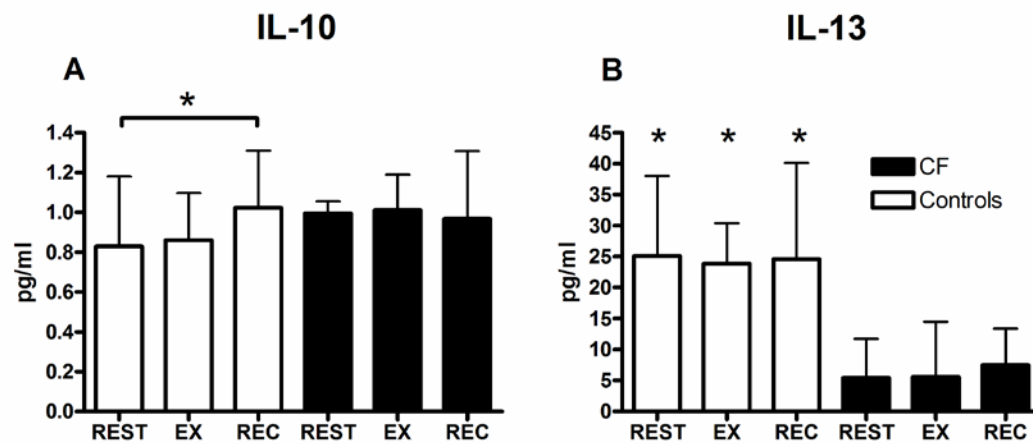


Figure 3. Serum concentration of (A) IL-10 and (B) IL-13 in children with CF and healthy controls. CF: cystic fibrosis. Values are expressed in median and interquartile range. *Significant at $p \leq 0.05$. *above graph indicates significant difference between groups at that time point.

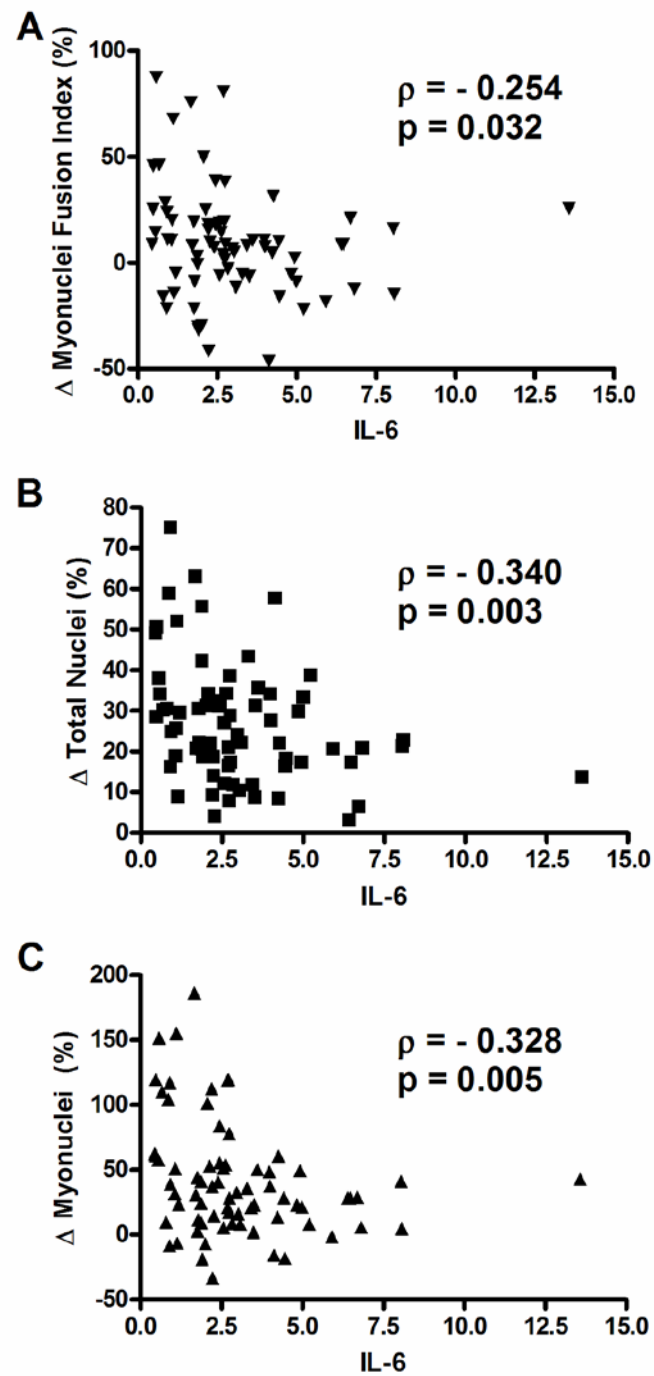


Figure 4. Relationship between IL-6 and Δ in (A) myonuclei fusion index, (B) total nuclei, and (C) myonuclei. IL-6 concentrations are expressed as pg/ml.

CHAPTER 5: DISUCSSION

5.1 Objectives

The specific objectives of the studies in this thesis were to:

- (1) Determine the effects of systemic factors from children with CF and healthy controls on myoblast proliferation, and determine the effect of post-exercise systemic factors on proliferation *in vitro*.
- (2) Determine the effects of systemic factors from children with CF and healthy controls on myoblast differentiation, and determine the effect of post-exercise systemic factors on differentiation *in vitro*.
- (3) Determine the effect of post-exercise systemic factors from children with CF and healthy controls on differentiation of myoblasts exposed to an inflammatory mediator *in vitro*.

5.2 Main findings

The main findings in this thesis were that myoblasts exposed to resting systemic factors from children with CF displayed greater proliferation and lower differentiation phenotype than myoblasts exposed to systemic factors from healthy controls. Systemic factors taken post-exercise did not have a consistent effect on proliferation or any effect on differentiation. There was a negative change in differentiation when myoblasts were treated with resting systemic factors from children with CF with LPS when compared to non-LPS condition. Conversely, there was a positive change in differentiation when myoblasts were treated with resting

systemic factor from healthy controls with LPS when compared to non-LPS condition. The effects of LPS were reversed or blunted when exposed to post-exercise systemic factors from CF and healthy controls.

5.3 The effects of systemic factors on proliferation phenotype

It was hypothesized that proliferation would be greater in myoblasts treated with CF serum. Both study 1 (Chapter 2) and study 2 (Chapter 3) supported this hypothesis, as evidenced by the findings that myoblasts treated with CF serum resulted in greater number of total nuclei, a marker of proliferation, compared to myoblasts treated with control serum. Findings from study 1 and 2 differed with respect to the effect of exercise on proliferation phenotype. In study 1, EX and REC control serum had greater myoblast proliferation compared to REST serum, while CF post-exercise serum had no effect on proliferation. In study 2, there was no post-exercise effect of serum on proliferation phenotypes with either CF or control serum. Thus, study 2 was unable to confirm the findings of study 1. This discrepancy may be linked to differences in methods used in each study.

First, pooled samples were used in study 1, while individual samples were used in study 2. It is important to note that only ~3 to 5 mL of serum was collected from each participant, therefore, samples in study 1 were pooled as a precaution so as to reserve serum samples for future experiments. Running repeated proliferation phenotype experiments using pooled serum may have reduced the variability in study 1. The individual samples used in study 2 displayed greater

variability, which might also be partly responsible for the observation of no significant effects of post-exercise serum on proliferation.

Second, the number of participants in study 1 differed from that of study 2. In study 1, eleven children with CF and eleven healthy control participants had their serum pooled for experiments. Serum from twelve children with CF and twelve healthy control participants were used in study 2. The difference in sample size was based on the inclusion criteria for study 1, which required all participants to have serum collected at each time point (i.e., REST, EX, REC). As such, 1 participant with CF and their matched healthy control were excluded due to the CF participant missing the REC sample. This time point was treated as missing data for study 2. It is possible that the exclusion of the healthy matched control allowed for the exercise effect in study 1.

Finally, the number of days myoblasts were allowed to proliferate before analyses differed between studies 1 and 2. After treatment, cells were allowed to proliferate for two days before analyses in study 1, and three days in study 2. It is plausible that three days after treatments was too long to detect an exercise effect. Specifically, 3 days of growth media may have washed away differences in proliferation rates induced by one hour of EX and REC serum exposure.

Although each of the aforementioned factors may explain the discrepancies between studies 1 and 2, the extent of their contribution to the observed differences remains unknown. Despite these differences, the finding that exercise serum from children with CF did not change proliferation was consistent across studies,

indicating that exercise did not exacerbate the elevated levels of proliferation observed with resting CF serum. The consistent, robust increase in myoblast proliferation observed with CF serum provides confidence in our finding that systemic factors from children with known systemic inflammation can alter early stages of skeletal muscle development in C2C12 myoblasts.

The cause of the higher proliferation with CF serum is unclear; however, the findings of studies 1 and 2 suggest this may involve IL-6, as discussed in subsequent sections. Among the other circulating factors that might be involved is the growth mediator, IGF-1, which is a potent mediator of skeletal muscle growth and is involved in inducing myoblast proliferation (Velloso, 2008; Yu et al., 2015; Rabinovsky et al., 2003; Florini et al., 1996). An earlier publication based on data collection from participants in this thesis revealed no differences in the resting the post-exercise plasma concentrations of IGF-1 between our CF and healthy participants (Nguyen et al., 2012)(data shown, statistical analyses not shown), suggesting IGF-1 is not likely responsible for the proliferation rates with CF serum. Moreover, patient with CF are known to have impaired growth (Cystic Fibrosis Canada, 2013; Cystic Fibrosis Canada, 2015) as well as lower circulating IGF-1 than healthy individuals (Laursen et al., 1995; Laursen et al., 1999; Taylor et al., 1997). Thus, it would be more likely that healthy controls would present with higher levels of IGF-1, and in turn, higher myoblast proliferation; this, however, was not observed.

High glucose combined with high levels of insulin (Grabiec et al., 2014), or insulin alone can induce greater myoblast proliferation (Yu et al., 2013; Conejo & Lorenzo, 2001). It is plausible that glucose and insulin levels in CF might explain our observation, particularly in light of the fact that patients with CF can suffer from CF-related diabetes (Moran et al., 1998), which presents clinically as hyperglycemia (Adler et al., 2011; Holl et al., 1995). However, CF-related diabetes is also associated with reduced insulin secretion from the pancreas (Moran et al., 1998). Indeed, a separate publication on six of the participants with CF and healthy controls presented in this thesis suggested that both circulating glucose and insulin levels were similar between groups (Nguyen et al., 2014). This would indicate that hyperglycemia and/or hyperinsulinemia are not likely responsible for the increased proliferation observed with CF serum. However, this cannot be confirmed since the assessments were based on only half of the participants included in this thesis. It is also important to note that none of the CF or control participants in this thesis had any form of diabetes.

Only a handful of studies have examined the effects of serum on myoblast myogenesis *in vitro* (Conboy et al., 2005; Chromiak et al., 1991; Corrick et al., 2015), and even fewer have assessed myoblast proliferation (Conboy et al., 2005; Chromiak et al., 1991). One such study was conducted by Conboy *et al.*, (2005) and reported that using satellite cells and resting serum from old mice led to less satellite cell activation, as measured by the percentage of Delta⁺ cells, compared to satellite cells treated with resting serum from young mice (Conboy et al., 2005).

This indicated that systemic factors from old mice had impaired proliferation. It is unknown whether the old were experiencing chronic systemic inflammation. However, it is known that aging is seen with increased plasma levels of TNF- α (Bruunsgaard et al., 1999; Bruunsgaard et al., 2000; Dobbs et al., 1999), IL-6 (Dobbs et al., 1999), and CRP (Ballou et al., 1996; Bruunsgaard et al., 2000). If the old mice from Conboy *et al.* study did indeed have chronic systemic inflammation coupled with reduced proliferation (Conboy et al., 2005), this is in contrast to finding from this thesis that found greater proliferation from inflamed CF serum. In another study, Chromiak *et al.* (1991) examined the effects of serum collected after exercise training from rats on myoblast proliferation *in vitro*. Exercise training consisted of 28 days of a 60 cm climb up a vertical grid with progressive increase in weights. Serum were taken from trained and untrained rats and placed on L6 rat myoblast and proliferation assessed using [3H]thymidine incorporation. The authors reported that serum taken 7 days after training displayed greater proliferation compared to serum from untrained mice (Chromiak et al., 1991). These results lend support to the potent effects of exercise training on systemic factors that can increase myoblast proliferation *in vitro*. It is difficult to compare our findings from that of Chromiak *et al.*, since their study examined the effects of exercise training while this thesis examined the effects of an acute bout of exercise. To this author's knowledge, there are no other studies that have examined the effects of serum from pediatric clinical or healthy populations on myoblast proliferation *in vitro*, nor have there been any studies examining the effects of

serum from an acute bout of exercise on proliferation. Therefore, the findings of this thesis serve to fill this gap, suggesting that serum from children with a known inflammatory condition caused greater myoblast proliferation, and exercise serum from healthy children may increase this proliferation from rest. Given that proliferation can have an effect on differentiation, which is the next stage of skeletal muscle development, we next examined the effects of resting and exercise systemic factors on myoblast differentiation.

5.4 The effects of systemic factors on differentiation phenotype

Since serum from children with CF caused greater myoblast proliferation, it was hypothesized that CF serum would result in lower myoblast differentiation. Although it is well established that in a healthy system, an expansion of myoblasts is required for myoblast differentiation (Shefer et al., 2006; Yin et al., 2013), it has also been established that pro-inflammatory mediators can induce greater proliferation at the expense of lower differentiation *in vitro* (Dogra et al., 2006). This hypothesis was examined in study 2, which confirmed that myoblasts treated with CF serum demonstrated reduced differentiation, as measured by myonuclei fusion index, compared to myoblasts treated with control serum. Moreover, exercise serum did not have an appreciable effect on differentiation.

One of the objective of study 3 was to verify the differentiation results from study 2. A supplementary analysis of differentiation of myoblasts treated with CF and control serum using study 3 data is presented in Appendix 2. Interestingly, study 3 did not confirm the findings of study 2. More specifically, there were no

group level differences for myoblast differentiation (CF: 23.5 ± 1.7 %, Controls: 19.0 ± 1.6 %, $p = 0.24$); however, there was an interaction effect, which indicated that myoblasts treated with CF serum had higher MFI at REST and REC time points compared to myoblasts treated with control serum. The discrepancies between studies 2 and 3 may be related to methodological differences. In study 2, myotubes were identified using actin. Myotubes, and thus myonuclei, were recognized by enclosed formations of longitudinal actin structures. The primary limitation of this method is that actin is also expressed by myoblasts, making it difficult to distinguish between myotubes and their predecessors. As such, a conservative approach was taken such that a minimum of 3 myonuclei surrounded by an obvious longitudinal formation of actin were required to be counted as a myonuclei. In study 3, myotubes were identified using embryonic myosin heavy chain (eMHC) which targeted myotubes more directly and simplified the process of identifying these structures. In this case, the criterion to include a nucleus as a myonuclei was set at a minimum of 2 myonuclei surrounded by longitudinal formation of eMHC.

Despite these differences, our findings may be indicative of potential impairments in myoblasts treated with CF serum. More specifically, these findings suggest systemic factors from children with CF may have had an enhanced ability to initiate myoblast-to-myoblast fusion to create a 2 myonuclei myotube; however, the progression to incorporating more myoblasts to an existing myotube to create a myotube with 3 myonuclei or more may be impaired. Interestingly, additional myoblasts fuse to existing myotubes at the interior region (Guerin & Kramer, 2009).

Taken together with the current results, this may highlight an important area for future investigation. A more simple explanation of our findings may be that myoblasts treated with CF serum display a time delay in myoblast differentiation since they proliferate to a greater extent. A switch from proliferation to differentiation signalling is required for commitment and progression to differentiation (Yin et al., 2013). Thus, if the proliferation signalling pathways are not terminated or suppressed, differentiation may be delayed.

A number of photographs of myoblasts treated with CF serum from study 2 demonstrated myoblast fusion, as indicated by the connection of actin between nuclei, but did not display obvious elongated actin structures resembling myotubes. Thus, these were not included as myonuclei. Instead, actin in these fused myoblasts displayed a chaotic structure and differed from the actin that was observed with myoblasts treated with control serum. Two representative photographs highlight these differences in Appendix 3. These observations may be indicative of difficulties with myotube elongation in myoblasts treated with CF serum compared to control serum, and should be investigated further. Myotube formation involves extensive cytoskeletal reorganization before and after myoblast fusion (Abmayr & Pavlath, 2012; Fulton et al., 1981). Although actin is known to play an integral role in cell-to-cell fusion, microtubules have been shown to play a more primary role in myotube elongation (Guerin et al., 2009).

The effect of systemic factors on myoblast differentiation *in vitro* has been examined by Corrick *et al.* (2015) Serum from six burn injury patients and healthy

controls (aged ~35 yrs) were collected and placed on satellite cells collected from a separate set of healthy individuals. Myoblasts treated with serum from burn patients displayed less myoblast differentiation, as measured by fusion index, and myotube size was reduced by ~33% as compared to myoblasts treated with control serum (Corrick et al., 2015). Interestingly, serum from burn patients contained higher levels of pro-inflammatory markers of IL-1 β , IL-1, IL-6, and TNF- α (Merritt et al., 2012), suggesting that systemic factors can impair myoblast differentiation. These findings are consistent with findings from study 2, where elevated pro-inflammatory systemic factors observed in children with CF resulted in impaired myoblast differentiation.

5.5 The effects of systemic factors on myogenic protein and mRNA expression

In study 1 and 2, protein and mRNA expression relating to myogenic programming were assessed to further explore the between group differences observed in our phenotype experiments. Our mRNA data from study 2 were not included in the final manuscript, but are displayed in Appendix 4. From both studies, protein expression of proliferation (STAT3, p-STAT3, SOCS3), as well as mRNA expression of proliferation (Pax7, SOCS3, Ki67), differentiation (myogenin) and anti-apoptosis (HSP70) markers were measured. Our findings suggest no between group differences in any of the aforementioned markers, with the exception of myogenin mRNA (Appendix 4-2), which was higher on day 0 and 3 of differentiation in myoblasts treated with CF REST serum, and higher on day 0 with

CF EX serum compared to controls serum. This would indicate that the systemic factors from children with CF are promoting differentiation to a greater extent than systemic factors in control serum. However, this was not reflected in our MFI results (measured at day 6) that revealed less differentiation with CF serum (study 2). Thus, our protein and mRNA analyses does not explain the between group difference observed in proliferation or differentiation phenotype experiments.

5.6 The anti-inflammatory effects of exercise

In study 3, LPS was used to provide a potent inflammatory insult to myoblasts, and quantify the downstream effects on differentiation. LPS was selected based on earlier studies demonstrating greater concentrations in patients with CF (Wilmott et al., 1994), as well as the result of our pilot work on the effects of LPS on proliferation. More specifically, pilot work supported the use of 10,000 ng/mL of LPS to induce a proliferation phenotype response (Appendix 5). Moreover, we found that LPS induced greater proliferation in myoblasts, but that this effect was inhibited with exercise serum from healthy children (Appendix 6). The concept of a rescue effect was then extended to myoblast differentiation. It must be noted that this concentration may be higher than physiologically reported levels (i.e., up to 50 pg/ml has been reported in CF patients)(Wilmott et al., 1994); however, our results illustrate the positive effects of exercise even in the presence of markedly elevated levels of LPS, which further lends support to the potency of the anti-inflammatory exercise response.

It was hypothesized that the effects of an inflammatory mediator on myoblast differentiation would only be blunted when myoblasts were exposed to post-exercise systemic factors from healthy control participants, and not from children with CF. Surprisingly, our study (Chapter 4) showed that REC serum from children with CF reversed the effects of LPS. In addition, post-exercise serum from healthy controls blunted the effects of LPS. Thus, our hypothesis was only partly supported by our data. This anti-inflammatory effect of exercise was more pronounced when data were reported as the change (Δ) values as opposed to absolute values. The Δ MFI revealed the effect of LPS was reduced with EX and REC serum from healthy controls, but reversed only with REC serum from CF. It is intriguing to note that this rescue effect was observed regardless of the initial effect of LPS with resting serum. That is, while Δ MFI was negative with REST CF serum and positive with REST control serum, the effect of LPS was blunted or reversed with post-exercise serum in both cases. This suggests a robust anti-inflammatory exercise response. The anti-inflammatory effect of exercise extended to Δ eMHC with CF serum, where REC serum was able to reverse the effects of LPS. Although a similar pattern in Δ eMHC and Δ MFI was observed for myoblasts treated with control serum, Δ eMHC was not significantly different between REST, EX or REC. Overall, our results indicate that systemic post-exercise factors can provide anti-inflammatory protection from LPS, even when the resting environment is consistent with a pro-inflammatory state, as seen in youth with CF.

Exercise also led to an increase in circulating levels of the anti-inflammatory cytokine IL-10 at REC from REST in control participants. This increase in IL-10 was not observed in children with CF, which may be partly related to the effects of chronic inflammation in inhibiting a switch from a pro-inflammatory to an anti-inflammatory state. Indeed, there is evidence to suggested increased production of pro-inflammatory cytokines, such as IL-6 and IL-8 (a neutrophil attractant), and reduced production of IL-10 by respiratory epithelial cells in CF lungs (Bonfield et al., 1999). Given that IL-6 is known to induce the production of IL-10 (Steensberg et al., 2003), and that our CF participants presented with higher circulating IL-6, it was expected that these participants would consequently also present with higher levels of IL-10. However, IL-10 concentrations were similar in healthy controls and CF. IL-10 is thought to be responsible for the anti-inflammatory properties of exercise (Petersen & Pedersen, 2005). The fact that there were no between group differences in IL-10 and no relationship between any of the differentiation markers and IL-10, suggests that IL-10 may not be the primary mediator of our anti-inflammatory effect. Thus the anti-inflammatory mediator responsible for reversing and blunting the effects of LPS on myoblast differentiation remains unknown, and requires additional investigation.

5.7 Interleukin 6

In study 1 (Chapter 2), children with CF had higher concentrations of IL-6 than controls. This was consistent with the finding of higher proliferation in myoblasts treated with CF serum. Moreover, the effects of exercise on IL-6 levels

in controls mirrored the exercise effect on proliferation of myoblasts exposed to control serum. Similarly, in youth with CF, exercise did not have any effect on either levels of IL-6 in CF participants or proliferation of myoblasts exposed to CF serum. The striking similarities in IL-6 concentrations and myoblast proliferation were further explored in a supplementary analysis (Appendix 7) examining the effects of human recombinant IL-6 on myoblast proliferation. The observed positive association between IL-6 and proliferation from our supplementary analysis led us to examine the relationship between IL-6, proliferation, and differentiation in study 2 (Chapter 3). In order to perform correlation analyses, individual serum sample were used in study 2. We found that IL-6 was positively related to proliferation, but negatively related to differentiation. The proliferation results were consistent with the findings of earlier studies of IL-6 involvement in myoblast proliferation (Serrano et al., 2008; Wang et al., 2008). However, we did not expect a negative relationship between IL-6 and differentiation, but expected a positive relationship since there is evidence to suggest that IL-6 is either required for myotube formation and fusion (Hoene et al., 2013) or has no effect on myoblast fusion (Wang et al., 2008). Another unexpected result of our work was the negative relationship between IL-6 and the effects of LPS on myoblast differentiation (Chapter 4). To the author's knowledge, there are no available studies suggesting a link between IL-6 and the ability of LPS to alter myoblast fusion. A reoccurring connection between IL-6 and myoblast proliferation and differentiation was consistently reported throughout this thesis. Therefore, IL-6 may be responsible for the myoblasts response observed.

However, it must also be noted that IL-6 was measured in plasma samples, while myoblasts were treated with serum. The extent to which this may affect our findings is unclear.

5.8 Clinical implications

The extent to which the findings of this thesis extend to the clinical setting is somewhat speculative, and limited by the fact that all experiments were completed with an *in vitro* model. Despite this limitation, this series of studies highlight the direct impact of systemic factors on early and late skeletal muscle development. The fact that CF serum can impair skeletal muscle development would suggest that systemic factors in CF may be contributing to the impaired growth observed in patients (Cystic Fibrosis Canada, 2013; Cystic Fibrosis Canada, 2015). It is important to note that the sample of CF patients in this thesis were relatively healthy, as indicated by their good pulmonary function (Nguyen et al., 2012). Thus, systemic factors can impair muscle development even in patients with more mild disease burden. These results are particularly alarming since skeletal muscle mass accounts for 30-40% of body weight (Kim et al., 2015; Kim et al., 2006), and survival has been linked to the degree of underweight in these youth (Kraemer et al., 1978).

Reduction of systemic inflammation, and specifically IL-6, may reverse impaired skeletal muscle development in CF. This may be achieved with chronic exercise. Indeed, reduced levels of various resting pro-inflammatory cytokines (Abd El-Kader et al., 2013; Wang et al., 2014; Lamina et al., 2014; Lakka et al.,

2005; Ho et al., 2013), including IL-6 (Abd El-Kader et al., 2013) have been reported after an exercise training program. In addition, promoting exercise to reduce systemic inflammation and maintain muscle development aligns well with the concept of “Exercise is Medicine”, which is already widely encouraged in CF due to the wide range of benefits associated with maintaining an active lifestyle (Wilkes et al., 2009). Since serum collected after an acute bout of exercise did not have any negative effects on myoblasts, it seems unlikely that exercise-induced changes in systemic factors would further impair development. Moreover, the exercise-induced anti-inflammatory protection from LPS may represent an additional protective mechanism for skeletal muscle, especially given the chronic pulmonary bacterial infections (Amin & Ratjen, 2008) and elevated concentrations of circulating LPS reported in these patients (Wilmott et al., 1994).

5.9 Limitations

The primary limitation of this thesis is the use of a murine cell line with human systemic factors. The genome of IL-6 between humans and mice is highly, but not completely conserved (Tanabe et al., 1988; Hammacher et al., 1994). Although C2C12 myoblasts are responsive to human recombinant GH (Frost et al., 2002; Sadowski et al., 2001) and IL-6 (Hammacher et al., 1994), it is impossible to determine whether C2C12 myoblasts are responsive to all mediators in human serum. As such, the results of this thesis should be verified with human myoblasts. Nevertheless, the inclusion of serum from healthy controls in the thesis provides an important comparator, and lends confidence to our findings.

Myoblasts in our studies were exposed to serum acutely, and we did not assess the effects of chronic exposure on myoblasts. It must be noted that chronic exposure to systemic inflammation may alter the sensitivity of myoblasts to these factors *in vivo* (Scheele et al., 2012). Given that serum from children with CF had higher resting IL-6 (Nguyen et al., 2012), it is possible that chronic exposure may desensitize satellite cells to IL-6 and blunt its effect *in vivo*. Indeed, satellite cells that have differentiated into myocytes in obese individuals with and without type 2 diabetes demonstrate abnormal responses to IL-6 (Scheele et al., 2012).

Finally, the use of a healthy myoblast model may represent an additional limitation of our studies. The CFTR channel, which is mutated in CF patients, is expressed in skeletal muscle. Its functions involve the release of ATP during acidosis in contracting skeletal muscle. Blockage of the CFTR in healthy skeletal muscle abolishes ATP release during contraction (Tu et al., 2012). This manifests as impaired skeletal muscle metabolism, and specifically, as lower resting ATP/phosphocreatine ratio and higher pH values following exercise in CF patients (Wells et al., 2011). It is not clear whether this impairment extends to satellite cells derived from CF patients, or how systemic factors would in turn affect CF myoblasts. To the author's knowledge, there is no known CF myoblasts cell line available for commercial use, and engineering this line was beyond the scope of our research. Therefore, we were unable to examine the effects of systemic factors on a CF-specific skeletal muscle cell line. Despite this limitation, the findings of this

thesis revealed that systemic factors derived from a pro-inflammatory environment had a significant impact on muscle development even in a healthy model.

5.10 Novelty of Findings

This thesis represents the first efforts towards understanding the impact of resting and post-exercise systemic factors from children with and without CF on skeletal muscle development *in vitro*. The key novel findings and the contributions of this thesis to advancing the state of knowledge in this field are summarized as follows:

1. Chapter 2: This is the first study to compare the effects of pooled pediatric CF and healthy control resting and post-exercise serum on myoblast proliferation. Findings suggest that CF serum induced greater myoblast proliferation, with no effect of exercise. In contrast, post-exercise control serum elicited greater myoblast proliferation. Proliferation and IL-6 concentrations showed similar profiles.
2. Chapter 3: This is the first study to compare the effects of individual pediatric CF and healthy control resting and post-exercise serum on myoblast proliferation and differentiation. Findings confirmed our previous study that CF serum can induce greater proliferation in myoblasts. In contrast, CF serum resulted in lower myoblast differentiation. Neither CF nor control post-exercise serum had an effect on myoblast proliferation or differentiation.

Higher concentration of IL-6 was associated with higher proliferation and reduced differentiation.

3. Chapter 4: This is the first study to compare the anti-inflammatory effects of individual pediatric CF and healthy control post-exercise serum on myoblast differentiation following LPS treatment. LPS with CF resting serum resulted in a negative change in differentiation; conversely, LPS with control resting serum resulted in a positive change in differentiation. Post-exercise serum from CF reversed the effects of LPS on myoblast differentiation, while post-exercise serum from controls blunted the effects of LPS. Higher concentration of IL-6 was linked with more negative changes in myoblasts differentiation with LPS treatment.

5.11 Future studies

The findings of this thesis suggest that additional studies examining the effects of systemic factors on tissue development are a worthwhile endeavour. Given that observed relationship between IL-6 and myoblast proliferation and differentiation, it is important that future work more closely assess the role of IL-6 in development. Establishing the role of IL-6 in CF may involve neutralizing IL-6 to determine whether myoblasts would display similar results to that of control serum. It would also be relevant to examine the function of the developed myotubes by measuring contraction forces, in order to determine whether systemic factors from CF serum induce a concomitant impairment in myotube function. Moreover,

examining the effects of serum collected following an exercise training program on myoblasts may help establish the effectiveness of exercise as a therapeutic measure to promote skeletal muscle development in youth with CF.

In order to gain a more holistic understanding of skeletal muscle development in children with CF, an examination of both systemic factors and skeletal muscle tissue in CF is warranted. Replicating the experimental work from this thesis using CF-derived cells, such as primary myoblasts derived from pediatric CF patients or mice, may allow for a better understanding of the combined effects of pathologic systemic factors (Tirakitsoontorn et al., 2001; Nguyen et al., 2012) and intrinsically impaired skeletal muscle tissue (Wells et al., 2011) on growth and development in this population.

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APPENDIX

APPENDIX 1

Physiological Reports

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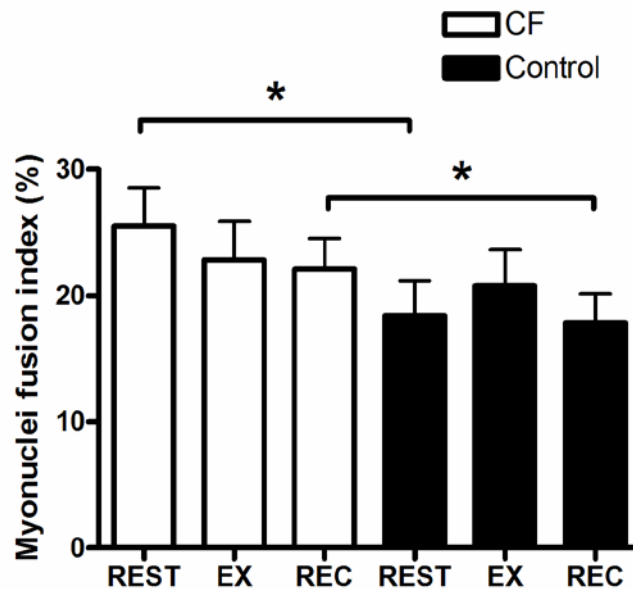
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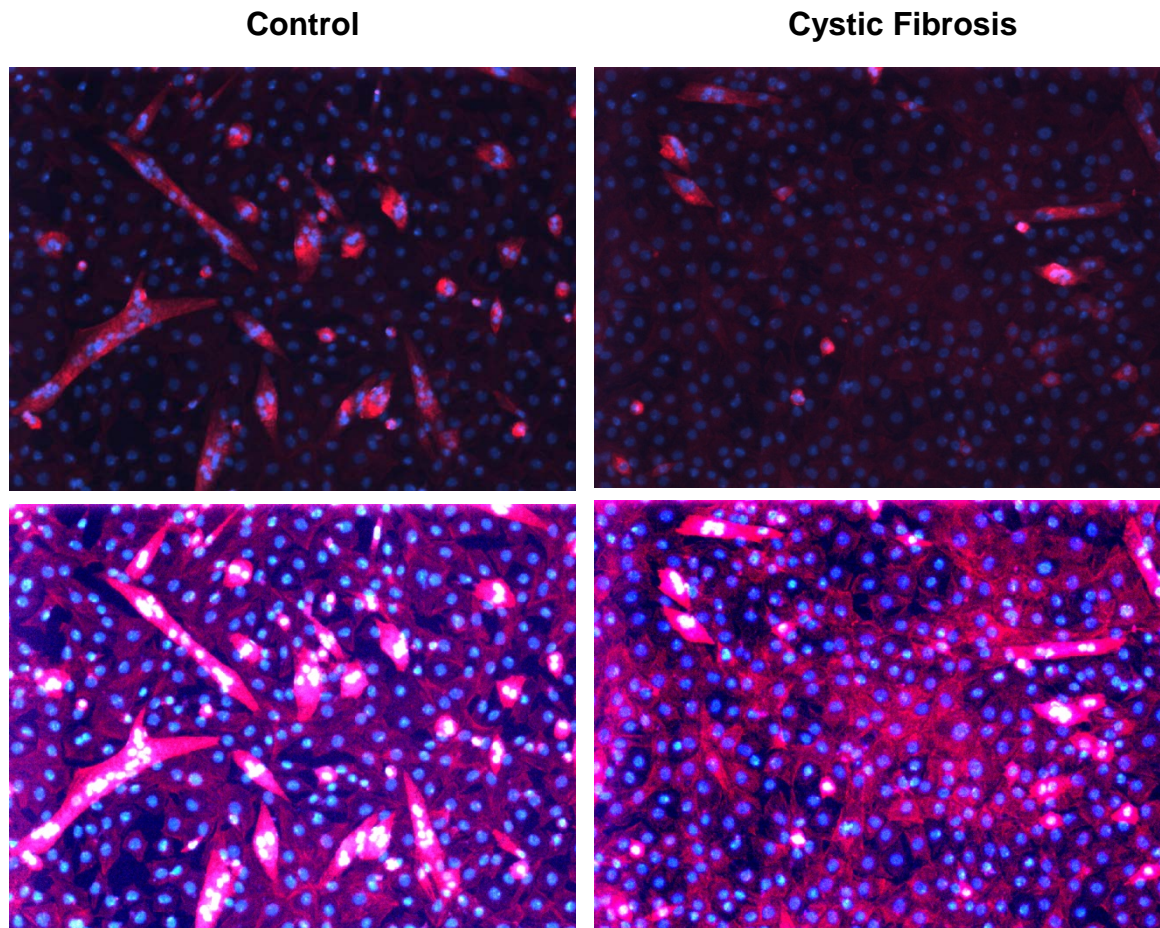
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APPENDIX 2



Appendix 2-1. Comparison of myonuclei fusion index (MFI) in myoblasts treated with serum from children with CF and healthy controls. Data analyzed were serum without LPS conditions collected from study 3 (Chapter 4). Myoblasts treated with CF serum had higher MFI at REST and REC compared to myoblasts treated with control serum. No group differences were observed (CF: $23.5 \pm 1.7\%$, Controls: $19.0 \pm 1.6\%$, $p = 0.24$). CF: cystic fibrosis. REST: serum samples collected before exercise, EX: serum samples collected immediately following 2 x 30 min bouts of cycling at 50% of peak mechanical power. REC: serum samples collected 1 hour after exercise. Values are expressed in mean \pm SEM. *Significance, $p \leq 0.05$.

APPENDIX 3

Appendix 3-1. Photographs of actin stained myoblasts treated with CF and control serum. Top panels are the original photographs from study 2 (Chapter 3). Bottom panels are the original photographs from study 2, but adjusted for visual comparison of actin organization. Myoblasts treated with CF serum were observed to have greater degree of advanced cell-to-cell actin connection, but lack of elongation and proper formation of myotubes compared to myoblasts treated with control serum.

APPENDIX 4

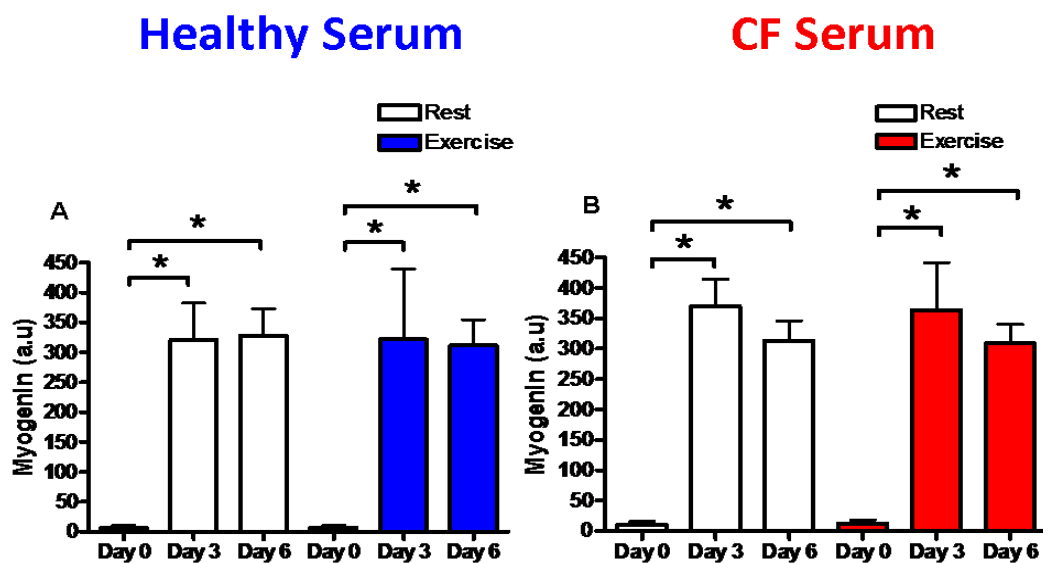
Title: mRNA experiments from PhD study 2 (Chapter 3)

Objective: Determine whether myoblasts treated with CF serum have reduced mRNA expression of differentiation, and altered proliferation- and apoptosis-related genes.

Method: C2C12 myoblasts were seeded in 100 mm plates at 2.5×10^5 cells per well and allowed to grow until ~70% confluence. Cells were then treated with 5% serum from children with CF (n=12) and serum from healthy biologically age-matched controls (n=12), supplemented with 1% penicillin/streptomycin in DMEM at a 1 mL volume for 1 hour. Serum samples collected before exercise (REST) and immediately following 2 x 30 min bouts of cycling at 50% of peak mechanical power (EX) was used. After 1 hour of treatment, cells were washed and induced to differentiation with differentiation media (2% horse serum supplemented with 1% penicillin/streptomycin in DMEM). After 3 days and after 6 days of differentiation cells were harvested using Trizol. mRNA was isolated from samples for PCR analysis. Myogenin (marker for differentiation)(Smith et al., 1994), Ki67 (marker for proliferation)(Scholzen & Gerdes, 2000; Sellathurai et al., 2013) and HSP70 (marker for anti-apoptosis)(Xiao et al., 2011) were measured. GAPDH was used as the housekeeping gene.

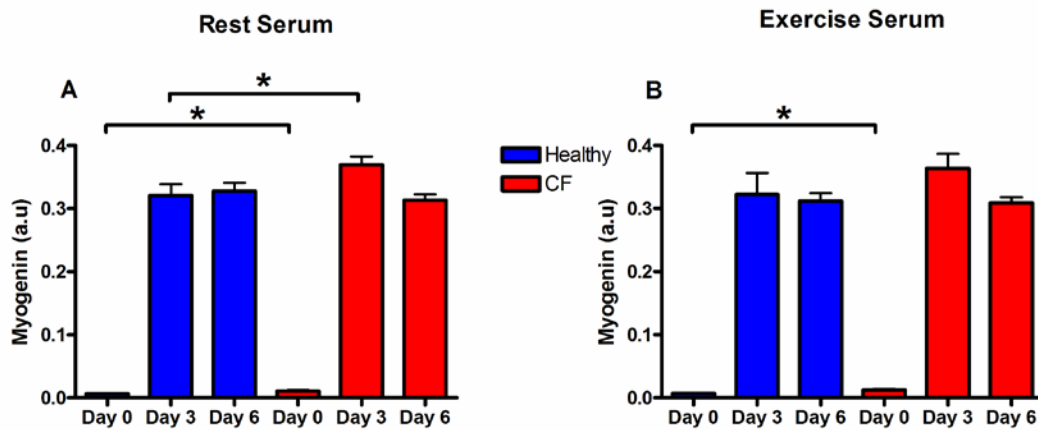
Statistics: Friedman tests were used to determine time effects. Wilcoxon Signed rank tests were used to determine differences between myoblasts treated with REST and EX serum in each group. Mann-Whitney U tests were used to determine differences between groups.

Results:



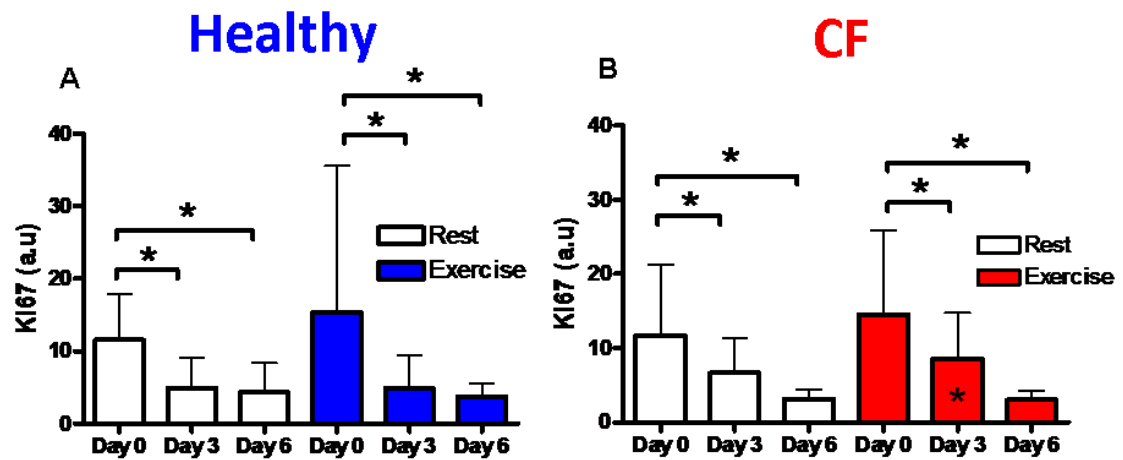
Appendix 4-1. Myogenin gene expression in myoblasts treated with (A) healthy control serum or (B) CF serum. Days represent the number of days C2C12 cells were induced to differentiate. Values are expressed as mean \pm SD of $2^{(-\Delta CT)}$. Both groups had similar increased in myogenin expression for REST and EX serum.

*Significant difference.

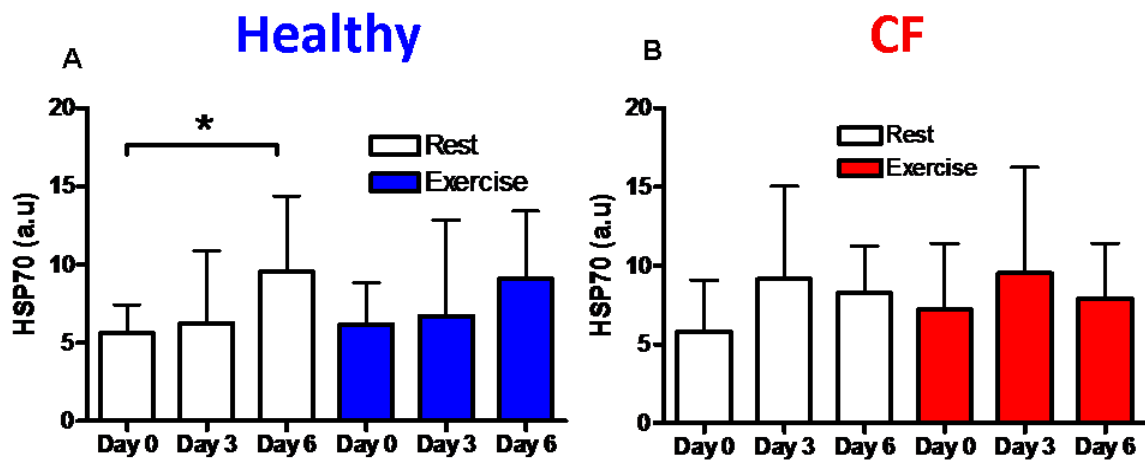


Appendix 4-2. Comparison of myogenin gene expression in (A) myoblasts treated with healthy control or CF REST serum and (B) myoblasts treated with healthy control or CF EX serum. Days represent the number of days C2C12 cells were induced to differentiate. Values are expressed as mean \pm SD of $2^{(-\Delta CT)}$. Myoblasts treated with CF REST serum expressed myogenin to a greater extent on Day 0 and Day 3, while CF EX expressed myogenin to a greater extent on Day 0 compared to myoblasts treated with healthy control serum. However, this increased expression of myogenin with CF serum disappeared at Day 6.

*Significant difference.



Appendix 4-3. Ki67 gene expression in myoblasts treated with (A) healthy control serum or (B) CF serum. Days represent the number of days C2C12 cells were induced to differentiate. Values are expressed as mean \pm SD of $2^{(-\Delta CT)}$. Both groups had similar increased in Ki67 expression for REST and EX serum. Ki67 was more highly expressed in myoblasts treated with CF EX serum than with CF REST serum, indicating that exercise can alter a marker of proliferation in myoblasts. There were no differences between groups. * above bars represent significant differences compared to Day 0. * within bars represent significant differences between REST and EX serum treatment for that day.



Appendix 4-4. HSP70 gene expression in myoblasts treated with (A) healthy control serum or (B) CF serum. Days represent the number of days C2C12 cells were induced to differentiate. Values are expressed as mean \pm SD of $2^{(-\Delta CT)}$. An increase in HSP70 was seen only in myoblasts treated with healthy control REST serum. No changes were observed with CF serum. There were no differences between groups. *Significant difference.

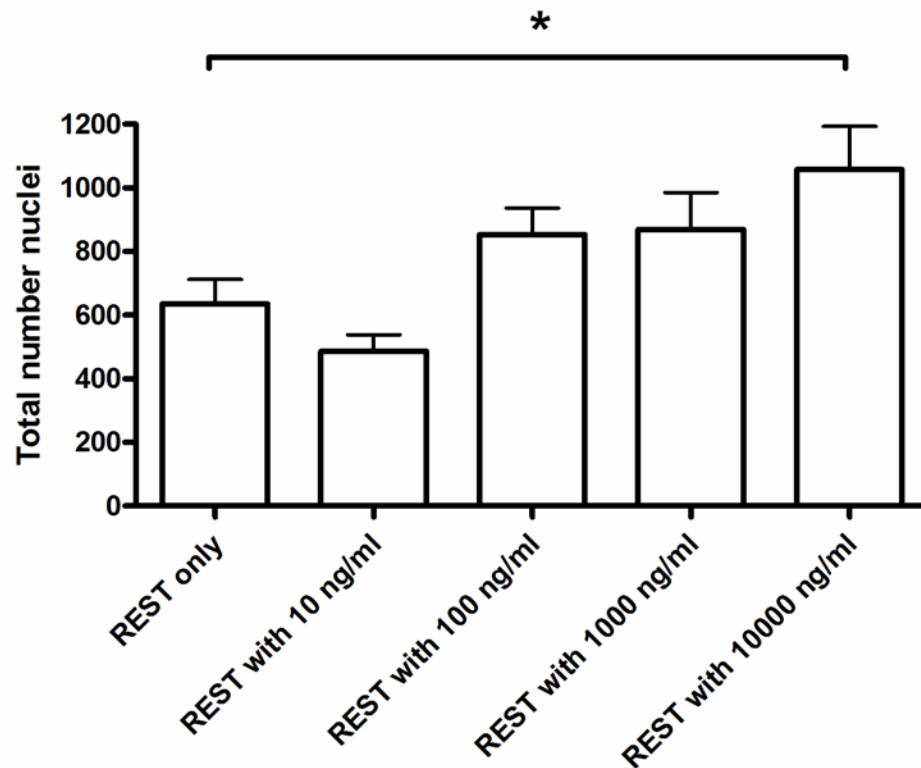
APPENDIX 5

Objective: Determine at which concentration of lipopolysaccharide (LPS) will effect myoblast proliferation.

Serum samples: Healthy pre-pubertal males (n=10) resting serum (REST). Individual samples were pooled for analysis.

Method: C2C12 myoblasts were seeded in 96-well plates at 1000 cells per well, and allowed to adhere and grow in growth media for 24 hours. Myoblasts were treated with 5% REST serum in DMEM supplemented with 1% penicillin/streptomycin and spiked with 0, 10, 100, 1000, or 10,000 ng/ml of LPS. Each condition was completed in replicates of 8. Myoblasts were treated for 1 hour and incubated at 37°C with 5% CO₂. After incubation, treatment media was replaced with growth media and then incubated at 37°C with 5% CO₂ for 3 days (fresh growth media was placed on cells after 2 days). On day 3, myoblasts were fixed with 2% PFA and stained with DAPI. Five photographs were taken at 5 random areas of each well and the number of nuclei was determined using the NIS-Element AR 3.2 64-bit (Nikon Instrument Inc., Melville, NY, U.S.A) program.

Statistics: 1-way repeated measures ANOVA with post hoc Tukey's HSD, where appropriate. Significance set at $p \leq 0.05$.

Results:

Appendix 5-1. Total number of nuclei after LPS treatment. Proliferation in myoblasts treated with REST serum with 10,000 ng/ml LPS was significantly greater compared to myoblasts treated with REST serum without LPS. Thus, 10,000 ng/ml of LPS was chosen for subsequent experiments.

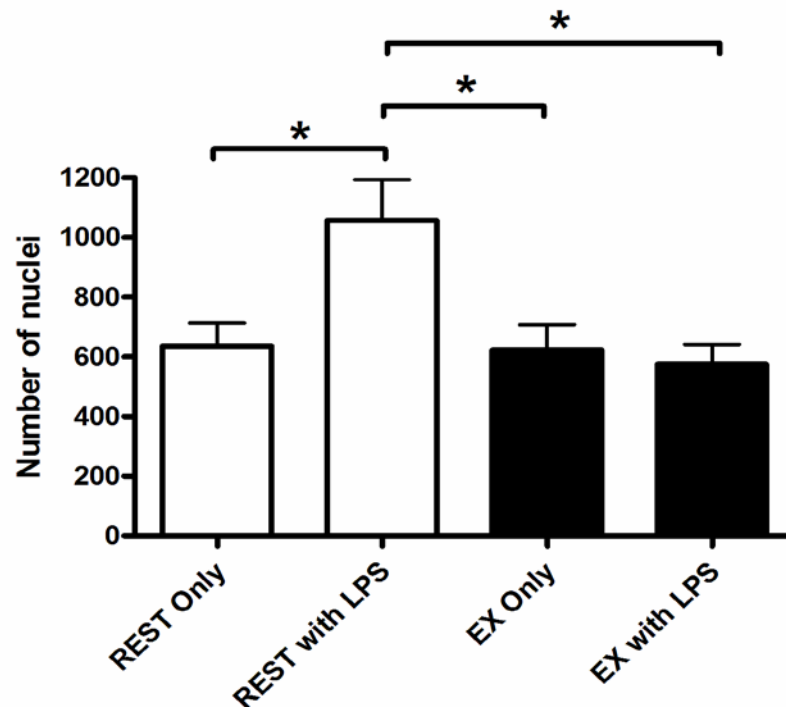
APPENDIX 6

Objective: Determine whether exercise serum from healthy children can rescue myoblasts spiked with LPS from an increase in myoblast proliferation.

Serum samples: Healthy pre-pubertal males (n=10) cycled at 50% of their peak mechanical power for 2 x 30-min bouts. Serum collected before exercise (REST) and after exercise (EX) were used for cell experiments. Individual samples were pooled for analysis.

Method: C2C12 myoblasts were seeded in 96-well plates at 1,000 cells per well, and allowed to adhere and grow in growth media for 24 hours. Myoblasts were treated with 5% serum in DMEM supplemented with 1% penicillin/streptomycin. REST and EX serum with and without 10,000 ng/ml of LPS was used as treatment media. Each condition was completed in replicates of 8. Myoblasts were treated for 1 hour and incubated at 37°C with 5% CO₂. After incubation, treatment media was replaced with growth media and then incubated at 37°C with 5% CO₂ for 3 days (fresh growth media was placed on cells after 2 days). On day 3, myoblasts were fixed with 2% PFA and stained with DAPI. Five photographs were taken at 5 random areas of each well and the number of nuclei was determined using the NIS-Element AR 3.2 64-bit (Nikon Instrument Inc., Melville, NY, U.S.A) program.

Statistics: 2-way repeated measures ANOVA with post hoc Tukey's HSD, where appropriate. Significance set at $p \leq 0.05$.

Results:

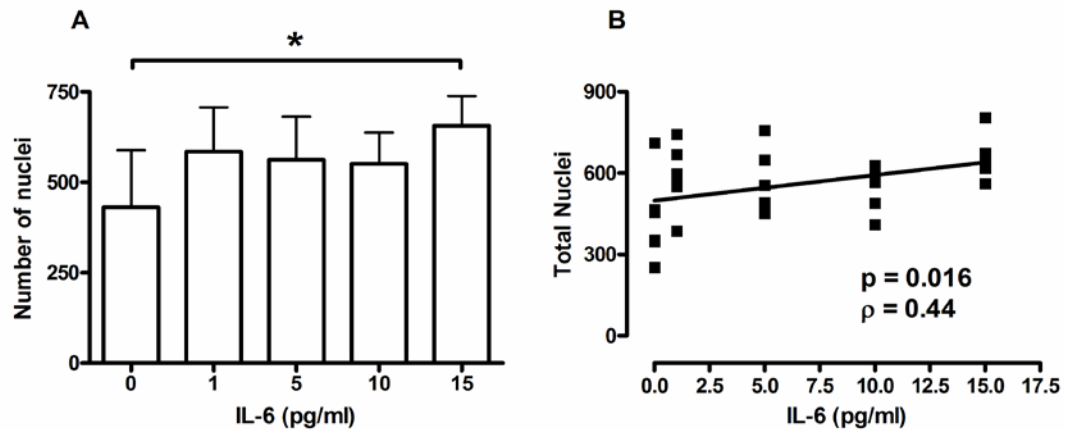
Appendix 6-1. Effects of LPS, REST and EX serum on myoblast proliferation. REST serum with LPS caused a greater increase in myoblast proliferation compared to myoblasts treated with REST serum only. This increase in proliferation was not observed in EX serum with LPS. EX serum from healthy children inhibited the effects of LPS on myoblast proliferation, indicating an anti-inflammatory capacity.

APPENDIX 7

Objective: Determine whether human recombinant IL-6 would cause greater myoblast proliferation, and the extent of the relationship between these two outcomes.

Method: Experimental setup, staining, and analysis were identical to those described for the proliferation phenotype experiment in study 1 (Chapter 2) using human serum, except treatment media consisted of DMEM supplemented with IL-6 in the following concentrations: 0, 1, 5, 10, or 15 pg/ml. Each condition was completed in replicates of 6. The concentrations were chosen to reflect the range of plasma IL-6 concentration observed in our participants. IL-6 concentration in our healthy group ranged from 0.4 – 3.4 pg/ml, while the CF group ranged from 0.8 – 10.2 pg/ml (chapter 2).

Statistics: One-way repeated measures ANOVA with post hoc Tukey's HSD, where appropriate. Spearman rank order correlation for total number of nuclei and IL-6. Significance set at $p \leq 0.05$.



Appendix 7-1. Effects of human recombinant IL-6 on myoblast proliferation. (A) Mean myoblast proliferation after IL-6 treatment. (B) Relationship between IL-6 concentration and proliferation. Proliferation was greater in myoblasts treated with 15 pg/ml of IL-6 compared to 0 pg/ml. No differences were observed with any other IL-6 concentration. There was a positive relationship between IL-6 concentration and proliferation. * indicates significant difference between conditions, $p \leq 0.05$.