Mechanisms of muscle damage: analysis of the myotube response to the cancer derived-secretome

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ABSTRACT

Cancer cachexia is a complex paraneoplastic syndrome affecting up to 80% of advanced cancer patients and directly contributes to up to 30% of cancer related deaths. Cancer cachexia is characterized by the loss of skeletal muscle mass and systemic inflammation. It is a complex condition with many factors contributing to its onset and progression, many of which still need to be fully understood. Using an *in vitro* model of cancer cachexia, we demonstrate that in response to the cachectic cancer secretome, C2C12 myotubes upregulate the protein expression of proinflammatory cytokines and immune cell chemoattractants. Further, we observe an upregulation in inflammatory and immune response related genes, including *Cxcl1* and *Timp1* in the treated myotubes. These results implicate myofibers as key contributors to the inflammatory muscle environment in cachexia and suggest that immune cells, specifically neutrophils, may be recruited to the muscle environment and contribute to the progression of cachexia. Interestingly, both the LLC and MC38 cancer models have different cachectic phenotypes *in vitro* and *in vivo*, suggesting different mechanisms of muscle wasting in different cancer models, highlighting the diversity of the cachectic phenotype. We also show that the cachectic cancer secretome does not directly cause myotube membrane damage, suggesting that the muscle damage observed in cachexia is not a result of tumor derived factors and rather due to other cell interactions occurring *in vivo*.

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LIST OF ABBREVIATIONS

AKT	Protein kinase B
AMPKα1	Protein kinase AMP-activated catalytic subunit alpha 1
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
CCL	Chmokine C-C motif ligand
CD14	Cluster of differentiation 14
CM	Conditioned media
CSA	Cross-sectional area
CXCL	Chemokine C-X-C motif ligand
DAMP	Damage-associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DM	Differentiation media
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
FAP	Fibro-adipogenic progenitors
FC	Fold change
Flt3	FMS-like tyrosine kinase 3
FSTL-1	Follistatin-like 1
G-CSF	Granulocyte colony-stimulating factor
GDF15	Growth differentiation factor 15
GFAL	GNDF family receptor alpha like
GM	Growth media
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GO	Gene ontology
HGF	Hepatocyte growth factor
HI-FBS	Heat inactivated-fetal bovine serum
HI-HS	Heat inactivated-horse serum
HMGB1	High mobility group box 1
HRP	Horseradish peroxidase
IFN	Interferon
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor-binding protein
IgG	Immunoglobulin G
IL	Interleukin
IL6R	Interleukin 6 receptor
KO	Knock out
LIF	Leukemia inhibitory factor
LLC	Lewis Lung Carcinoma
LTA	Left tibialis anterior
Ly6G	Lymphocyte antigen 6 family member G
M-CSF	Macrophage colony-stimulating factor
MRF	Myogenic regulatory factor
mTOR	Mammalian target of rapamycin

mTORC1	Mammalian target of rapamycin complex 1
MuRF1	Muscle RING-finger protein-1
MuSC	Muscle stem cell
MYF5	Myogenic factor 5
MyHC	Myosin heavy chain
MYOD	Myoblast determination protein 1
NK	Natural killer
OCT	Optimal cutting temperature
OPN	Osteopontin
P/S	Penicillin/streptomycin
PAX7	Paired box 7
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
ROS	Reactic oxygen species
scRNA-seq	Single-cell ribonucleic acid sequencing
TA	Tibialis anterior
TAM	Tumour associated macrophage
TIMP1	Tissue inhibitor of metalloproteinase 1
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
TRAF6	Tumour necrosis factor receptor-associated factor 6
UC	Unconditioned media
vSMC	Vascular smooth muscle cells
WGA	Wheat germ agglutinin
WISP-1	WNT1-inducible signaling pathway protein 1
WT	Wild type

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1. INTRODUCTION

1.1. Skeletal muscle and interorgan crosstalk

Skeletal muscle is a vital organ that is essential for movement, breathing and whole-body metabolism, specifically energy regulation and protein homeostasis (Argilés et al., 2016; McPherron et al., 2013; Wolfe, 2006). Skeletal muscle can store large amounts of glycogen, which is broken down into glucose in order to meet the muscles energetic needs (Argilés et al., 2016). This ability to store glycogen is unique to the muscle, liver and kidneys, making skeletal muscle an important metabolic regulator to ensure that glucose is readily available for contraction when needed (Argilés et al., 2016). Skeletal muscle is also the body's largest protein reservoir, which can be accessed in cases where there is not enough glucose to satisfy the body's energetic needs (Argilés et al., 2016). Muscle proteins are continuously being degraded and synthesized, and the balance between this degradation and synthesis determines the total muscle protein content (Witard et al., 2016). In normal metabolic conditions, protein synthesis replenishes any protein that is broken down, however in cases of severe metabolic stress, there is an excess of protein degradation, resulting muscle protein content and muscle mass loss (Argilés et al., 2016).

Myokines are cytokines and peptides secreted by muscle fibers that act in an autocrine, paracrine and / or endocrine manner to mediate intra- and inter-organ communication (Eckel, 2019; Severinsen & Pedersen, 2020). Myokines have important roles in regulating homeostatic and metabolic functions in the muscle and other organs (Lee & Jun, 2019; Severinsen & Pedersen, 2020). For example, myokines work in an autocrine fashion to regulate myogenesis and muscle metabolism and in an endocrine fashion to regulate various homeostatic functions such as appetite, lipolysis and bone formation to name a few (Severinsen & Pedersen, 2020). Acute and chronic conditions can result in metabolic changes and loss of homeostasis in the

muscle resulting in muscle atrophy, and this can be accompanied by myokine expression changes (Argilés et al., 2016; Piccirillo, 2019; Severinsen & Pedersen, 2020). Changes in the expression of myokines related to muscle homeostasis and myogenesis have effects on muscle mass maintenance (Piccirillo, 2019). One myokine that has a negative effect when upregulated is myostatin, which inhibits differentiation and negatively regulates skeletal muscle size by inhibiting the mTOR pathway (Trendelenburg et al., 2009). Additionally, downregulation of the myokine FSTL-1, which is involved in muscle vascularization, was showed to be associated with loss of muscle mass (de Castro et al., 2021; Ouchi et al., 2008). To date, over 3000 myokines have been identified, however little is known about their function, as they are not fully characterized and few studies have focused on the role of myokines in muscle atrophy (Lee & Jun, 2019; Piccirillo, 2019).

1.2. Skeletal muscle regeneration

Following damage and injury, skeletal muscle can repair itself through the process of myogenic differentiation (Howard et al., 2020). Muscle resident stem cells, or satellite cells, are a population of quiescent cells located between the basal lamina and sarcolemma that become activated following muscle injury (Howard et al., 2020; Mauro, 1961). The activated satellite cells enter the cell cycle to proliferate and differentiate into myogenic progenitors (Howard et al., 2020; Jin et al., 2018). These cells continue to differentiate and fuse with each other or to existing myofibers to repair the injured muscle (Chazaud, 2020; Howard et al., 2020; Jin et al., 2018). This transition from activated satellite cell to myofiber involves dynamic changes to the protein expression profile of these cells (Forcina et al., 2020). There are four Myogenic Regulatory Factors (MRFs), MYF5, Myogenin, MYOD and MRF4, which are transcription factors that regulate the process of differentiation and fusion (Hernández-Hernández et al., 2017; Relaix et al., 2021). These transcription factors contain a basic helix-loop-helix domain which

allows them to bind to the regulatory sequence of their target genes (Hernández-Hernández et al., 2017). They are each expressed at different stages of differentiation to coordinate this process (Hernández-Hernández et al., 2017).

Quiescent satellite cells express the transcription factors PAX7 and MYF5 (Beauchamp et al., 2000). Once activated, committed satellite cells will downregulate PAX7 and upregulate MYOD and enter the differentiation pathway (Cornelison & Wold, 1997; Olguin et al., 2007). As the cells begin to differentiate, myogenin is upregulated, which induces MYF5 downregulation (Cornelison & Wold, 1997; Deato et al., 2008). The combined expression of Myogenin and MYOD induces MRF4 expression, which allows for the expression of late muscle differentiation genes and allows for fusion and myofiber formation (Hinterberger et al., 1991). In the mature fiber, MRF4 expression persists, however myogenin and MYOD are downregulated (Hinterberger et al., 1991). During regeneration, a subset of the activated satellite cells downregulate MYOD, but retain high levels of PAX7 and return to quiescence to maintain the satellite cell pool in a process called self-renewal (Chazaud, 2020; Howard et al., 2020; Olguin & Olwin, 2004).

The process of muscle regeneration and the activity of satellite cells is regulated by the muscle environment that is made up of the myofiber, immune cells, endothelial cells and fibro-adipogenic progenitors (FAPs), which are surrounded by an extracellular matrix (ECM) (Mashinchian et al., 2018). The niche plays an important role in tightly regulating satellite cell quiescence and activation in response to injury in order to coordinate the process of muscle repair and maintain the satellite cell pool (Howard et al., 2020; Relaix et al., 2021). The ECM is composed of fibers such as laminin and collagen which are important in regulating both satellite cell quiescence and activation (Mashinchian et al., 2018; Relaix et al., 2021). Satellite cells are

able to produce Collagen V which maintains their quiescence and is an important component of the ECM (Baghdadi et al., 2018). Deletion of the *Col5a1* gene, which encodes for Collagen V, results in precocious differentiation and depletion of the satellite cell pool, underlining the importance of the ECM in regulating satellite cell quiescence (Baghdadi et al., 2018). Endothelial cells and satellite cells are often located near each other in the muscle environment and interact with each other to coordinate myogenesis and angiogenesis (Christov et al., 2007; Mashinchian et al., 2018; Relaix et al., 2021). Endothelial cells support the proliferation of satellite cells, and differentiating myogenic progenitors support angiogenesis, allowing for capillarization in regenerating muscle (Christov et al., 2007). Additionally, pericytes, an endothelial associated cell, secrete angiopoietin-1 which maintains satellite cells in a state of quiescence (Kostallari et al., 2015). Depletion of these cells results in an increase in cycling satellite cells (Kostallari et al., 2015). FAPs support satellite cells through the process of differentiation by generating transient fibrosis to maintain the structure of the regenerating muscle (Collins & Kardon, 2021). They also play a role in maintaining both skeletal muscle mass and the satellite cell pool (Wosczyna et al., 2019).

Inflammation and immune cell recruitment is a critical process for proper muscle regeneration (Chazaud, 2020; Howard et al., 2020; Tidball, 2005). The infiltration of immune cells following muscle damage is essential to remove necrotic tissue and aid in the regulation of satellite cell activation and differentiation (Fig. A) (Chazaud, 2020; Howard et al., 2020; Tidball, 2005). Following muscle damage, muscle resident macrophages secrete chemoattractants such as CXCL1 and CCL2, and myofibers release damage-associated molecular patterns (DAMPs) such as HMGB1, which contributes to the activation and recruitment of immune cells (Ho et al., 2022; Howard et al., 2020). In addition to this, in response to damage, muscle-resident mast cells are

activated and secrete proinflammatory mediators such as TNF-α and IL-1β resulting in the recruitment of mast cells, neutrophils and other immune cells to the damaged tissue as well as increased expression of E-selectin on vascular endothelium to facilitate immune cell adhesion (Butterfield et al., 2006; Howard et al., 2020; W. Yang & Hu, 2018). The activated endothelium releases IL-8 and IL-6 which attracts neutrophils, the first cells to infiltrate damaged tissue (Butterfield et al., 2006; Howard et al., 2020; Tidball, 2005). These cells are important for clearing out damaged muscle fibers and recruiting monocytes to the site of injury through the secretion of IL-8 and IL-1 (C. F. P. Teixeira et al., 2003; W. Yang & Hu, 2018). In fact, neutrophil depletion results in impaired clearing of the necrotic tissue following muscle injury (C. F. P. Teixeira et al., 2003). The recruited monocytes differentiate into macrophages, which are first polarized into an M1 pro-inflammatory phenotype (Howard et al., 2020). These macrophages inhibit myogenic differentiation and secrete proinflammatory cytokines and reactive oxygen species (ROS) to remove damaged fibers and debris and recruit additional immune cells (Nguyen & Tidball, 2003). Following this, there is an expansion of M2 anti-inflammatory macrophages which are important in resolving the inflammation through secretion of anti-inflammatory cytokines and supporting muscle growth and differentiation (Arnold et al., 2007; W. Yang & Hu, 2018). The switch from a predominantly M1 to M2 macrophage population is essential, as dysregulation in this transition was shown to result in decreased regeneration and muscle growth (Mounier et al., 2013). A recent study found that AMPKα1, a regulator of energy homeostasis, is involved in the transition from the M1 to M2 macrophage phenotype (Mounier et al., 2013). Mice with AMPKα1 macrophage knock out (KO) had impaired regeneration and a lack of switching from the M1 to M2 phenotype (Mounier et al., 2013). As such, coordination of these processes and the resolution of inflammation is an integral

part of proper muscle regeneration and the return to homeostasis (Chazaud, 2020; Jin et al., 2018; Mounier et al., 2013).

1.3. Cancer cachexia

Cancer cachexia is a complex paraneoplastic and metabolic syndrome characterized by the loss of body and skeletal muscle mass accompanied by systemic inflammation (Aoyagi et al., 2015; K. Fearon et al., 2011, 2011; Muscaritoli et al., 2006). Central to the pathophysiology of cancer cachexia is a negative protein and energy balance which is driven by abnormal metabolism and results in weight loss greater than 5% over 6 months (K. Fearon et al., 2011; K. C. H. Fearon, 2008). This disorder affects up to 80% of advanced cancer patients and directly contributes to more than 30% of cancer related deaths (AlSudais et al., 2022; Onesti & Guttridge, 2014). Cachectic patients have an elevated mortality rate due to immobility, cardiac and respiratory failure and reduced effectiveness of cancer treatment as a result of the loss of skeletal muscle mass (K. C. H. Fearon, 2008). Although all cancers have the capacity to induce cachexia, gastric, pancreatic, lung, colorectal and head and neck cancers have the highest incidence of cachexia (K. C. H. Fearon, 2008; Lim et al., 2020). There are currently no treatments for cachexia, as there can be multiple mechanisms of muscle wasting involved, which has made the identification of suitable treatments difficult (K. Fearon et al., 2011)

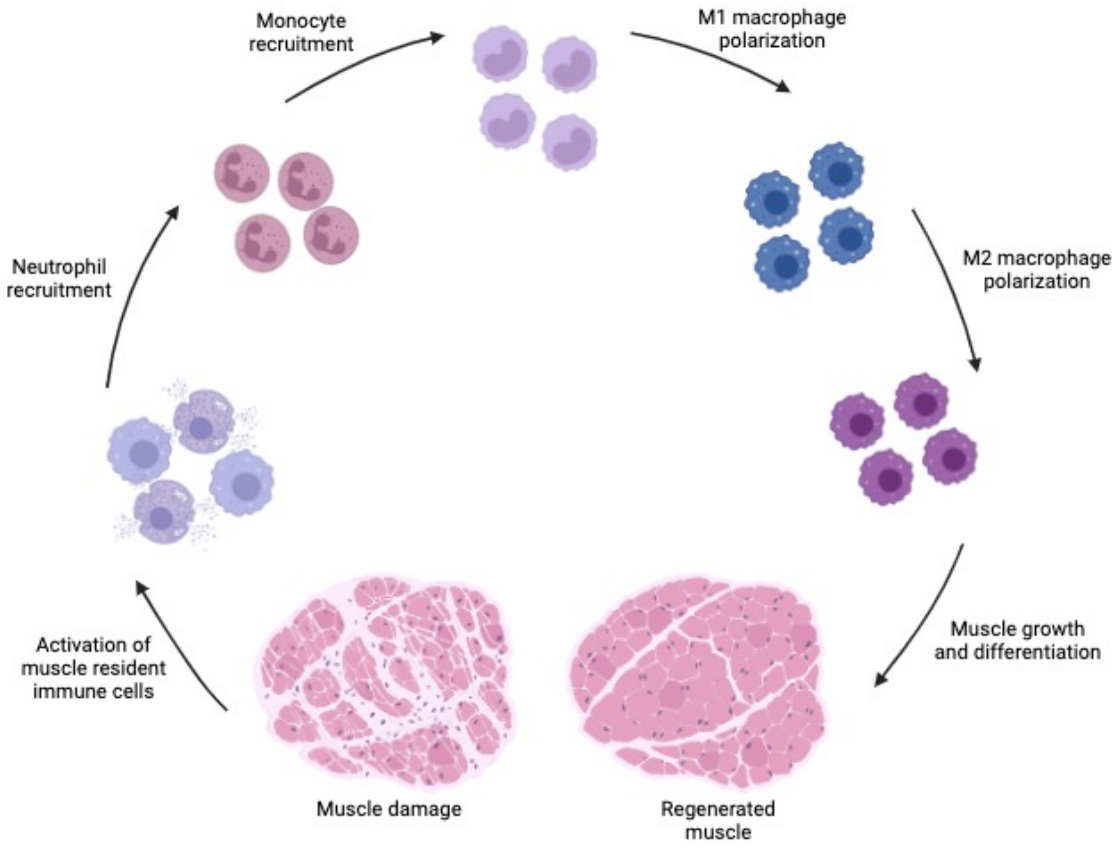

Figure A. Immune cell recruitment in skeletal muscle regeneration. Schematic representation of the order of immune cell recruitment and activation during skeletal muscle regeneration. Following damage, muscle resident mast cells and macrophages become activated and release pro-inflammatory cytokines leading to the recruitment and activation of neutrophils. Neutrophils release IL-1 and IL-8 recruiting monocytes to the site of injury. Monocytes differentiate into macrophages and are first polarized to an M1 pro-inflammatory phenotype to clear out damaged tissue, then M2 anti-inflammatory macrophage phenotype to promote muscle growth and differentiation.

1.4. Inflammation and cancer cachexia

Transient inflammation is important for the process of skeletal muscle repair, however chronic inflammation in the muscle environment is detrimental to muscle homeostasis as persistent pro-inflammatory signaling promotes wasting by inhibiting protein synthesis and increasing protein breakdown (Chazaud, 2020; Howard et al., 2020; Jin et al., 2018; Webster et al., 2020). Systemic inflammation is a hallmark of cachexia and contributes to the muscle atrophy seen with this disorder (AlSudais et al., 2022; Petruzzelli & Wagner, 2016). Pro-inflammatory cytokines TNF-α, IL-6, IL-1 and IFN-γ are elevated in cachexia and contribute to muscle wasting by increasing protein degradation, inhibiting protein synthesis and inhibiting muscle regeneration, resulting in the net loss of skeletal muscle mass (Aoyagi et al., 2015; Chazaud, 2020; He et al., 2013; Jiang & Clemens, 2006; Y.-P. Li & Reid, 2000; Petruzzelli & Wagner, 2016; Robert et al., 2012; Tisdale, 2009).

Protein degradation in skeletal muscle is increased through the activation of the ubiquitin-proteasome and autophagy-lysosome pathways, which degrade myofibrillar and regulatory proteins and cytoplasmic constituents (Law, 2022; Onesti & Guttridge, 2014; Sin et al., 2019). The autophagy-lysosome pathway is a catabolic system which degrades damaged organelles and macromolecules in the cell (Sandri, 2016). This pathway involves multiple steps which are regulated by autophagy genes, some of which have been shown to be upregulated in cachexia (Sandri, 2016). The ubiquitin-proteasome pathway allows for targeted degradation of ubiquitinated proteins (Sandri, 2016). E1, E2, and E3 enzymes are involved in the process of ubiquitination; E1 is the ubiquitin activating enzyme, E3 is the ubiquitin ligase which catalyzes the transfer of ubiquitin from the E2 enzyme to the target protein (Sandri, 2016). Following ubiquitination, the protein is degraded by the proteasome (Sandri, 2016). TRAF6 and MuRF1 are two E3 ubiquitin ligases shown to be involved in cancer cachexia (Neyroud et al., 2023; Paul et

al., 2010). Skeletal muscle TRAF6 KO mice inoculated with Lewis Lung Carcinoma (LLC) tumors had very little change in myofiber cross sectional area, indicating a role for TRAF6 in regulating muscle wasting in cachexia (Paul et al., 2010). Similarly, cachexia was induced in wild type (WT) mice inoculated with KPC tumors, however MuRF1 KO mice were spared, demonstrating a role of this ubiquitin ligase in skeletal muscle atrophy in cachexia (Neyroud et al., 2023).

Protein synthesis is decreased through inhibition of the mTOR pathway and targeted degradation of translation initiation factors, preventing protein translation (Lagirand-Cantaloube et al., 2008; Law, 2022; Tisdale, 2009). The Akt-mTORC1 pathway is important in regulating skeletal muscle mass and function, and activation of this pathway increases protein synthesis (Geremia et al., 2022). In cachexia, there is a decrease in Akt-mTORC1 activation which contributes to muscle wasting (Geremia et al., 2022).

Muscle satellite cell activity is also inhibited, leading to defects in muscle regeneration (AlSudais et al., 2022; He et al., 2013; Law, 2022; Marchildon et al., 2015; Sin et al., 2019). Marchildon et al. showed that PAX7+ satellite cells are increased in the uninjured tibialis anterior (TA) muscle of LLC tumor bearing mice (Marchildon et al., 2015). They also showed that following injury to the cachectic muscle, fibrosis and immune infiltration was increased and regeneration was reduced when compared to control mice (Marchildon et al., 2015). Additionally, the satellite cells in cachectic muscle maintain a high level of PAX7 and low levels of MyoD and myogenin, which prevents them from differentiating, as downregulation of PAX7 is required for differentiation (He et al., 2013). Through these mechanisms, pro-inflammatory cytokines induce a metabolic shift increasing catabolic signaling while reducing anabolic

signaling, resulting in negative energy balance, protein breakdown and muscle atrophy (Aoyagi et al., 2015; Law, 2022; Tisdale, 2009).

1.5. Skeletal muscle changes in cancer cachexia

1.5.1. Inflammatory muscle environment

Chronic inflammation in the muscle environment is seen in cachexia and directly contributes to muscle wasting (AlSudais et al., 2022; Petruzzelli & Wagner, 2016). The source of the inflammation and pro-inflammatory cytokines has yet to be fully understood and is likely due to both tumor and host derived factors (Onesti & Guttridge, 2014). Research has shown that pro-inflammatory cytokines are released by both the tumor and immune cells which are activated in response to the body's anti-tumor response (Onesti & Guttridge, 2014). Recent research into myokine expression has suggested that skeletal muscle may secrete pro-inflammatory and / or pro-cachectic factors in response to cachectic tumors (AlSudais et al., 2022). Studies have shown increased muscle expression of myostatin, a myokine which prevents muscle growth, in animal models of cachexia (Costelli et al., 2008). It has been shown that inhibiting myostatin signaling in multiple models of cachexia prevents further loss of skeletal muscle mass and reverts any prior loss seen, likely due to ubiquitin proteasome pathway inhibition and satellite cell activation (X. Zhou et al., 2010). These findings suggest that the muscle itself may change its myokine expression in response to cachectic tumors, and these changes may contribute in part to the inflammation and muscle wasting seen in cachexia.

1.5.2. Skeletal muscle immune cell infiltration and cachexia

Immune dysfunction is seen in cachexia, however the role of the immune system in cachexia is not well understood (Ferrara et al., 2022). Recent studies have shown that there is a change in the immune cell populations present in the muscle in the context of cachexia (Anoveros-Barrera et al., 2019; Leal et al., 2021; Martin & Freyssenet, 2021). A study conducted

by Salazar-Degracia et al. found that there was an increased number of inflammatory cells in the gastrocnemius and diaphragm of cachectic rats relative to the control, however their study did not look at the specific cell populations that were affected (Salazar-Degracia et al., 2018). One study looking at the role of macrophages in cancer cachexia found that the number of M2 macrophages are increased in the muscle of cachectic patients (Shukla et al., 2020). They also found that macrophage depletion restored body weight relative to control tumor bearing mice (Shukla et al., 2020). Studies focusing on changes in other immune cell populations in the muscle with cachexia as well as the timeline of infiltration with respect to muscle wasting has not been explored.

1.5.3. Muscle damage and cachexia

Muscle damage can occur due to a wide variety of stimuli such as exercise, injury or disease (Tidball, 2017). Recent studies have shown that skeletal muscle becomes damaged in cachexia (He et al., 2013; Iwata et al., 2016; Talbert et al., 2014; Talbert & Guttridge, 2016). The cachectic muscle shows an increase in IgG and Evans blue staining (markers of membrane damage), indicating that sarcolemmal perturbations occur in cachexia in both human and animal models (He et al., 2013; Talbert et al., 2014; Talbert & Guttridge, 2016). In addition, electron microscope imaging of human cachectic and non-cachectic individuals found membrane alterations in the cachectic individuals which worsened with increased weight loss (He et al., 2013). Although we know that this damage is not the result of tumor cells infiltrating into the muscle, the specific cause of this damage is not known (He et al., 2013; Talbert & Guttridge, 2016).

1.6. Communication pathways and cachexia

Cancer cachexia is a complex syndrome which involves communication between the tumor and host cells (Wang et al., 2024). In cachexia, the tumor secretes factors which will

communicate with host cells to induce changes in these populations, such as metabolic reprogramming, inflammatory responses and alterations in inter-organ communication, ultimately leading to the onset and progression of cachexia (Wang et al., 2024). The tumor derived factors that contribute to cachexia are not only secreted by the cancer cells, but also by cells located in the tumor microenvironment (TME) such as stromal cells and immune cells (Wang et al., 2024). The cancer cells in the tumor secrete proinflammatory cytokines and also release extracellular vesicles which generate an inflammatory response in the host (Hu et al., 2019; Wang et al., 2024). Additionally, cells in the TME such as macrophages, neutrophils and cancer associated fibroblasts (CAFs), also release proinflammatory cytokines that contribute to the inflammatory environment in the tumor and systemically in the host (Wang et al., 2024). It is also known changes to skeletal muscle myokine expression occur in cachexia and are thought to play a role in its progression (Wang et al., 2024; Webster et al., 2020). Systemic changes in communication pathways are thought to be altered in cachexia, leading to dysfunctional signaling, however the scope of these changes are not currently known or very well understood (Wang et al., 2024).

One of the challenges in understanding these pathways is uncovering the source of the inflammation and what is being expressed by each cell type, as different cell populations can express the same factors (Wang et al., 2024). For example, skeletal muscle, tumor associated macrophages (TAMs), CAFs and cancer cells can all secrete IL-6, which we know is elevated in cachexia, however it is unclear if all of these cell types, one, or a combination of these cell populations are secreting IL-6 in the context of cachexia (Guo et al., 2023; Ong et al., 2012; Zimmers et al., 2016). Zimmers et al. looked at the IL-6 communication pathway in cachexia and found that tumor derived IL-6 contributes to muscle atrophy and that muscle atrophy is reduced

in mice bearing IL-6 KO tumors (Rupert et al., 2021). However, they found that tumor IL-6 KO did not spare adipose tissue wasting, and that skeletal muscle derived IL6R was signaling to adipose tissue and contributing to lipolysis (Rupert et al., 2021). This study highlights the complexity of the communication pathways in cachexia and how due to the complicated nature of these pathways, the source of many cachexia inducing factors and muscle wasting is currently unknown. Muscle wasting can be driven directly by tumor secreted factors as has been shown *in vitro* with models of cachexia (AlSudais et al., 2022). However, it is possible that other mechanisms of wasting are also present, as the degree of cachexia *in vitro* is not always as severe as observed *in vivo* (Jackman et al., 2017). Additionally, many of the studies that have focused on identifying factors involved in the pathogenesis of cachexia have used neutralizing antibodies or receptor knockouts (Kim-Muller et al., 2023; Wu et al., 2023; X. Yang et al., 2024). Although these studies have been useful in identifying factors that contribute to muscle wasting in cachexia, such as GDF15 and LIF, these studies do not identify the source of these cachexia inducing factors (Kim-Muller et al., 2023; X. Yang et al., 2024). As such, the main sources of many cachexia inducing factors and the source of muscle wasting is currently unknown.

2. RATIONALE

Cancer cachexia is a complex metabolic syndrome that affects up to 80% of advanced cancer patients and directly contributes to more than 30% of cancer related deaths (AlSudais et al., 2022; Onesti & Guttridge, 2014). Systemic inflammation is a hallmark of cachexia and this inflammation in the muscle environment leads to muscle wasting through dysregulated protein homeostasis and impaired regeneration (AlSudais et al., 2022; Petruzzelli & Wagner, 2016). The communication pathways that are involved in cachexia are complex and poorly understood. Understanding the myofiber response in various models of cachexia will help to uncover myofiber derived cachexia inducing factors and will also help determine the similarity of the

myofiber response to different cachexia causing cancers. This will further our understanding of the role of the myofiber in cachexia and in muscle wasting

3. HYPOTHESIS

Changes in cytokine expression are observed in cachexia, however the cellular source of these mediators and their impact on myofiber integrity are unknown (Webster et al., 2020). I hypothesize that in cachexia, myofibers respond to the tumor secretome by upregulating the expression of pro-inflammatory and immune response mediators, contributing to the recruitment of inflammatory cells and muscle wasting in cachexia.

4. OBJECTIVES

1) Validate the cachectic phenotype of multiple mouse cancer models *in vivo*
2) Validate the cachectic phenotype of multiple mouse cancer models *in vitro*
3) Assess changes in cytokine expression of myotubes exposed to different cancer secretomes
4) Assess changes in myotube gene expression following exposure to cachectic and non-cachectic cancer cell secretomes

5. MATERIALS AND METHODS

5.1. Animal care

All animals used in this study were maintained at 22°C with 30% relative humidity on a 12 hour light / dark cycle and were provided food and water *ad libitum*. All experiments involving animals were approved by the University of Ottawa Animal Care Committee and conducted in accordance with the guidelines set by the Canadian Council on Animal Care.

5.2. *In vivo* assessment of cancer cachexia

For the *in vivo* model of cachexia, $5x10^5$ LLC or MC38 cells in 1X phosphate buffered saline (PBS) were injected subcutaneously in both flanks of 12-15 week old female C57BL/6 mice (Charles River Laboratories). Sham mice were injected with 100 μL of 1X PBS. Mice were

subjected to weekly EchoMRI (Animal Behaviour and Physiology Core, University of Ottawa) to assess changes in body weight, lean mass and fat mass over the course of tumor growth. Mice were sacrificed 3.5 weeks after tumor inoculation and the TA muscle was collected, embedded in optimal cutting temperature (OCT) compound and flash frozen in liquid nitrogen cooled isopentane. 7mm thick TA muscle sections were fixed on charged slides using the HM252NX cryostat for immunohistochemistry. Tumors were collected and weighed to assess tumor free body weight and lean mass.

5.3. Cell culture

C2C12 myoblasts (ATCC) were grown in growth media (GM), composed of Dulbecco's Modified Eagles Medium (DMEM, Wisent, 319-005-CL) supplemented with 10% heat inactivated fetal bovine serum (HI-FBS) and 1% penicillin/streptomycin (P/S). Cultures were maintained at sub confluent densities and were passaged every two days. To passage the cells, cultures were washed with 1X PBS and 1mL of 0.25% trypsin /10cm plate was added. Cells were incubated at 37°C for five minutes and fresh GM was added to inactivate the trypsin. To induce differentiation, once cells reached confluence, they were switched to differentiation media (DM), composed of DMEM supplemented with 2% heat-inactivated horse serum (HI-HS) and 1% P/S. LLC (ATCC), EL4 (From Dr. Marc-André Langlois, University of Ottawa), MC38 (from Dr. Robert Korneluk, CHEO), B16 (from Dr. Barbara Vanderhyden, University of Ottawa), ID8 (from Dr. Barbara Vanderhyden, University of Ottawa), CT2A (from Dr. Robert Korneluk, CHEO), and GL261 (from Dr. Robert Korneluk, CHEO) cancer cell lines were cultured in GM and passaged every two days. All cells were maintained at 37°C and 5% CO_2.

5.4. Conditioned media

Cancer cells were seeded in GM according to seeding densities in Table 1. Cells were cultured for 48 hours to reach 90% confluence and fresh GM was added. 48 hours later the conditioned media (CM) was collected and centrifuged twice at 500g for 5 minutes.

Table 1. Seeding densities for cancer cell lines for conditioned media protocol

Cancer cell line	**Seeding density (cells/10cm plate)**
MC38, B16	$6x10^5$
LLC, EL4, ID8	$8x10^5$
CT2A	$1.2x10^6$
GL261	$1.6x10^6$

5.5. *In vitro* model of cancer cachexia

C2C12 myoblasts were seeded in GM ($8x10^4$/6 well plate, $4x10^5$/10cm plate) and cultured for 48 hours to reach 90% confluency. After 48 hours, cells were switched to DM for 96 hours to allow myotubes to form. Myotubes were treated for 48 hours with a 1:1 mix of DM and CM. The control condition was treated with a 1:1 mix of DM and GM.

5.6. Myotube isolation

To isolate myotubes, differentiating cultures were washed twice with 1X PBS and then treated with 1mL of 0.15% trypsin /10cm plate for 5 minutes at room temperature. The plate was gently washed with 2mL of GM and lifted myotubes were collected. Collected myotubes were centrifuged at 1200g for 3 minutes and media was aspirated. Cells were washed with 1X PBS before being centrifuged again at 1200g for 3 minutes and media was aspirated. Cell pellets were stored at -80℃.

5.7. Immunofluorescence

C2C12s were cultured in 6 well plates following the *in vitro model of cancer cachexia*. Cells were washed twice with 1X PBS for five minutes and fixed in ice cold methanol for 15 minutes at room temperature. Cells were washed twice with 1X PBS and permeabilized for 15 minutes with 1X PBS containing 0.5% Triton-X-100. Cells were incubated overnight at 4°C with the anti-myosin heavy chain primary antibody (MF20, DSHB, 1:50) in 1X PBS with 0.1% Triton-X-100. Cells were washed three times with 1X PBS with 0.1% Triton-X-100 for five minutes and then incubated for 1 hour at room temperature with Cy3 AffiniPure donkey anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoResearch Laboratories, 715-165-150). Cells were washed three times with 1X PBS with 0.1% Triton-X-100 for five minutes and incubated with DAPI (0.5µg/mL) for 1 minute. Cells were washed twice with 1X PBS for five minutes. Images were taken of five random fields of view using the 10x objective on the Zeiss Axio Observer 7 microscope (Cell Biology & Image Acquisition Core Facility, University of Ottawa) for downstream quantification.

TA cross sections were incubated for 1 hour at room temperature in Alexa Fluor 488 conjugated wheat germ agglutinin (WGA) (Invitrogen, W11261, 1:300) in 1X PBS with 1% w/v bovine serum albumin (BSA). Sections were briefly washed in 1X PBS and incubated with DAPI (0.5µg/mL) for 10 minutes. Sections were briefly washed in 1X PBS and left to air dry at room temperature. Slides were mounted with DAKO mounting media (Agilent, S3023). Sections were imaged using the 20x objective on the Zeiss Axio Observer 7 microscope (Cell Biology & Acquisition Core, University of Ottawa) for downstream quantification.

5.8. Proteome profiler mouse XL cytokine array

Protein lysates from C2C12 myotubes were prepared using Lysis Buffer 17 (R&D Systems, 895943) supplemented with 10µg/mL of Aprotinin (Tocris, 4139), 10µg/mL of

Leupeptin (Tocris, 1167) and 10µg/mL of Pepstatin (Tocris, 1190) following the manufacturer's protocol. Protein concentration was estimated using Coomasie Plus (Bradford) assay. Proteome profiler mouse XL cytokine arrays (R&D Systems, ARY028) were prepared following the manufacturer's protocol and were incubated overnight at 4°C with 100µg of protein from each trial (300µg total protein/membrane). Incubation with the detection antibody and streptavidin-horseradish peroxidase (HRP) was performed following the manufacturer's protocol. This was followed by a chemiluminescence reaction using Clarity Western ECL Substrate (BioRad, 1705601). The ChemiDoc MP Imaging system and Image Lab Software (BioRad) was used to detect chemiluminescence. Pixel densities of detected spots on each membrane were measured and analyzed using the Quick spots software (HLImage++, Western Vision Software, https://www.wvision.com/QuickSpots.html). Pixel densities are presented as a log2 fold change (FC) relative to the control conditions.

5.9. RNA isolation, sequencing and analysis

C2C12 myotube RNA was isolated using the RNeasy mini kit (Quiagen, 74104) following the manufacturer's protocol. Total RNA was sent to Genome Quebec for stranded poly-A enriched library preparation and sequencing on the Illumina NovaSeq PE100. 25 million reads were sequenced per sample.

FASTQ files from the RNA-seq were processed using a standard pipeline. Briefly, UseGalaxy (The Galaxy Community et al., 2022) was used to process the sequencing data. FastQC (v.0.12.1) was used to run a quality check on the FASTQ files. The Illumina Nextera adapter was trimmed from sequences using Trimmomatic (v.0.38). FastQC (v.0.12.1) was repeated to quality check the trimmed files. Reads were aligned to the mouse genome (GRCm39 assembly) using Salmon quant (v.1.10.1). Differentially expressed genes were determined using

DESeq2 (v.1.40.2). The differentially expressed genes were annotated using the gencode vM33 annotation.

5.10. Creatine kinase activity assay

C2C12 myotubes were cultured following the *in vitro model of cancer cachexia* using conditioned media from LLC, EL4, MC38 or ID8 cancer cells or control. Media was collected for the Creatine Kinase Activity Assay (Abcam, ab155901) at two different timepoints. The first sample was collected from the initial 1:1 mixture of DM and CM right before it was put on the myotubes, the second was collected at the end of the 48h treatment period. The media samples were stored at -80°C. Creatine kinase activity was measured as per the manufacturer protocols.

5.11. Evans Blue damage assay

C2C12 myotubes were cultured following the *in vitro model of cancer cachexia* using conditioned media from LLC, EL4, MC38 or ID8 cancer cells or control. After 48h treatment with conditioned media, cells were washed once with 1X PBS. The positive control was permeabilized with 1mL of ice-cold methanol and then washed once with 1X PBS. A 0.05% w/v Evans Blue solution was prepared in a 1:1 mix of 5% w/v BSA in 1X PBS and DMEM and filtered through a 0.2µm filter. 1mL of the Evans Blue solution was added to the myotubes and incubated for 10 minutes at room temperature. Cells were fixed in 4% PFA for 10 minutes. Cells were washed once with 1X PBS for five minutes and incubated with DAPI (0.5µg/mL) for 1 minute. Cells were washed twice with 1X PBS for five minutes. Images were taken of 10 random fields of view using the 20x objective on the Zeiss AxioObserver D1 microscope (Cell Biology & Image Acquisition Core Facility, University of Ottawa) for downstream quantification.

5.12. Image analysis

Myotube diameter analysis was performed using Fiji (FIJI Is Just ImageJ). Diameter measurements were taken at 3 points on each myotube (both ends and the middle) from 5 images in each condition.

To assess TA cross sectional area (CSA), Cellpose (Stringer et al., 2021) was used to segment the myofibers. Using Fiji (FIJI Is Just ImageJ), any myofibers that were missed by Cellpose were segmented. Fiji was used to measure the CSA of each myofiber.

Evans Blue positive area was assessed using Fiji (FIJI Is Just ImageJ). Positive control images were used to set a reference intensity which was normalized across all images. Following normalization, images were thresholded, and Evans Blue positive area was quantified.

5.13. Statistical analysis

To determine significance between multiple conditions, a one-way ANOVA was used and if significant differences were found, a multiple comparisons analysis was performed using Tukey's post hoc test. To determine significance between multiple conditions and timepoints, a two-way ANOVA was used. All statistical analysis and graphical representation were performed using GraphPad Prism 10.2.2. Significance is indicated as *($P<0.05$), **($P<0.01$), ***($P<0.001$), ****($P<0.0001$). All experiments represent a minimum of three replicates and are shown as mean ±SD.

6. RESULTS

6.1. LLC and MC38 are cachectic *in vivo*

LLC (Lewis Lung Carcinoma) is a cell line commonly used in the study of cancer cachexia and whose cachectic phenotype has been validated by our lab and others (AlSudais et al., 2022; Talbert et al., 2017; G. Zhang et al., 2017). MC38 (colon adenocarcinoma) is a cell line whose cachectic phenotype is debated in the literature. Some studies have found it to be

cachectic *in vivo*, while other have not seen a cachectic phenotype using this model (Huot et al., 2021; Rohm et al., 2016; Schäfer et al., 2016; Dolly et al., 2023). These differences in phenotype are likely attributed to differences in the experimental protocols used in each study, where the period of tumor growth, number of cells injected, and measures of cachexia all differ.

To assess the cachectic phenotype of LLC and MC38 *in vivo* using the experimental protocol established by our lab, 3 female mice aged 12-15 weeks per condition were inoculated subcutaneously on each flank with $5x10^5$ LLC or MC38 cells; 3 female control mice were injected subcutaneously with PBS. The tumors were allowed to grow for 3.5 weeks before mice were sacrificed, and the left tibialis anterior (LTA) muscle was collected for analysis. Weekly EchoMRIs were performed to assess changes in body mass and composition in response to LLC and MC38 tumor growth. As tumor mass is a confounding factor when assessing body weight and lean mass, tumor mass was subtracted from end point body weight and lean mass in models of cachexia to obtain tumor free body weight and lean mass respectively.

Comparison of tumor free body weight at endpoint to starting body weight revealed a significant decrease in tumor free body weight over the course of 3.5 weeks in MC38 and LLC tumor bearing mice relative to control (Fig. 1A). Both the LLC and MC38 tumor bearing mice exhibited a decrease in body weight, whereas the control mice increased in weight (Fig. 1A). Analysis of change in fat mass over time measured by the EchoMRI showed a significant decrease in fat mass in the LLC tumor bearing mice compared with control (Fig. 1B). Although the MC38 tumor bearing mice trended towards a decrease in fat mass relative to control, it is not significant (Fig. 1B). Comparison of tumor free lean mass at endpoint to starting lean mass found a significant decreased in LLC and MC38 tumor bearing mice compared to controls, which increased in lean mass at endpoint (Fig. 1C). LLC and MC38 tumor bearing mice showed a trend

towards a decreased LTA weight compared to control mice, however this decrease was not significant (Fig. 1D). Cross sectional area analysis of the muscle fibers in the LTA showed no significant differences between any of the conditions, indicating no significant change in myofiber size in the tumor bearing mice compared to controls (Fig. 1E, F). Although there are no significant differences in the CSA of tumor bearing mice, there is a negative correlation between tumor mass and cross-sectional area (Fig. 1G).

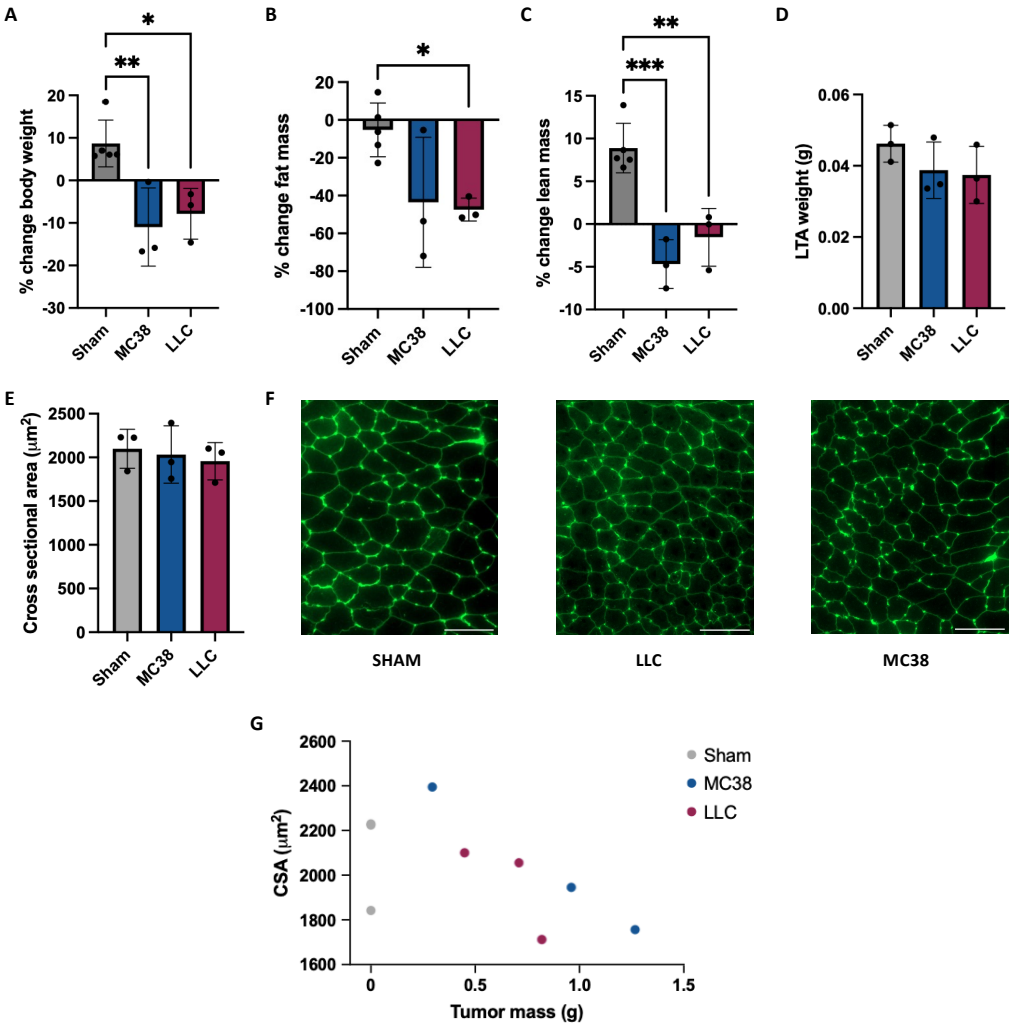

Figure 1. MC38 and LLC are cachectic *in vivo*. Female mice aged 12-15 weeks were inoculated subcutaneously with 5x10^5 LLC or MC38 cells per flank. Control mice were injected subcutaneously with 1X PBS. Tumors were allowed to grow for 3.5 weeks. (A) Percent change in tumor free body weight in Sham, LLC and MC38 tumor bearing mice. (B) Percent change in fat mass mass in Sham, LLC and MC38 tumor bearing mice. (C) Percent change in tumour free lean mass in Sham, LLC and MC38 tumor bearing mice. (D) Cross sectional area of LTA myofibers in Sham, LLC and MC38 tumor bearing mice. (E) LTA weight at endpoint of Sham, LLC and MC38 tumor bearing mice. (F) Wheat germ agglutinin immunostaining (green) of the myofiber membrane border in Sham, LLC and MC38 tumor bearing mice. Scale bar = 100 μm. (G) Plot of tumour mass vs. cross sectional area in Sham, LLC and MC38 tumor bearing mice. (n=3) for MC38 and LLC, (n=5) for Sham. Data presented as mean ±SD. Conditions compared using 1-way ANOVA, * ($P<0.05$), ** ($P<0.01$), *** ($P < 0.001$).

Although there is no significant change in cross sectional area, which is the most common measure of cachexia, there are significant decreases in tumor free body weight and tumor free lean mass in LLC and MC38 tumor bearing mice. Fat mass was also significantly decreased in LLC tumor bearing mice. Altogether, these results suggest that the MC38 and LLC models are cachectic *in vivo* using our experimental protocols, as we observe significant decreases in body weight and lean mass, which are indicative of cachexia.

6.2. LLC cancer secretome induces changes in C2C12 myotube cytokine expression

To understand how myofibers respond to a cachectic milieu, we used an *in vitro* model of cancer cachexia in which C2C12 mouse myoblasts are differentiated for 96 hours, then treated for 48 hours with a 1:1 mix of differentiation media to conditioned media (CM) from either LLC (CM-LLC) or EL4 (CM-EL4) cancer cells (Fig. 2A). To assess cachexia related changes in the cytokine expression of myotubes, LLC was used as the cachectic cancer model and EL4 was used as a non-cachectic cancer control. Unconditioned medium (UC) consisting of a 1:1 mix of differentiation media and tumor cell growth media was used as an additional media control. As shown previously, incubation with CM from LLC cells resulted in a significant decrease in myotube diameter relative to the control condition (Fig. 2B) (AlSudais et al., 2022). Incubation with CM from EL4 cells also resulted in a significant decrease in myotube diameter relative to the control condition, however these myotubes were significantly larger than the LLC CM-treated condition (Fig. 2B).

Cytokine protein expression by myotube whole cell extracts was assessed in each condition with the Proteome Profiler Mouse XL Cytokine Array (R&D Systems; ARY028) (Fig. 2C). First, the effect of treatment with LLC conditioned medium on myotubes was compared to EL4 conditioned medium and unconditioned medium (Fig. 2D). Of the top 10 significantly

upregulated cytokines in LLC CM-treated myotubes relative to UC, 7 are pro-inflammatory cytokines (CCL11, CXCL5, GDF-15, IL-6, CXCL9, IL-33, GM-CSF). Of the top 10 significantly upregulated cytokines in LLC CM-treated myotubes relative to EL4 CM-treated myotubes, 5 of them are pro-inflammatory cytokines (CXCL5, CCL11, M-CSF, GDF-15, IL-6) (Fig 3A, B). To identify cytokines which are upregulated uniquely in the context of cachexia, the top 10 cytokines upregulated LLC CM-treated myotubes relative to UC and upregulated in LLC CM-treated myotubes relative to EL4 CM-treated myotubes were identified. Among these 10 cytokines, 6 are pro-inflammatory cytokines (CXCL5, CCL11, GDF-15, IL-6, GM-CSF, Leptin) (Fig. 3C). This suggests that in the cachectic environment, myotubes begin to express pro-inflammatory cytokines, which is predicted to contribute to the chronic inflammation and muscle wasting in cachexia.

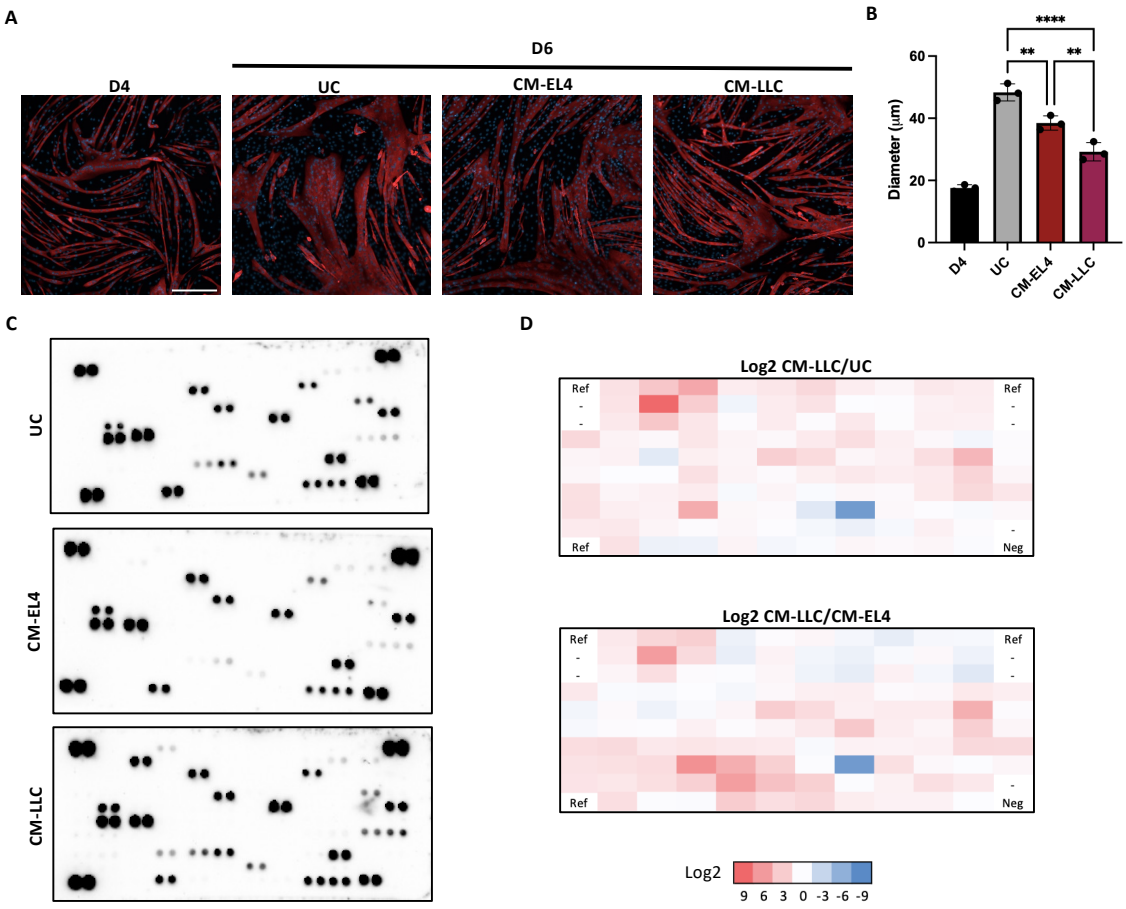

Figure 2. The cachectic cancer secretome decreases the diameter and the alters cytokine expression of C2C12 myotubes. (A) Myosin heavy chain immunostaining (red) of C2C12 myotubes on day 4 of differentiation (D4) and day 6 of differentiation (D6) after treatment with unconditioned medium (UC), or conditioned medium (CM) from EL4 or LLC cells (CM-EL4 and CM-LLC) for 2 days. Nuclei are stained with DAPI (blue). Scale bar = 300 µm. (B) Average myotube diameter of C2C12 myotubes as cultured in (A) (n=3). Data presented as mean ±SD. Conditions compared using 1-way ANOVA, ** ($P < 0.005$), **** ($P < 0.0001$). (C) C2C12 myotubes as cultured in (A) were isolated. 100 µg of protein from 3 different trials were pooled and cytokine expression was assessed using the Proteome Profiler Mouse XL Cytokine Array (R&D Systems; ARY028). (D) Expression heat map for CM-LLC array presented as Log2 fold change (FC) relative to UC and CM-EL4.

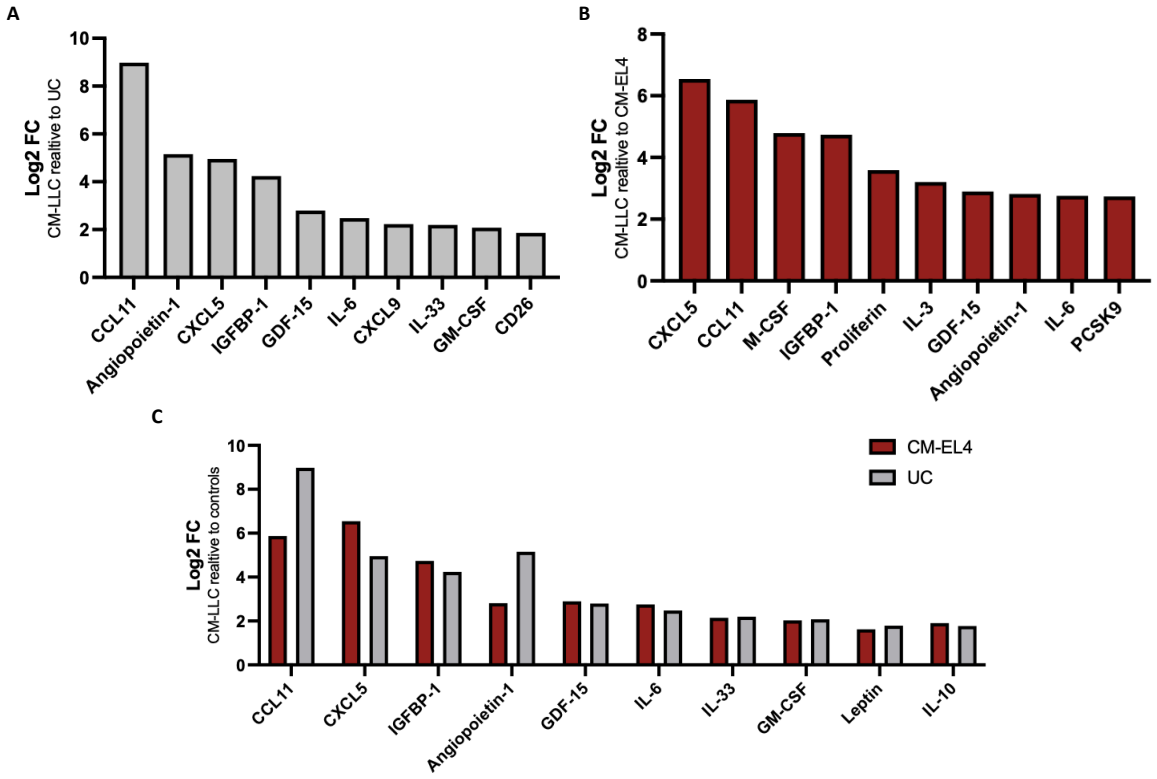

Figure 3. Myotubes overexpress pro-inflammatory cytokines in a cachectic environment. (A) Top 10 upregulated cytokines in LLC CM-treated myotubes compared to UC presented as log2FC. (B) Top 10 upregulated cytokines in LLC CM-treated myotubes compared to EL4 CM-treated myotubes presented as log2FC. (C) Top 10 cytokines upregulated in LLC CM-treated myotubes relative to both EL4 CM-treated myotubes and UC presented as log2FC.

To determine which cells in the muscle environment are targeted by the top 10 upregulated cytokines in LLC CM-treated myotubes relative to EL4 CM-treated myotubes and UC, the expression of the cognate receptors for these cytokines was assessed in the muscle environment using scRNA-seq (single-cell RNA sequencing) data generated by the Wiper-Bergeron and De Lisio laboratories (A. Brown, unpublished). A brief description of the cytokines, their receptors and some of their main functions are described in Table 2.

Table 2. Upregulated cytokines in LLC CM-treated myotubes relative to EL4 CM-treated myotubes and UC and their respective receptors and functions

Cytokine	Receptors	Functions
CCL11	*Ccr2, Ccr3, Ccr5* (Hughes & Nibbs, 2018; A. L. Teixeira et al., 2018)	Immune cell recruitment. Associated with inflammatory diseases (Kindstedt et al., 2017)
CXCL5	*Cxcr2* (Hughes & Nibbs, 2018; Y. Yang et al., 2017)	Neutrophil recruitment (S.-L. Zhou et al., 2012)
IGFBP-1	N/A	Binds to soluble IGF proteins (Bae et al., 2013)
Angiopoietin-1	*Tek* (Saharinen et al., 2015)	Suppresses blood vessel leakage, inhibits vascular inflammation, stimulates angiogenesis and vessel remodeling (Brindle et al., 2006)
GDF-15	*Gndf* (Wischhusen et al., 2020)	Associated with cachexia and contributes to muscle wasting (Kim-Muller et al., 2023; Wischhusen et al., 2020)
IL-6	*Il6ra*, *Il6st* (O'Shea et al., 2023; Wolf et al., 2014)	Produced in response to infection and tissue injury. Dysregulation involved in chronic inflammation (Tanaka et al., 2014)
IL-33	*Il1rl1* (Brunner et al., 2024)	Plays a role in innate and adaptive immune response (Drake & Kita, 2017)
GM-CSF	*Csfra, Csfrb* (Y. Shi et al., 2006)	Hematopoietic growth factor.

		Recruits neutrophils, monocytes and lymphocytes (Y. Shi et al., 2006)
Leptin	*Lepr* (Francisco et al., 2018)	Regulates energy metabolism and immune cell recruitment (Francisco et al., 2018)
IL-10	*Il10ra, Il10rb* (Iyer & Cheng, 2012; Shouval et al., 2014)	Attenuates immune response and maintains homeostasis (Iyer & Cheng, 2012; Shouval et al., 2014)

This data shows that in the muscle environment, *Ccr2* and *Ccr5* are expressed in monocytes / macrophages and T cells / Natural killer (NK) cells and *Cxcr2* is highly expressed in neutrophils (Fig. 4). *Il6ra* is expressed in FAPs monocytes / macrophages, neutrophils and T cells / NK cells and *Il6st* is broadly expressed across many cell types in the muscle environment (Fig. 4). Given that *Il6ra* can only signal upon forming a receptor complex with *Il6st*, IL-6 would signal through cells co-expressing these genes, which includes FAPs, monocytes / macrophages and T cells / NK cells (Fig. 4). Additionally, *Il6ra* is capable of being cleaved by metalloproteinases which allows for trans-signalling in cells expressing *Il6st* (Scumacher et al., 2015). Thus, it is possible that MuSCs (muscle stem cells), endothelial cells, vSMCs (vascular smooth muscle cells) / pericytes, tenocytes, neural / glial cells and schwann cells would also be able to respond to IL-6 signalling (Fig. 4). *Csfra* and *Csfrb* are highly expressed in monocytes / macrophages and neutrophils (Fig. 4). *Lepr* is expressed in tenocytes and *Il10ra* and *Il10rb* are expressed across a wide variety of different immune cells (Fig. 4). *Tek* is highly expressed in MuSCs / progenitors, endothelial cells and FAPs (Fig. 4). *Ccr3, Gndf* and *Il1rl1* are not significantly expressed in the muscle environment (Fig. 4) These results suggest that in the context of cachexia, myokine expression shifts towards an inflammatory phenotype and the

muscle begins to express immune cell chemoattractants, which may recruit neutrophils and other inflammatory cell types to the muscle environment.

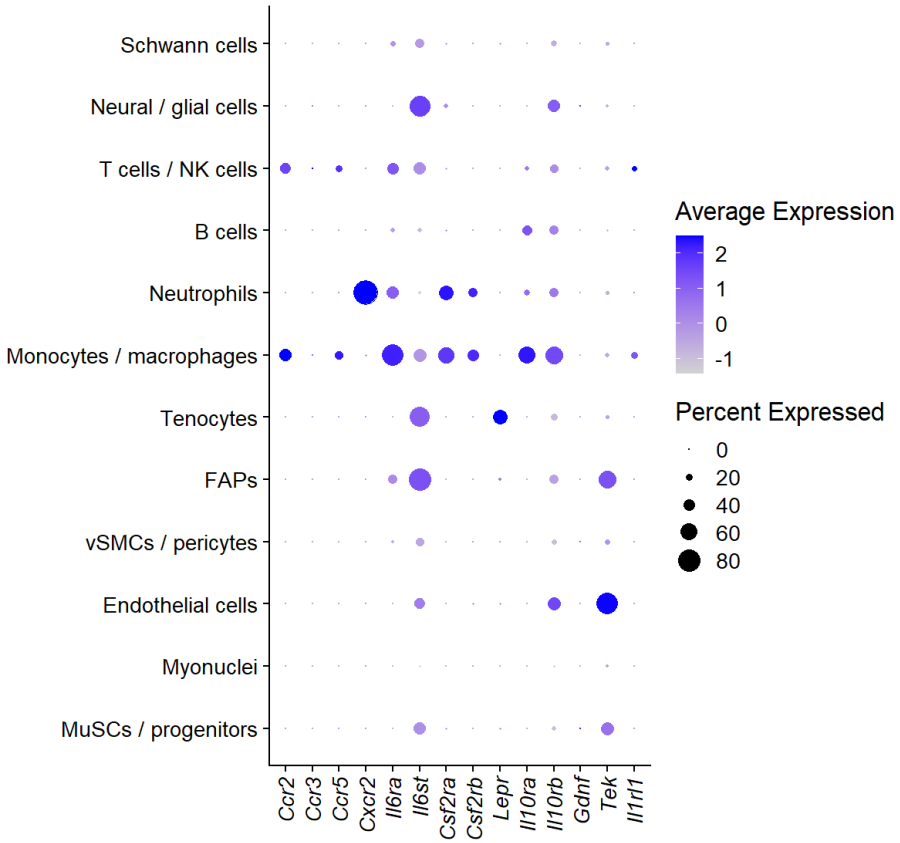

Figure 4. Expression of the cognate receptors in the muscle environment for the top 10 upregulated cytokines in LLC CM-treated myotubes relative to EL4 CM-treated myotubes and UC. Data obtained from scRNA-seq of healthy muscle of 15-week-old male mice performed by our lab (A. Brown, unpublished). Receptor ligands can be found in Table 2.

6.3. Multiple cancer secretomes induce changes to C2C12 myotube cytokine expression

Although LLC is a good model of cancer cachexia, there are other types of cancers, such as pancreatic and colon cancers, that also cause cachexia (Leal et al., 2021; Nosacka et al., 2020). It has also been shown that different types of cancers will affect the severity of the cachectic phenotype (K. C. H. Fearon, 2008). Due to these differences in the presentation of cachexia, we wanted to determine if the myotube response to the cachectic cancer secretome was consistent across different types of cancers or differed based on the cancer type. To validate the cachectic phenotype and effect on myotube cytokine expression, C2C12 myotubes were treated with conditioned media from MC38, CT2A (glioblastoma), B16 (melanoma), ID8 (ovarian cancer) and GL261 (glioma) cancer cells.

Incubation with CM from MC38 (CM-MC38), CT2A (CM-CT2A), ID8 (CM-ID8) or GL261 (CM-GL261) did not result in any significant change in myotube diameter relative to UC (Fig. 5A, B). Incubation with CM from B16 (CM-B16) resulted in a significant decrease in myotube diameter relative to UC (Fig. 5A, B). This suggests that of the 5 cancer cell lines screened, only the B16 cell line models a cachectic cancer *in vitro.* This is an interesting finding as we found that the MC38 model induces cachexia *in vivo* (Fig. 1). This suggests that the MC38 model indirectly induces cachexia, perhaps by targeting a different muscle-resident cell population rather than myofibers to induce muscle wasting.

Cytokine protein expression in each condition was assessed with the Proteome Profiler Mouse XL Cytokine Array (R&D Systems; ARY028) using whole cell extracts from treated myotubes. Analysis of the cytokine arrays found the most significant expression changes in MC38 and B16 CM-treated myotubes relative to UC (Fig. 5C). 105 of the 111 cytokines screened were upregulated in MC38 CM-treated myotubes relative to UC and 94 were

upregulated in B16 CM-treated myotubes relative to UC (Fig. 5C). Of the 7 significantly upregulated cytokines in ID8 CM-treated myotubes compared to UC, 4 are pro-inflammatory (Serpin-E1, osteopontin (OPN), WISP-1, pentraxin-2) (Fig. 6A). GL261 and CT2A CM-treated myotubes both have 5 significantly upregulated cytokines compared to UC, 2 of which are pro-inflammatory (CM-GL261: OPN, CCL5; CM-CT2A: OPN, WISP-1) (Fig. 6B, C). These results suggest that in response to a non-cachectic cancer secretome, the myotubes do not have a very significant change in their cytokine expression, and relatively few of the upregulated cytokines are inflammatory in nature.

Of the top 10 upregulated cytokines in MC38 CM-treated myotubes, 7 are pro-inflammatory (CCL11, CD14, tissue factor, IL-1β, GDF-15, CCL17, CXCL9), whereas for B16 CM-treated myotubes, only 4 are pro-inflammatory (GDF-15, leptin, E-selectin, CCL6) (Fig. 6D, E). To determine which cells in the muscle environment might be targeted by these upregulated cytokines, the expression of the cognate receptors for these cytokines was assessed in the muscle environment. A brief description of the upregulated cytokines in MC38 CM-treated myotubes, their receptors and some of their main functions are described in Table 3.

Table 3. Top 10 upregulated cytokines in MC38 CM-treated myotubes relative to UC and their respective receptors and functions

Cytokine	Receptors	Functions
CCL11	*Ccr2, Ccr3, Ccr5* (Hughes & Nibbs, 2018; A. L. Teixeira et al., 2018)	Immune cell recruitment. Associated with inflammatory diseases (Kindstedt et al., 2017)
HGF	*Met* (Cecchi et al., 2011)	Regulates skeletal muscle differentiation and transition to M2 macrophage phenotype (Choi et al., 2019; Zheng et al., 2016)
CD14	N/A	Co-receptor for TLRs (Sharygin et al., 2023)

Tissue factor	N/A	Primary initiator of blood coagulation (Mackman, 2006)
IL-1β	*Il1r1* (Kaneko et al., 2019)	Mediator of inflammatory and pathogen response. Exacerbates damage in tissue injury (Lopez-Castejon & Brough, 2011)
GDF-15	*Gndf* (Wischhusen et al., 2020)	Associated with cachexia and contributes to muscle wasting (Kim-Muller et al., 2023; Wischhusen et al., 2020)
G-CSF	*Csf3* (Panopoulos & Watowich, 2008)	Regulates granulopoiesis and neutrophil development (Panopoulos & Watowich, 2008)
Cystatin C	*Tgfbr2* (Sokol et al., 2005; Tzavlaki & Moustakas, 2020)	Ubiquitously expressed inhibitor of lysosomal and cysteine proteinases (Fernando & Polkinghorne, 2020)
CCL17	*Ccr4* (Scheu et al., 2017)	Recruits immune cells (Ye et al., 2022)
CXCL9	*Cxcr3* (Tokunaga et al., 2018)	Immune cell differentiation, activation, and migration (Tokunaga et al., 2018)

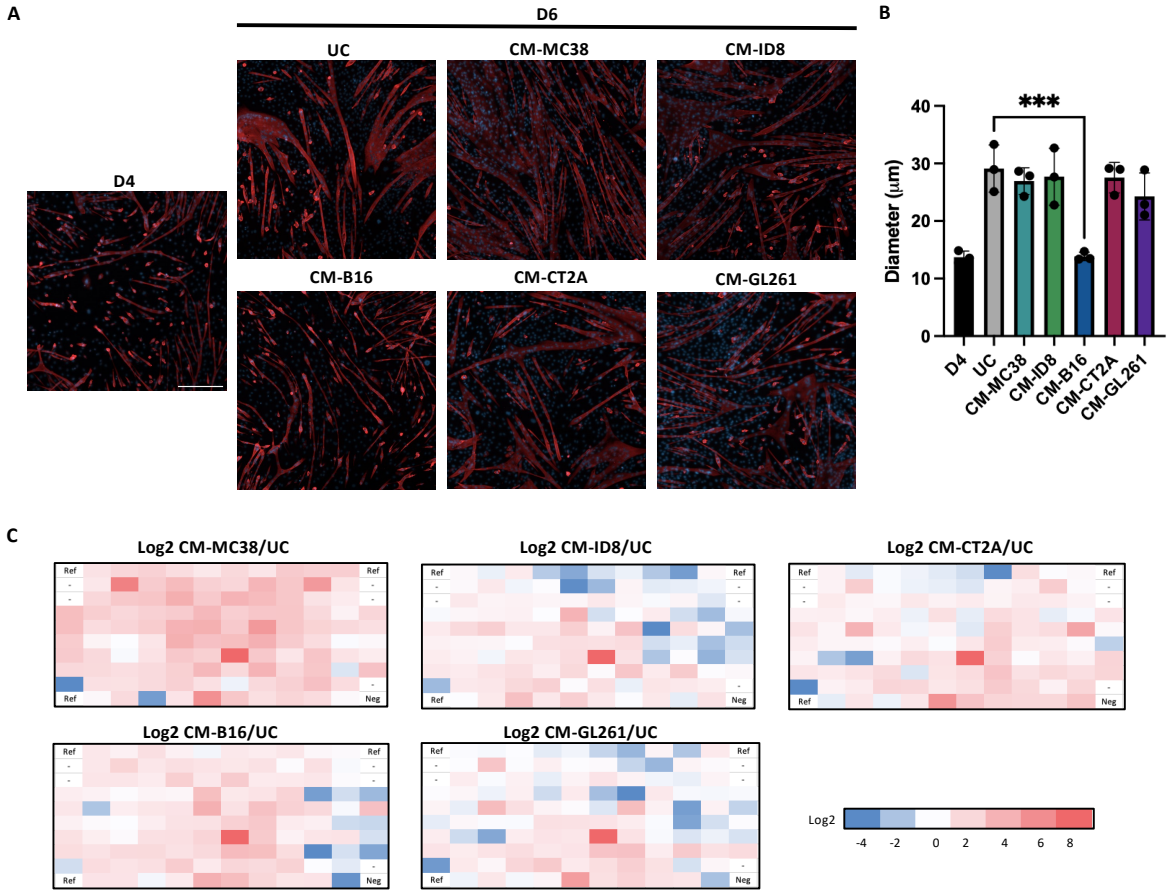

Figure 5. Multiple cancer secretomes alter the cytokine expression of C2C12 myotubes. (A) Myosin heavy chain immunostaining (red) of C2C12 myotubes on day 4 of differentiation (D4) and day 6 of differentiation (D6) after treatment with unconditioned medium (UC), or conditioned medium (CM) from MC38, ID8, B16, GL261 or CT2A cells (CM-MC38, CM-ID8, CM-B16, CM-GL261 and CM-CT2A) for 2 days. Nuclei are stained with DAPI (blue). Scale bar = 300 µm. (B) Average myotube diameter of C2C12 myotubes as cultured in (A) (n=3). Data presented as mean ± SD. Conditions compared using 1-way ANOVA, *** ($P < 0.001$). (C) C2C12 myotubes as cultured in (A) were collected. 3 trials were pooled and a Proteome Profiler Mouse XL Cytokine Array (R&D Systems; ARY028) was used to assess cytokine expression. (B) Expression heat map for CM-MC38, CM-ID8, CM-CT2A, CM-B16 and CM-GL261 arrays presented as log2FC relative to UC.

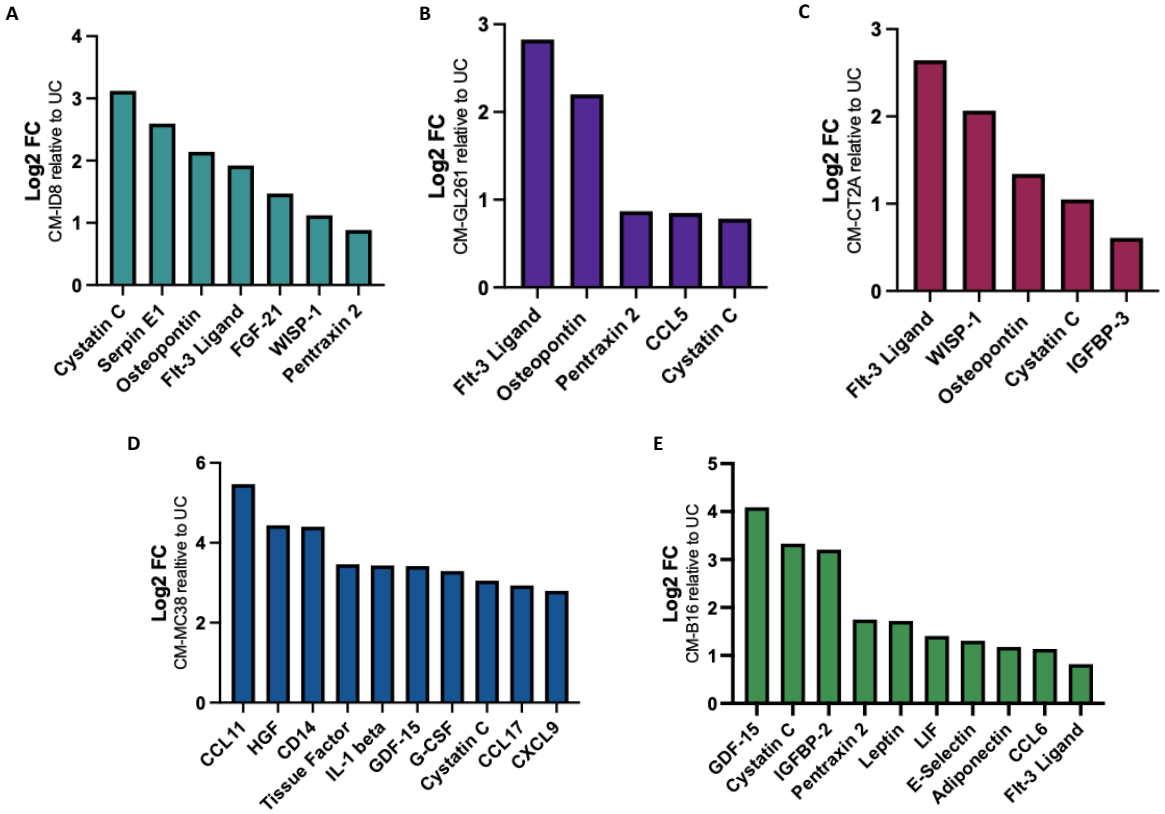

Figure 6. Myotubes overexpress pro-inflammatory cytokines and immune cell chemoattractants in response to B16 and MC38 CM. (A) Significantly upregulated cytokines in ID8 CM-treated myotubes compared to UC presented as log2FC. (B) Significantly upregulated cytokines in GL261 CM-treated myotubes compared to UC presented as log2FC. (C) Significantly upregulated cytokines in CT2A CM-treated myotubes compared to UC presented as log2FC. (D) Top 10 upregulated cytokines in MC38 CM-treated myotubes compared to UC presented as log2FC. (E) Top 10 upregulated cytokines in B16 CM-treated myotubes compared to UC presented as log2FC.

scRNA-seq data generated by the Wiper-Bergeron and De Lisio laboratories was used to assess the expression of the receptors found in Table 3 (A. Brown, unpublished). *Ccr2* and *Ccr5* are expressed in monocytes / macrophages and T cells / NK cells and *Met* is primarily expressed in tenocytes (Fig. 7). *Il1r1* is mainly expressed in MuSCs / progenitors, vSMCs / pericytes and FAPs (Fig. 7). *Tgfrb2* is expressed across a large variety of cell types and *Cxcr3* is expressed in T cells / NK cells (Fig. 7). *Ccr3, Gndf* and *Csf3* are not significantly expressed in the muscle environment (Fig. 7). These findings suggest that in response to the cachectic MC38 cancer secretome, the myotubes are targeting immune cells and other non-immune cell populations, even in the absence of an observable cachectic phenotype. This shows that although there is no wasting effect *in vitro*, there is still an effect at the level of cytokine protein expression, whereby the myotubes begin to express pro-inflammatory cytokines which target other cells in the muscle environment.

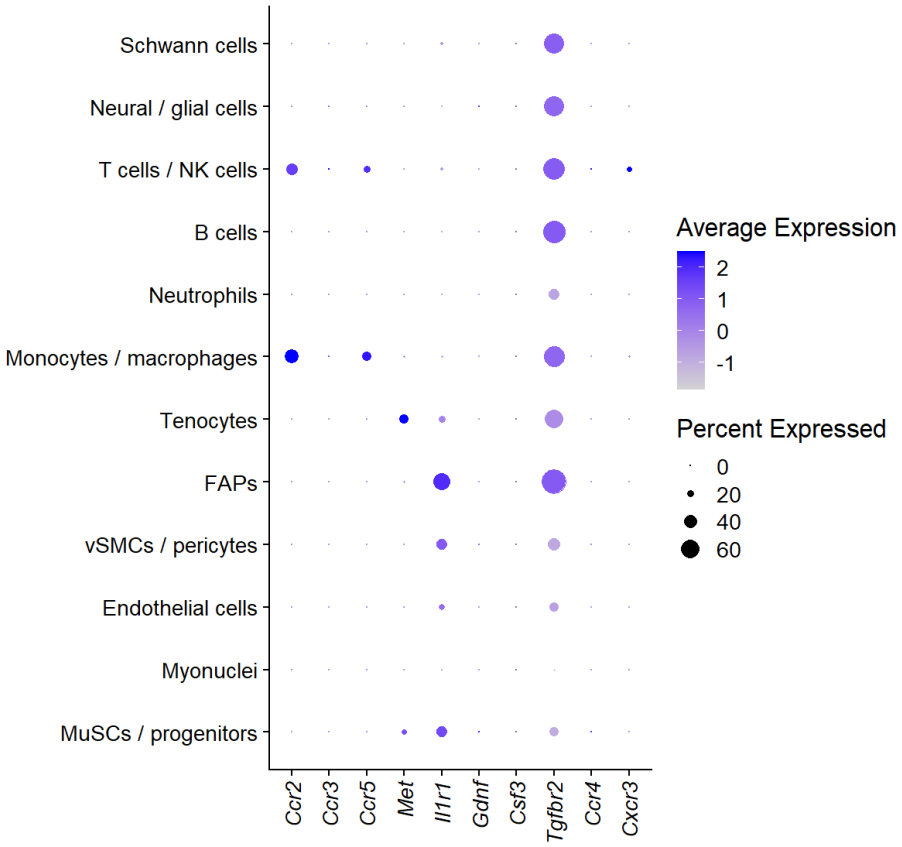

Figure 7. Expression of the cognate receptors in the muscle environment for the top 10 upregulated cytokines in MC38 CM-treated myotubes relative to UC. Data obtained from scRNA-seq of healthy muscle of 15-week-old male mice performed by our lab (A. Brown, unpublished). Receptor ligands can be found in Table 3.

To determine which cells in the muscle environment might be targeted by the cytokines upregulated in the B16 CM-treated myotubes, the expression of the cognate receptors in the muscle environment was assessed. A brief description of the upregulated cytokines, their receptors and some of their main functions are described in Table 4.

Table 4. Top 10 upregulated cytokines in B16 CM-treated myotubes relative to UC and their respective receptors and functions

Cytokine	Receptors	Functions
GDF-15	*Gndf* (Wischhusen et al., 2020)	Associated with cachexia and contributes to muscle wasting (Kim-Muller et al., 2023; Wischhusen et al., 2020)
Cystatin C	*Tgfbr2* (Sokol et al., 2005; Tzavlaki & Moustakas, 2020)	Ubiquitously expressed inhibitor of lysosomal and cysteine proteinases (Fernando & Polkinghorne, 2020)
IGFBP-2	N/A	Regulates IGF activity (T. Li et al., 2020)
Pentraxin 2	*Fcgr1, Fcgr3* (Lu et al., 2018)	Anti-inflammatory plasma protein (Basturk et al., 2020; Nakagawa et al., 2016)
Leptin	*Lepr* (Francisco et al., 2018)	Regulates energy metabolism and immune cell recruitment (Francisco et al., 2018)
LIF	*Lifr* (Yue et al., 2015)	Part of the IL-6 superfamily. Involved in inflammatory response (Yue et al., 2015)
E-selectin	N/A	Mediates cell attachment to the endothelium (Barthel et al., 2007)
Adiponectin	*Adipor1, Adipor2* (Khoramipour et al., 2021)	Increases skeletal muscle insulin sensitivity and glucose uptake (Khoramipour et al., 2021)
CCL6	*Ccr1* (Nosacka et al., 2020)	Chemotaxis of macrophages and monocytes (Nosacka et al., 2020)

Flt3-Ligand	*Flt3* (Drexler & Quentmeier, 2004)	Stimulates proliferation of stem cells, progenitor cells, dendritic cells and NK cells (Gilliland & Griffin, 2002)

scRNA-seq data generated by the Wiper-Bergeron and De Lisio laboratories was used to assess the expression of the receptors found in Table 4 (A. Brown, unpublished). *Tgfbr2*, *Il6st, Adipor1* and *Adipor2* are expressed across a wide variety of immune and non-immune cell types (Fig. 8). *Fcgr3* and *Ccr1* are highly expressed in neutrophils and are also expressed in monocytes / macrophages (Fig. 8). *Fcgr1* is expressed in monocytes / macrophages and *Lepr* is expressed in tenocytes (Fig. 8). *Gdnf* and *Flt3* are not significantly expressed in the muscle environment (Fig. 8). These results show that, similarly to what is seen in the LLC and MC38 CM-treated myotubes, myotube exposure to the cachectic B16 CM results in the upregulation in cytokines which interact with other cell types in the muscle environment, including neutrophils and other immune cells.

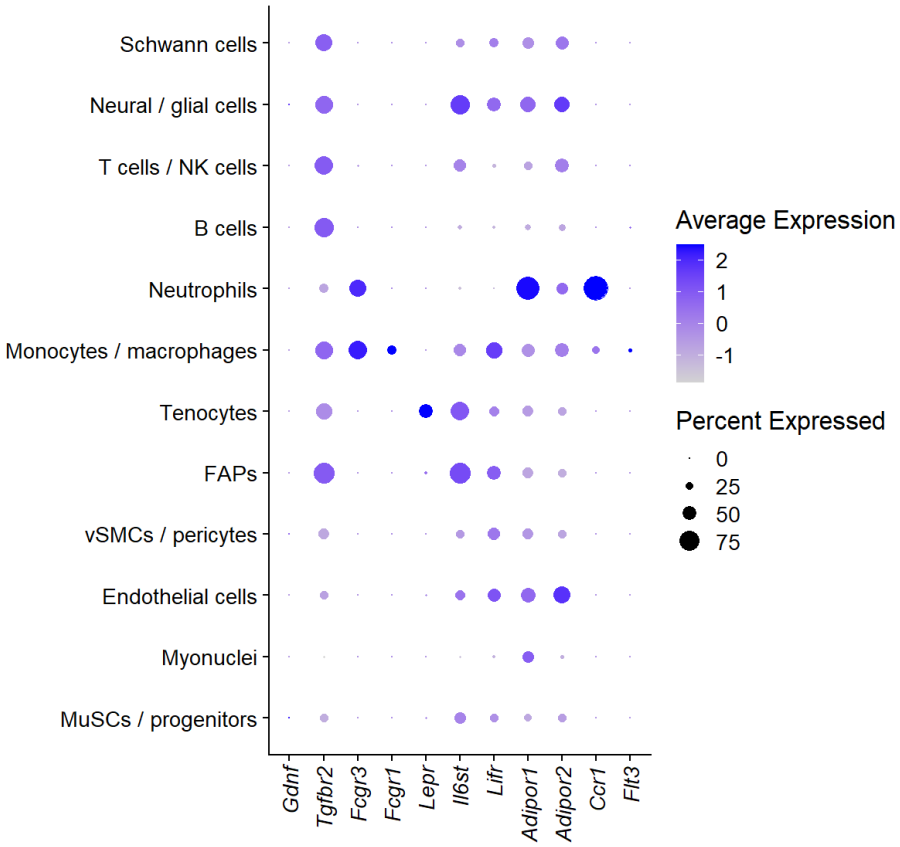

Figure 8. Expression of the cognate receptors in the muscle environment for the top 10 upregulated cytokines in B16 CM-treated myotubes relative to UC. Data obtained from scRNA-seq of healthy muscle of 15-week-old male mice performed by our lab (A. Brown, unpublished). Receptor ligands can be found in Table 4.

These results further validate the results acquired from the LLC CM-treated myotube analysis, suggesting that in a cachectic environment, myotube cytokine expression shifts towards an inflammatory phenotype and the muscle begins to express immune cell chemoattractants. This is especially interesting in the case of MC38 CM-treated myotubes. We showed that this cell line is cachectic *in vivo,* however these myotubes do not exhibit a cachectic phenotype as measured by myotube diameter *in vitro.* Additionally, they exhibit a change in cytokine expression like that seen in the cachectic LLC condition, even though there is no change in their myotube diameter. These results suggest that in the MC38 model, the tumor derived factors are not directly causing myotube atrophy but are inducing changes in cytokine expression. This suggests that the wasting we see *in vivo* is likely secondary to changes in the muscle environment and is mediated by other factors such as immune cell recruitment.

6.4. Myotubes exposed to the cachectic cancer secretome upregulate genes related to inflammation and the immune response

To better understand the gene expression changes in myotubes exposed to a cachectic vs. non-cachectic environment, we performed RNA-sequencing on C2C12 myotubes treated with conditioned media from cachectic and non-cachectic cancers using the *in vitro* model of cachexia. Myotubes were treated with LLC or MC38 CM to model cachectic conditions, EL4 or ID8 CM to model non-cachectic conditions and UC was used as the control.

To determine which genes were upregulated in a cachectic condition, we compared LLC CM-treated myotubes to the control condition. This analysis revealed 757 significantly upregulated and 867 significantly downregulated genes in LLC CM-treated myotubes relative to control (Fig. 9A). Gene ontology (GO) term enrichment analysis of the significantly upregulated genes in LLC CM-treated myotubes relative to control revealed enrichment of genes associated

with cell motility, adhesion and cytokine response (Fig. 9B). Specific genes upregulated in LLC CM-treated myotubes relative to control include many inflammatory cytokines such as *Ccl11, Cxcl5, Ccl8, Ccl9* and *Ccl7.* Interestingly, *Pax7* was upregulated in the treated myotubes. PAX7 is a transcription factor expressed in satellite cells and is downregulated to initiate the differentiation process (von Maltzahn et al., 2013). GO term enrichment of the downregulated genes in LLC CM-treated myotubes showed a significant enrichment of genes associated with sarcomere organization, myofibril assembly and muscle organization and development (Fig. 9C). Additionally, specific downregulated genes include *Lgr6, Lgr5, Wnt11* and *Stmn2,* which are involved in skeletal muscle differentiation and organization (Gros et al., 2009; Kitakaze et al., 2023; Krus et al., 2022; Leung et al., 2020).

The MC38 CM-treated myotubes had 88 significantly upregulated genes and 43 significantly downregulated genes relative to control (Fig. 9D). GO term enrichment analysis of significantly upregulated genes in MC38 CM-treated myotubes compared to control revealed significant enrichment in genes with a role in cytokine, viral and immune response (Fig. 9E). This is reflected in the overexpressed genes which include those involved in viral-mediated inteferon response, such as *Ifi44, Ifit1, Oas2, Ddx60* and *Gbp3* (Liao et al., 2020; Miyashita et al., 2011; Pan et al., 2020; Peng et al., 2022; Pichlmair et al., 2011). GO term enrichment analysis of significantly downregulated genes in MC38 CM-treated myotubes relative to control showed enrichment of genes involved in actin-myosin contraction, myofibril assembly and muscle development, like the results seen in LLC CM-treated myotubes (Fig. 9F). As seen in the LLC CM-treated condition, genes which have roles in skeletal muscle differentiation and organization such as *Lgr6, Lgr* and *Stmn2* are downregulated (Gros et al., 2009; Kitakaze et al., 2023; Krus et al., 2022; Leung et al., 2020). Additionally, multiple myosin heavy chain (MyHC)

isoforms, which are required for muscle contraction such as *Myhc6, Myhc7,* and *Myhc7b*, are downregulated (Gao et al., 2024; Stuart et al., 2016).

To understand how myotubes respond to non-cachectic cancers and how this compares to the response seen in our cachectic conditions, we analyzed results obtained from EL4 and ID8 CM-treated myotubes. The EL4 CM-treated myotubes had 109 significantly upregulated genes and 96 significantly downregulated genes relative to the control condition (Fig. 10A). GO term enrichment analysis of significantly upregulated genes in EL4 CM-treated myotubes relative to control showed a significant upregulation of genes related to innate immune response, neutrophil chemotaxis, and cytokine response (Fig. 10B). Upregulated genes include several guanylate binding proteins, such as *Gbp6, Gbp4, Gbp10* and *Gbp9,* which are involved in mediating innate immune response (Praefcke, 2018; H. Shi et al., 2022; Tretina et al., 2019). Additionally, inflammatory cytokines *Cxcl5*, *Ccl11* and *Ccl7* were also upregulated. GO term enrichment analysis of the significantly downregulated genes in EL4 CM-treated myotubes relative to control did not yield any results. Specific downregulated genes were found to be involved in skeletal muscle differentiation and organization and include *Lrg6, Stmn2* and *Wnt11,* which are genes we found to be significantly downregulated in both the LLC and MC38 CM-treated conditions.

The ID8 CM-treated myotubes had 66 significantly upregulated genes and 42 significantly downregulated genes (Fig. 10C). GO term enrichment analysis of significantly upregulated genes in ID8 CM-treated myotubes relative to control found enrichment of genes involved in sterol and alcohol metabolism and biosynthesis, including *Cyp51* and *Dhcr24* (Bai et al., 2022; Lepesheva et al., 2008) (Fig. 10D). Additionally, *Gbp6, Gbp4, Gbp10* and *Gbp9* were upregulated, which was also seen in the EL4 CM-treated myotubes. GO term enrichment

analysis of the significantly downregulated genes in ID8 CM-treated myotubes relative to control yielded no results. Like the results seen in LLC, MC38 and EL4 CM-treated myotubes, significantly downregulated genes included those involved in skeletal muscle differentiation and organization such as *Lrg6, Stmn2* and *Wnt11*.

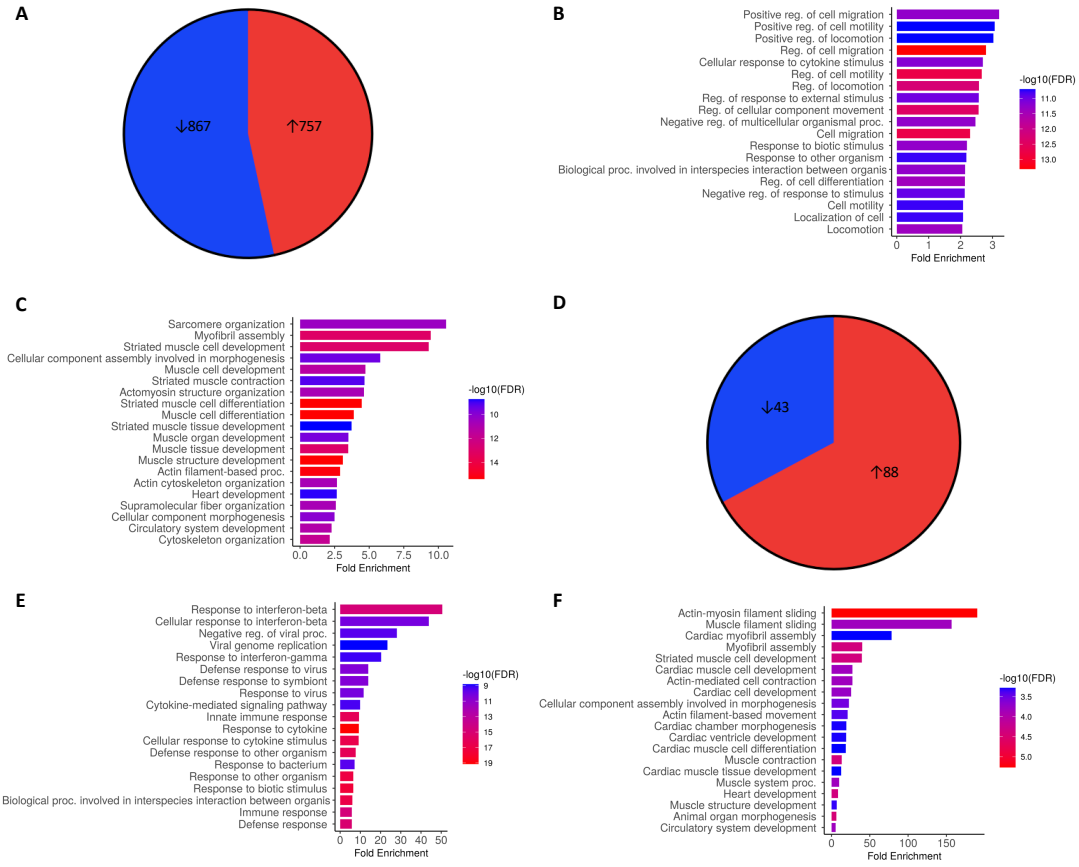

Figure 9. Myotubes exposed to the cachectic cancer secretome upregulate genes related to inflammation and immune response and downregulate genes related to myofiber assembly. (A) Number of significantly upregulated and downregulated genes in LLC CM-treated myotubes relative to control. (B) GO term enrichment analysis of significantly upregulated genes in LLC CM-treated myotubes relative to control. (C) GO term enrichment analysis of significantly downregulated genes in LLC CM-treated myotubes relative to control. (D) Number of significantly upregulated and downregulated genes in MC38 CM-treated myotubes relative to control. (E) GO term enrichment analysis of significantly upregulated genes in MC38 CM-treated myotubes relative to control. (F) GO term enrichment analysis of significantly downregulated genes in MC38 CM-treated myotubes relative to control.

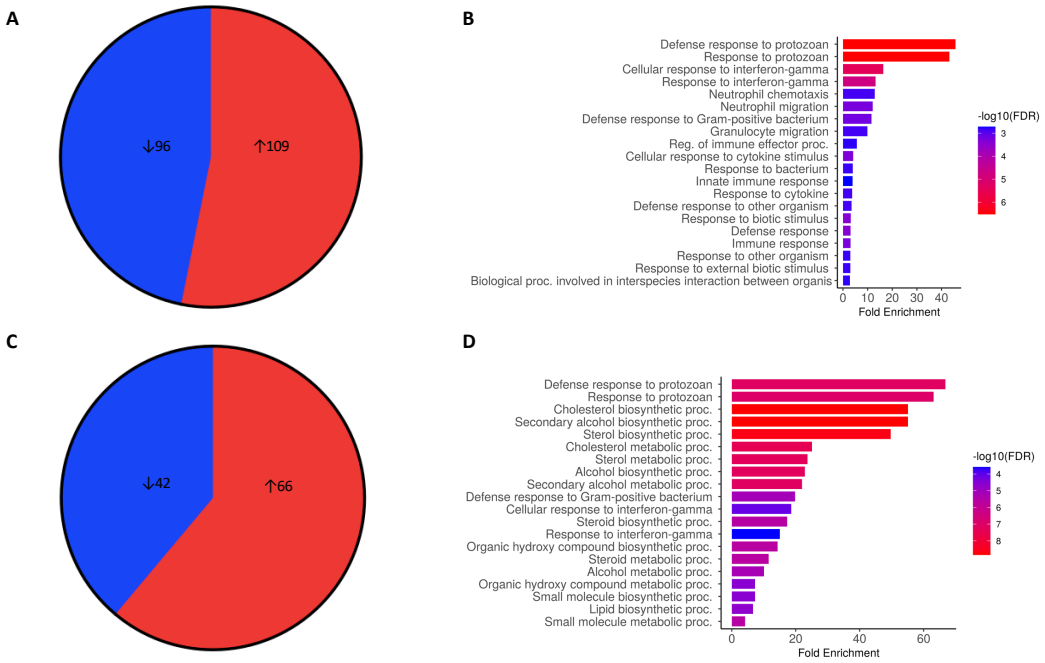

Figure 10. Myotubes exposed to the non-cachectic cancer secretome upregulate genes related to immune response and sterol biosynthesis and metabolism. (A) Number of significantly upregulated and downregulated genes in EL4 CM-treated myotubes relative to control. (B) GO term enrichment analysis of significantly upregulated genes in EL4 CM-treated myotubes relative to control. (C) Number of significantly upregulated and downregulated genes in ID8 CM-treated myotubes relative to control. (D) GO term enrichment analysis of significantly upregulated genes in ID8 CM-treated myotubes relative to control.

The results of the RNA sequencing analysis reveal that in all conditions there is significant upregulation of inflammatory genes and / or genes involved in the immune response and significant downregulation of genes involved in skeletal muscle differentiation and organization. However, the LLC CM-treated myotubes have the largest response with the highest number of significantly upregulated and downregulated genes. These results suggest that the upregulation of inflammatory and immune response-related genes and the downregulation of genes involved in skeletal muscle differentiation and organization might not be solely a response to the cachectic secretome and might occur simply as a result of cancer-derived factors. Thus, to identify which genes and pathways are involved specifically in the cachectic phenotype, we compared LLC CM-treated myotubes to EL4 CM-treated myotubes.

The LLC CM-treated myotubes had 244 significantly upregulated genes and 356 significantly downregulated genes compared to EL4 CM-treated myotubes (Fig. 11A). GO term enrichment analysis of the significantly upregulated genes in LLC CM-treated myotubes relative to EL4 CM-treated myotubes revealed an enrichment of genes related to sterol biosynthesis as well as immune response, cytokine response and inflammation (Fig. 11B). Significantly upregulated genes include pro-inflammatory genes *Ccl11, Hgf, Ccl7* and *Ccl8*. GO term enrichment analysis of significantly downregulated genes in LLC CM-treated myotubes relative to EL4 CM-treated myotubes showed an enrichment of genes related to myofibril organization, muscle differentiation and development (Fig. 11C). Specific downregulated genes include *Lgr6* and *Stmn2* as well as a few myosin heavy chain isoforms, however, *Wnt11* is not significantly downregulated in this analysis.

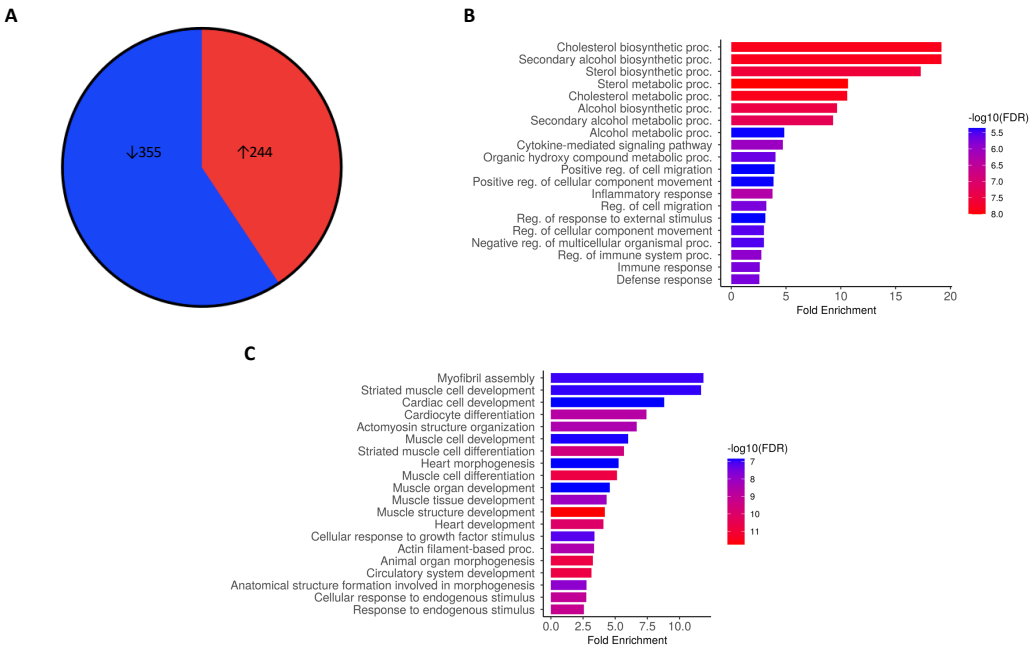

Figure 11. Myotubes exposed to the cachectic cancer secretome upregulate genes involved in immune response and sterol biosynthesis when compared to myotubes exposed to a non-cachectic cancer secretome. (A) Number of significantly upregulated and downregulated genes in LLC CM-treated myotubes relative to EL4 CM-treated myotubes. (B) GO term enrichment analysis of significantly upregulated genes in LLC CM-treated myotubes relative to EL4 CM-treated myotubes. (C) GO term enrichment analysis of significantly downregulated genes in LLC CM-treated myotubes relative to EL4 CM-treated myotubes

Overall, the RNA sequencing analysis shows that although there is an upregulation of genes related to inflammation and the immune response in all conditions, the degree of this response is greater in myotubes exposed to the cachectic conditioned medium. Comparison of the myotubes treated with cachectic LLC CM compared to the non-cachectic EL4 CM revealed that the LLC CM-treated myotubes have a significant upregulation of inflammatory cytokines and immune response genes relative to the EL4 CM-treated myotubes. Thus, although the upregulation of inflammatory cytokines might not be a uniquely cachectic response, the response is significantly higher and more pronounced in a cachectic condition, thus implicating this response in the cachectic phenotype. Furthermore, comparison of the gene sets upregulated in LLC CM-treated myotubes and MC38 CM-treated myotubes shows that, although they are both upregulating inflammatory and immune response genes, the gene sets are largely divergent. The LLC CM-treated myotubes primarily upregulate inflammatory cytokines and immune cell chemoattractants, whereas the MC38 CM-treated myotubes upregulate genes involved in the viral-mediated interferon response such as *Ifi44, Ifit1, Oas2, Ddx60* and *Gbp3*. This suggests that even within cachectic cancers, there are divergent myotube responses which may reflect alternative mechanisms of wasting.

6.5. Myotubes exposed to the cachectic cancer secretome upregulate secreted genes related to immune cell recruitment and activation

The RNA sequencing analysis performed previously found that there was differential expression of similar gene sets in all conditions, however the response was greater in response to the cachectic cancer secretome. To determine which genes expressed by myotubes in the cachectic conditions are involved in the cachectic phenotype, we identified genes uniquely upregulated in LLC or MC38 CM-treated myotubes. To determine which genes were unique to a given condition or were shared with other conditions, significantly upregulated genes in LLC,

EL4, MC38 and ID8 CM-treated myotubes relative to control were compared to each other (Fig. 12). This comparison revealed that the MC38 CM-treated myotubes shared 40% its significantly upregulated genes with the LLC CM-treated myotubes (Fig. 12). The EL4 CM-treated myotubes shared 77% of its significantly upregulated genes with other conditions and had the highest percentage of shared genes across all conditions (Fig. 12). Interestingly, it shared almost half of its upregulated genes with the LLC-CM treated myotubes (Fig. 12). The LLC CM-treated myotubes was the condition with the highest percentage of unique genes, with 82% of its significantly upregulated genes unique to its condition (Fig. 12). To determine which genes were involved in the cachectic response the 621 genes uniquely upregulated in LLC CM-treated myotubes, 32 genes uniquely upregulated in MC38 CM-treated myotubes and the 35 genes upregulated in both conditions were investigated further.

Myotubes treated with LLC CM have a cachectic phenotype *in vitro*, however myotubes treated with MC38 CM do not. As such, genes which are upregulated uniquely in MC38 CM-treated myotubes and / or those upregulated in both MC38 and LLC CM-treated myotubes are unique to the cachectic response but do not contribute to muscle wasting directly (i.e. do not lead to decreased myotube diameter *in vitro*). To assess which differentially expressed genes from CM-treated myotubes are secreted, and thus could target other cells types *in vivo*, we identified the predicted mouse secretome using a protocol developed by the Nielsen group (Robinson et al., 2019).

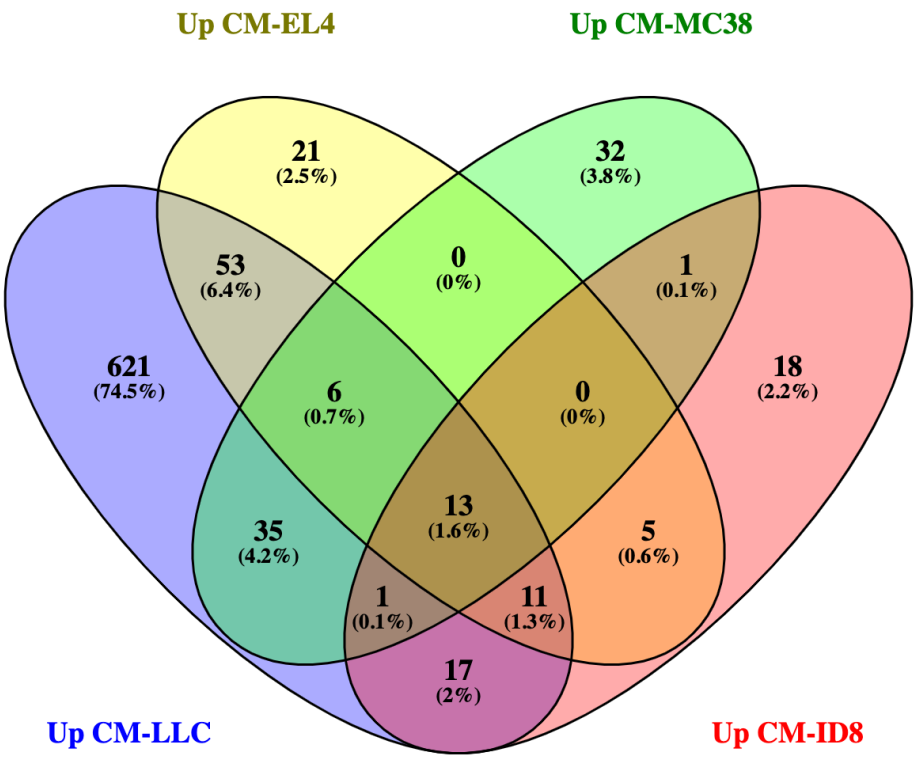

Figure 12. Comparison of the significantly upregulated genes in EL4, MC38, LLC and ID8 CM-treated myotubes. 621 genes are uniquely upregulated in LLC CM-treated myotubes, 32 genes are uniquely upregulated in MC38 CM-treated myotubes and 35 genes are uniquely upregulated in both LLC and MC38 CM-treated myotubes.

GO term enrichment analysis of the 35 genes upregulated in both LLC and MC38 CM-treated myotubes revealed that these genes are enriched in IFNβ and cytokine response (Fig. 13A). Comparison of these 35 genes with the predicted mouse secretome revealed 8 (30%) are expected to be secreted (Fig. 13B). These genes include *Ccl8, Adamtsl1, Cp, Timp1, Sulf1, Sdc1, Ctsd* and *B2m*. Of these 8 inferred secreted proteins, 6 have roles in mediating an inflammatory response and/or neutrophil recruitment/activation. *Ccl8* is a proinflammatory cytokine showed to recruit monocytes, NK cells and mast cells (Farmaki et al., 2020). *Cp* is a molecule involved in iron metabolism; however its expression is elevated in some cancer types and correlates with immune cell infiltration (Chen et al., 2021). It is also an acute phase protein and is expressed during infection and inflammation (Chen et al. 2021). *Timp1* directly activates neutrophils and is also involved in neutrophil recruitment (Schoeps et al., 2023). Elevated levels of TIMP1 are associated with the progression of inflammatory diseases involving neutrophils (Schoeps et al., 2021). *Sdc1* is a protein involved in regulating the state of neutrophil activation and, when it is released in the extracellular space, plays a role in recruiting and activating neutrophils (Gill et al., 2016; Sawant et al., 2016; Teng et al., 2012). *Ctsd* is a lysosomal protease, however when secreted it has been shown to promote inflammation by degrading ECM proteins which causes the release of chemokines that recruit leukocytes (Yadati et al., 2020). Abnormal serum levels of *B2m* are associated with certain conditions such as inflammation, infection, and certain cancers (Li et al., 2016). No current links have been established between secreted *Adamtsl1* and *Sulf1* in inflammation and immune response or in skeletal muscle function.

GO term enrichment analysis of the 621 genes uniquely upregulated in LLC CM-treated myotubes showed an enrichment of genes involved in cell migration (Fig. 13C). Comparison of these 621 upregulated genes to the predicted mouse secretome revealed that 60 (10%) are

expected to be secreted (Fig. 13D). Of these upregulated genes, many are pro-inflammatory cytokines such as *Il34, Ccl9, Il12a, Cxcl12, Cxcl1* and *Ccl2*. *Il34* supports monocyte development and differentiation into macrophages (Lelios et al., 2020). *Ccl9, Ccl2* and *Cxcl12* are involved in immune cell recruitment and *Ccl2* and *Cxcl12* are specifically implicated in neutrophil recruitment (Calì et al., 2022; Cambier et al., 2023; Gschwandtner et al., 2019; Łazarczyk et al., 2023; Reichel et al., 2009). *Il12a* has a role in regulating the immune response and T-cell and NK cell activation (Ullrich et al., 2020). *Cxcl1* is involved in the recruitment and activation of neutrophils (Capucetti et al., 2020; Sawant et al., 2016).

GO term enrichment analysis of the 32 genes uniquely upregulated in MC38 CM-treated myotubes showed an enrichment of genes relating to IFNβ and innate immune response (Fig. 13E). Comparison of these 32 genes to the predicted mouse secretome revealed that only one (3%), *Lgals3bp,* is inferred to be secreted (Fig. 13F). *Lgals3bp* is a gene encoding for a secreted hyperglycosylated protein shown to have a role in innate immunity (Capone et al., 2021). The role of this protein in mediating the immune response is complex, as it has been shown to have both inflammatory effects and immune inhibitory effects (Hong et al., 2019; Xu et al., 2019). As such, exposure of myotubes to the MC38 cell-derived secretome does not promote the expression of a myotube-derived secretome, in contrast to the effect of LLC CM.

As LLC CM-treated myotubes show a decrease in myotube diameter, we sought to identify which pathways might be involved in the wasting seen in this condition. To do so, we looked further into the 561 genes which are upregulated uniquely in the LLC CM-treated myotubes but are not secreted. GO term enrichment analysis of this gene set revealed an enrichment of genes related to negative regulation of protein phosphorylation, negative regulation of signal transduction and negative regulation of signaling (Fig. 13G). This suggests

that changes in signaling pathways and protein phosphorylation may play a role in the changes in myotube diameter seen in the LLC CM-treated myotubes. Altered phosphorylation and changes in signaling pathways such as the Akt-mTORC pathway are shown to be involved in cachexia (Geremia et al., 2022). It is possible that the mechanism of wasting seen *in vitro* in the LLC CM-treated myotubes is due to changes in phosphorylation and signaling pathways crucial for muscle mass maintenance.

This analysis shows that myotubes exposed to the cachectic cancer secretome upregulate genes related to inflammation and immune cell recruitment and activation, which are not upregulated in response to non-cachectic cancers. Additionally, a number of these genes are secreted suggesting that they are directly targeting and recruiting immune cells to the muscle environment in cachexia. As the targets of these secreted genes are immune cells, and we do not see the cachectic phenotype *in vitro* with the MC38 model, this suggests a potential role for immune cell recruitment in the progression and pathogenesis of cachexia *in vivo*.

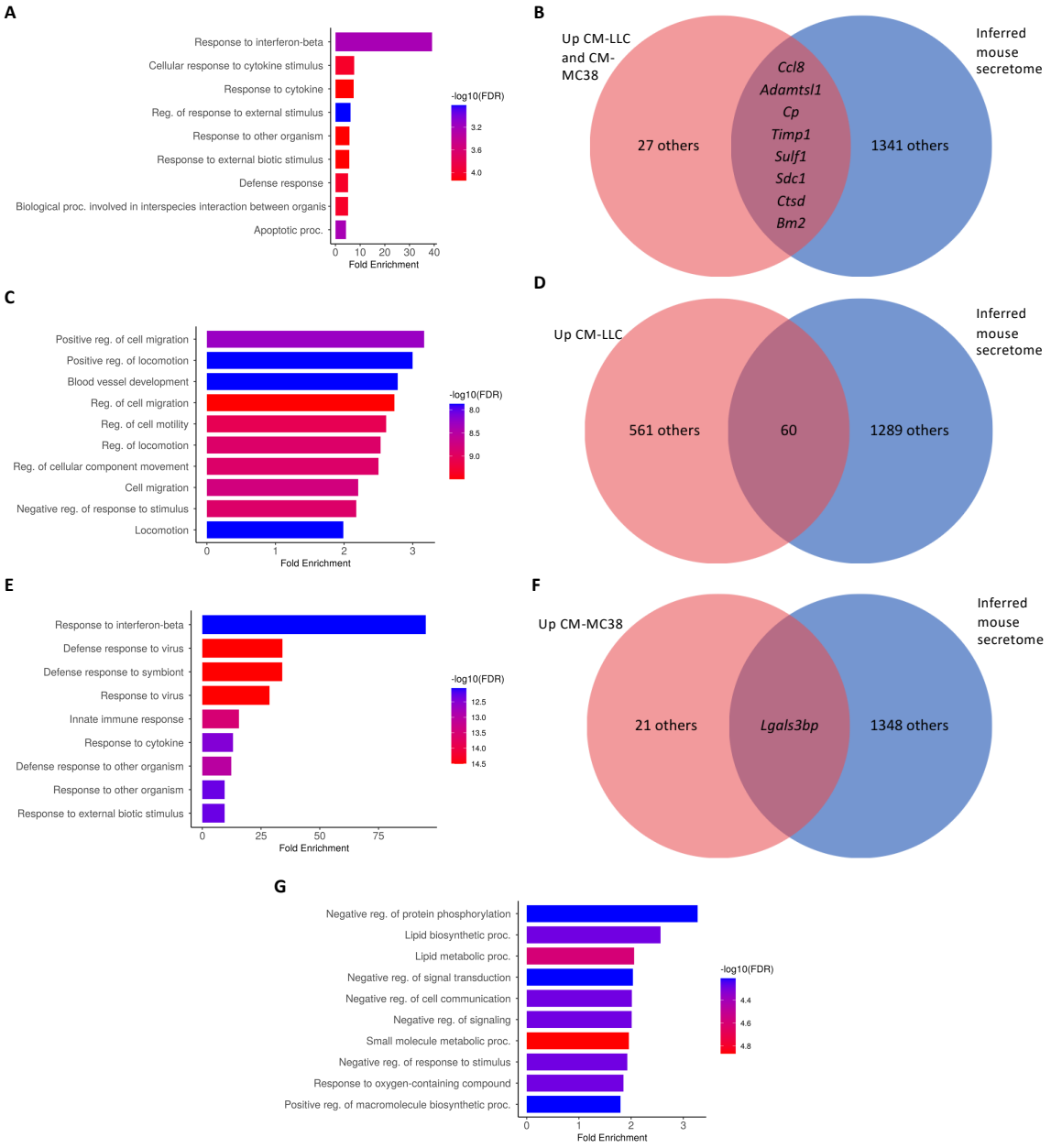

Figure 13. Myotubes exposed to the cachectic cancer secretome upregulate genes related to immune cell recruitment and activation. (A) GO term enrichment analysis of the 35 uniquely upregulated genes in MC38 and LLC CM-treated myotubes (B) Comparison of the 35 uniquely upregulated genes in MC38 and LLC CM-treated myotubes to the inferred mouse secretome. (C) GO term enrichment analysis of the 621 uniquely upregulated genes in LLC CM-treated myotubes. (D) Comparison of the 621 uniquely upregulated genes in LLC CM-treated myotubes to the inferred mouse secretome. (E) GO term enrichment analysis of the 32 uniquely upregulated genes in MC38 CM-treated myotubes. (F) Comparison of the 32 uniquely upregulated genes in MC38 CM-treated myotubes to the inferred mouse secretome. (G) GO term enrichment analysis of the 561 upregulated non-secreted genes in LLC CM-treated myotubes.

6.6. Myotubes exposed to the cachectic cancer secretome express secreted genes which target endothelial cells and some immune cells

To determine which cells in the muscle environment might be targeted by the 60 genes expected to be secreted only from the myotubes exposed to LLC CM, we mapped the receptors of each of these genes using Cellinker (Y. Zhang et al., 2021). This mapping revealed that the 60 secreted proteins may target 94 receptors. To find which cell populations these ligands might be targeting, we assessed the expression of these receptors in the muscle environment using scRNA-seq data from healthy hindlimb muscle from 15-week-old male mice (A. Brown, unpublished). This analysis found that the receptors for these secreted genes are enriched in the endothelial cell populations in the muscle (Fig. 14A). Some of the receptors are also expressed in some immune cell populations such as macrophages, neutrophils, and B-cells (Fig. 14A). This shows that many of the secreted proteins in the LLC CM-treated myotubes appear to target endothelial cells and some immune cell populations. This is not surprising as endothelial cells can respond to inflammatory signaling, and also express many adhesion molecules such as E-selectin and VCAM1 (Preuss et al., 2023).

Additionally, we sought to compare the similarity of the myotube response to LLC CM with the myofiber response to the LLC tumor. To do this, we compared the expression of the 60 genes expected to be secreted from LLC CM-treated myotubes to scRNA-seq data from the myonuclei of 15 week old male LLC tumor bearing mice after 3.5 weeks of tumor growth (A. Brown, unpublished). This analysis showed that there are 2 secreted genes which are similar in both conditions, *Cxcl12* and *Gpx3*. *Cxcl12* is involved in neutrophil recruitment and *Gpx3* is an antioxidant and plays a role in restricting inflammation (Calì et al., 2022; Cambier et al., 2023; Nirgude & Choudhary, 2021). This suggests a role for *Cxcl12*-mediated immune cell recruitment in the progression of cachexia *in vivo*, as the response is similar in both conditions. The

upregulation of *Gpx3* may be the result of a protective effect, as *Gpx3* is an antioxidant and plays a role in attenuating inflammation (Nirgude & Choudhary, 2021). It is possible the myofibers are upregulating this gene in an attempt to dampen the inflammatory signaling in the muscle environment.

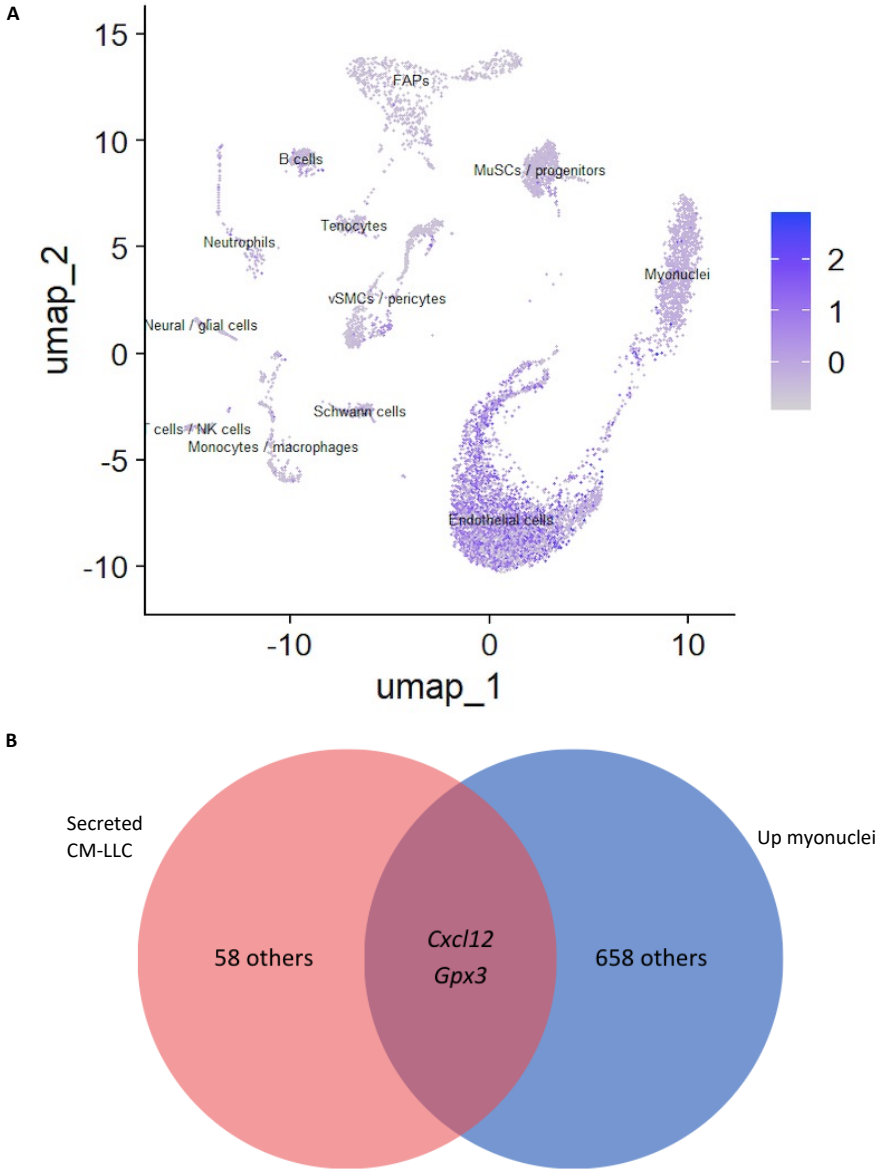

Figure 14. Secreted genes in myotubes treated with LLC CM target endothelial and immune cell populations and share similar secreted genes with myonuclei in the cachectic environment. (A) Feature plot showing the expression pattern of the 94 cognate receptors targeted by the 60 genes expected to be secreted only from LLC CM-treated myotubes. (B) Comparison of the 60 genes expected to be secreted from LLC CM-treated myotubes to the significantly upregulated genes in myonuclei of 15-week-old male LLC tumor bearing mice after 3.5 weeks of tumor growth.

6.8. The cancer secretome does not directly cause damage to C2C12 myotubes

Studies have shown that skeletal muscle becomes damaged in cachexia, with membrane damage evident using Evans Blue and IgG staining and electron microscopy imaging (He et al., 2013; Iwata et al., 2016; Talbert et al., 2014; Talbert & Guttridge, 2016). Although we have evidence that this damage occurs in cachexia, the cause of this damage is unknown. This damage might be the direct effect of the cancer secretome, or it may be a secondary effect caused by changes in the muscle environment. To assess if the cancer secretome directly induces membrane damage, we used both qualitative and quantitative measures of damage. Creatine kinase release from myotubes is a marker of membrane damage (Baird et al., 2012). We used the *in vitro* model of cachexia to assess myotube membrane integrity following exposure to CM from LLC, EL4, MC38 and ID8 cancer cell lines. We collected the first media sample from the initial 1:1 mixture of DM and CM before it was put on the myotubes (0h). We collected the second sample at the end of the 48h treatment period (48h). We assessed creatine kinase activity level at each timepoint as a marker of membrane damage. Additionally, we stained myotubes using Evans Blue dye, which is a membrane impermeable compound (Vijayaraghavareddy et al., 2017). As it is impermeable to the myotube membrane, there will only be evidence of Evans Blue stain if there is membrane damage.

Measure of creatine kinase activity in the media of myotubes treated with LLC, EL4, ID8 and MC38 CM or UC showed no significant differences in creatine kinase activity between the 0h and 48h timepoints of each condition (Fig. 15A). To further support this data, quantification of the percent Evans Blue positive area in myotubes treated with CM showed no significant differences between the control and each of the conditioned media treated conditions (Fig. 15B, C). We used a positive control permeabilized with methanol as a reference (Fig. 15C). Lack of significant difference in creatine kinase activity between the media collected at 0h and 48h hours

of treatment and in Evans Blue positive area suggests that the cancer secretome is not directly causing damage to the myotube membrane. As there is no evidence of damage *in vitro* following exposure to the cancer conditioned media, this suggests that there are other factors *in vivo* that are mediating the muscle damage seen in cachexia.

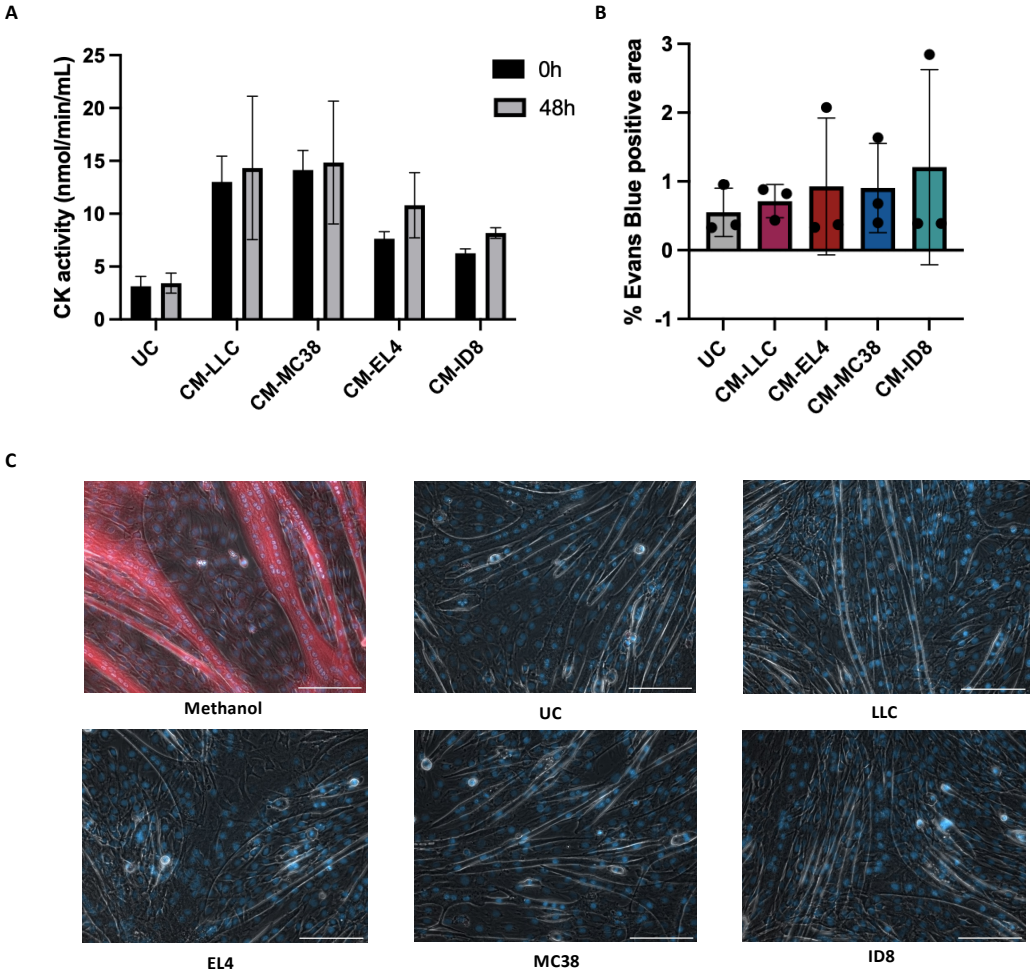

Figure 15. The cancer secretome does not directly induce membrane damage in myotubes. Myotubes were differentiated for 4 days and then treated for 48 hours with LLC, EL4, MC38 or ID8 conditioned media. Control was treated with growth media. (A) Measure of creatine kinase activity in the media at 0h of treatment and 48h of treatment with CM. (n=3). Data presented as mean ± SD. Conditions compared using 2-way ANOVA. (B) Percent Evans Blue positive area of myotubes treated for 48 hours with CM. (n=3). Data presented as mean ±SD. Conditions compared using 1-way ANOVA. (C) Evans Blue staining (red) and phase imaging of myotubes treated for 48 hours with CM. Nuclei are stained with DAPI (blue). Scale bar = 100µm.

8. DISCUSSION

In this study, we showed that in response to treatment with conditioned media from cachectic cancers, C2C12 myotubes upregulate the protein expression of proinflammatory cytokines and immune cell chemoattractants. We also demonstrate that in response to treatment with conditioned media, myotubes upregulate genes that are involved in inflammatory pathways and the immune response. We found that the MC38 cancer cells, which cause cachexia *in vivo*, do not affect myotube diameter *in vitro*, suggesting an indirect mechanism of muscle wasting in this model. Interestingly, even though there is no cachectic phenotype *in vitro,* there are still changes in cytokine protein expression and gene expression resembling the changes seen in the cachectic LLC condition. Furthermore, we demonstrate that the cancer secretome does not directly cause membrane damage to myotubes, suggesting that the mechanism of muscle damage seen in cachexia is not a direct effect of the tumor secretome.

We assessed the cachectic phenotype of LLC and MC38 cancer cell lines *in vivo,* and we found that both cell lines cause cachexia *in vivo* using our experimental protocols. The cachectic phenotype of the LLC model has been validated previously by our lab and others, and the results that we obtained support these findings (AlSudais et al., 2022; Talbert et al., 2017; G. Zhang et al., 2017). The cachectic phenotype of the MC38 model has been more elusive and different studies have obtained different results (Huot et al., 2021; Rohm et al., 2016; Schäfer et al., 2016; Dolly et al., 2023). These differences in phenotype are likely due to differences in the experimental protocols used in each study, where the period of tumor growth, number of cells injected, and measures of cachexia used differ. Based on our results, the MC38 tumor model is cachectic *in vivo* using the experimental protocol that we have established. The small sample number in our study is a limiting factors regarding our *in vivo* assessment of the cachectic phenotype. *In vivo* models have higher variability than *in vitro* models due to the biological

differences between each mouse. Cohort sizes of 3 mice are underpowered considering the differences in tumor growth and size observed. Differences in starting muscle mass in the mice used in a trial could also impact the observation of cachexia, as a mouse with a lower starting muscle mass may not lose as much lean mass as a mouse with a higher starting muscle mass. Differences in tumor size in each mouse also accounts for some additional variability in each sample. Although each mouse is inoculated with the same number of cells, the resulting tumors can vary in size and do influence the cachectic phenotype observed. The mice used in this study had large differences in tumor size, which accounts for some of the variability seen in the cross-sectional area. Both our LLC and MC38 tumor bearing mice had a negative correlation between tumor size and cross-sectional area, indicating that tumor size affects the cachectic phenotype. Having a larger sample size would allow us to include more mice that have similar tumor sizes and would allow for a more direct comparison of cross-sectional area across specimens.

Another factor to consider in this model are the sex differences in the presentation of the cachectic phenotype between male and female mice. In human patients, females appear to be more resistant to cachexia, with a higher percentage of cachectic patients being male, and the same appears to hold true in mouse models of cachexia (Zhong & Zimmers, 2020). These differences could arise for many different reasons such as differences in the response to inflammation and muscle fiber type (Zhong & Zimmers, 2020). A recent study found that in the C26 model of cancer cachexia, female mice have a reduction in fat mass and increased protein degradation, however their skeletal muscle mass remained relatively unaffected when compared to the male mice (Cabrera et al., 2023). We used female mice in our study as this is the sex our lab has previously used in its cachexia studies, however, given the sex differences in cachexia, it

would be important to repeat the *in vivo* experiments in male mice to validate the results in both sexes.

Using the *in vitro* model of cachexia, we showed that the LLC model causes a significant decrease in myotube diameter relative to the controls, however the MC38 model showed no effect. This finding is interesting as we found this model to be cachectic *in vivo*. These differences in the cachectic phenotype *in vitro* and *in vivo* suggest different mechanisms of wasting in each condition. As there is wasting *in vitro* in myotubes treated with LLC CM, this indicates that the LLC cancer secretome directly causes wasting in treated myotubes. One study found that the LLC secretome directly induces wasting in myotubes through the TLR4 receptor (G. Zhang et al., 2017). In the case of the MC38 model, we see no changes in the myotube diameter *in vitro*, suggesting that the cancer secretome is not directly causing wasting, but the wasting is an effect of other cell interactions which can only be observed *in vivo.*

This does constitute a limitation of the *in vitro* model of cachexia that we use, as it is only allows us to study the direct effect of the cancer secretome and fails to consider other cell interactions that may be occurring at the tumor level. *In vivo,* the tumor secretome may be distinct from that of cancer cells in isolation as tumors contain malignant cells but also include other cell types (endothelial, macrophages, cancer-associated fibroblasts) that likely also contribute to the tumor secretome. Additionally, this model does not allow us to study other cell interactions occurring in the muscle environment, such as the interaction between FAPs or infiltrating immune cells and the myotubes. To determine the mechanism of wasting in the MC38 model for example, it would be important to assess how the cancer cell-derived secretome affects other cell types in the muscle environment and if these changes contribute to muscle wasting in cachexia. Additionally, it would be important to consider how the cancer secretome-

mediated changes in the myofibers themselves may result in wasting through paracrine and / or endocrine interactions with other cell populations.

Results from the cytokine arrays showed cytokine expression changes in myotubes treated with the conditioned media from the cachectic LLC, MC38 and B16 models. After treatment with the cachectic conditioned media, there was increased expression of pro-inflammatory cytokines relative to control and non-cachectic cancer treated conditions. Systemic inflammation is a hallmark of cachexia, and chronic inflammation in the muscle environment in cachexia directly contributes to muscle wasting (AlSudais et al., 2022; Petruzzelli & Wagner, 2016). The source of the inflammation and pro-inflammatory cytokines has yet to be fully understood and is likely due to both tumor and host derived factors (Onesti & Guttridge, 2014). The results of the cytokine array suggest that myofibers may be a key source of the inflammation in the muscle environment in cachexia. This would indicate myofibers are acting in an autocrine and/or paracrine manner to drive inflammation in the muscle, effectively amplifying the tumor-derived signal. Thus, attenuating the muscle-derived inflammation would be an interesting option in the treatment of cachexia.

Another interesting finding from the arrays is the upregulation of immune cell chemoattractants such as CCL11, CXCL5, CCL6, CCL17 and IL-6. The upregulation of these cytokines by the muscle would suggest that immune cells are recruited to the muscle environment in cachexia and may play a role in the progression of this disorder. Although the upregulation of specific inflammatory cytokines differed between the LLC, MC38 and B16 CM-treated conditions, interestingly, GDF-15 was upregulated consistently in all 3 cases. GDF-15 has emerged as a biomarker of cachexia and is associated with poor prognosis (Ahmed et al., 2021; Ling et al., 2023). Different studies have shown that neutralizing GDF-15 or inhibiting the

GRAL receptor reverses cachexia in different models of the disease (Kim-Muller et al., 2023; Suriben et al., 2020). These studies both found that GDF-15 plays a role in mediating body and muscle mass loss in cachexia through an increase in lipolysis and that inhibiting the GDF-15-GFRAL pathway allows for restoration of body mass and improves muscle function (Kim-Muller et al., 2023; Suriben et al., 2020). GDF-15 is known to be expressed by skeletal muscle and the liver, however in the context of cachexia, whether GDF-15 is derived from skeletal muscle, liver or tumor is unknown (De Paepe et al., 2020; Wang et al., 2024). The cytokine arrays show that myotubes exposed to the LLC, MC38 and B16 cachectic cancer secretomes all have increased levels of GDF-15. This suggests that skeletal muscle may be the source of the increased serum GDF-15 in cachectic patients.

In addition to changes in cytokine protein expression, there were also changes observed at the level of gene expression in response to the cachectic secretome. RNA-seq of myotubes exposed to the LLC and MC38 cachectic cancer secretomes showed that they upregulate genes involved with inflammation and the immune response. These results support some of the observations made at the protein level with the cytokine arrays, where we found cachectic myotubes increase expression of pro-inflammatory cytokines and immune cell chemoattractants. Comparison of EL4-CM treated myotubes to control showed an upregulation in inflammatory genes, however, comparison of the LLC CM-treated myotubes to EL4 CM-treated myotubes revealed that many of the same inflammatory genes were upregulated. This indicates that the muscle may respond similarly in a cachectic vs. non-cachectic environment (i.e. upregulating inflammatory genes, down regulating genes related to muscle organization), but the magnitude of the response is greater in the cachectic conditions relative to the non-cachectic ones. This suggests that although the responses may appear similar at first glance, they are different in

magnitude. Furthermore, all the conditioned media treated conditions, regardless of the cachectic nature of the cancer, exhibited a significant decrease in genes related to the contractile apparatus and muscle organization and development such as *Wnt11, Lgr6* and *Stmn2*. This is interesting as we are seeing a change in gene expression which could explain wasting, even when no wasting is observed *in vitro*. The downregulation of these genes could be the result of a fusion or differentiation defect rather than myotube atrophy. Determining the fusion and differentiation indices of the myotubes in each condition would be able to help assess if the of genes related to the contractile apparatus and muscle organization are due to a fusion and / or differentiation defect. It is also possible that the downregulation of contractile genes is related to other factors such as nutrient deprivation or factors found in the conditioned media from each cancer type.

These findings are very significant in the context of research involving mediators of cachexia and underlines the importance of comparing the cachectic phenotype to a non-cachectic phenotype. The comparison of these phenotypes allows us to determine exactly which observations are related to cachexia rather than simply a response to cancer. Determining which genes and protein products are specifically the result of the cachectic phenotype is essential in the identification of cachexia causing factors and mediators of this condition.

Comparison of the myotube response to LLC and MC38 conditioned media shows that the specific pathways being targeted by the upregulated genes differ in each condition. The LLC CM-treated myotubes upregulate more inflammatory cytokines, whereas the MC38 CM-treated myotubes upregulate genes involved in a viral-mediated immune response and interferon signaling. This result is interesting as it suggests different mechanisms of wasting in response to each cachectic secretome and is supported by the observation that the MC38 conditioned media does not cause wasting *in vitro.* In the case of the LLC CM-treated myotubes, there is unique

upregulation of many different proinflammatory cytokines and immune cell chemoattractants such as *Cxcl1, Cxcl12, Ccl2* and *Ccl9*. These genes are involved in the progression of inflammation, as well as the recruitment of various immune cells such as neutrophils, lymphocytes and macrophages (Guyon, 2014; Jin et al., 2018; Sawant et al., 2016). A recent study found that treating C2C12 myoblasts with CXCL1 or CCL2 recombinant protein impaired differentiation (Hogan et al., 2018). MuSC dysfunction has been observed in cachexia due to persistent expression of PAX7 and the subsequent inability to properly commit to differentiation (He et al., 2013; Marchildon et al., 2015). By inhibiting the differentiation of MuSCs, this study suggests a potential role for CXCL1 and CCL2 in the MuSC dysfunction observed in cachexia. Hogan et al. also found that cachectic 531LN2 lung adenocarcinoma tumor bearing mice had increased levels of serum and TA CXCL1 (Hogan et al., 2018). They also showed that there was decreased efficiency of repair and increased neutrophil and M2 macrophage recruitment to the muscle environment relative to control following administration of CXCL1 recombinant protein to the hindlimb (Hogan et al., 2018). This suggests that muscle derived CXCL1 may recruit neutrophils into the muscle environment and contribute to MuSC dysfunction in cachexia.

Timp1 was elevated in both the MC38 and LLC conditioned media-treated myotubes. It has been shown that TIMP1 correlates with neutrophil activation and high systemic levels of TIMP1 are also present in inflammatory diseases involving neutrophils (Schoeps et al., 2023). Serum levels of TIMP1 are increased in individuals with inflammatory diseases such as kidney injury, pancreatitis and sepsis, and in individuals with Duchenne Muscular Dystrophy (Kumar et al., 2022; Schoeps et al., 2023). Increased levels of TIMP1 have been correlated with cachexia in patients with pancreatic cancer and are associated with a worse prognosis in virtually all cancer types (Prokopchuk et al., 2018, 2021). Furthermore, high levels of systemic TIMP1 induces

granulopoiesis and neutrophilia in mice (Kobuch et al., 2015). Taken into this context, the increased level of *Timp1* in the myotubes treated with the cachectic cancer secretome is significant, as it suggests that myofibers may be a source of the increased systemic levels of TIMP1 in cachexia. Studies on the role of TIMP1 in cachexia have only focused on pancreatic cancer, and our study suggests that it may be involved in other cachectic cancers such as lung and colon cancers. In addition to this, the association of TIMP1 with neutrophil activation further implicates these immune cells in the progression of cachexia. The links with CXCL1 and TIMP1 in the progression of cachexia should be explored further to determine the role they play in the onset of this disorder. Confirming that there is increased protein expression of TIMP1 and CXCL1 in the treated myotubes and evaluating the serum and muscle levels of these proteins in tumor bearing mice is an important first step to establish this link. Further studies should also focus on the correlation between CXCL1 and TIMP1 muscle levels and neutrophil recruitment to the muscle environment in tumor-bearing mice.

Much of the data obtained in this study both at the protein level and mRNA level have implicated neutrophils as a prime target of the muscle in cachexia. Our data suggests that in response to the cachectic cancer secretome, skeletal muscle upregulates genes associated with inflammation and immune cell chemoattractants. Our findings suggest that it is potentially muscle-derived factors rather than tumor-derived factors which perpetrate the inflammatory muscle environment and systemic levels of these proteins in cachexia. Furthermore, these proteins may recruit and activate neutrophils in the muscle of cachectic individuals which in turn may cause myofiber membrane damage. A few studies have focused on the role of neutrophils in cachexia and they have found that neutrophilia is associated with cachexia and a poor prognosis (Petruzzelli et al., 2022). Furthermore, a recent study found that depleting Ly6G-positive cells,

which includes neutrophils, reduces skeletal muscle wasting in a pancreatic cancer model of cachexia (Deyhle et al., 2022). Lastly, single cell RNA sequencing hindlimb muscle of cachectic mice at different timepoints of tumor growth recently conducted by our lab has shown an increased number of neutrophils in the muscle environment throughout the progression of cachexia, with the highest number seen at endpoint at 3.5 weeks (A. Brown, unpublished). Taken together, these results suggest that neutrophils are recruited to the muscle in cachexia, likely due to muscle-derived factors, and likely play a role in the progression of cachexia which remains to be explored.

Currently, there is no established course of treatment for cachexia, and treatment has mainly focused on alleviating symptoms (Tazi & Errihani, 2010). Treatment of cachexia targeting single, or a combination of pro-inflammatory cytokines have been investigated with mixed results in phase I and II clinical trials and have largely been unsuccessful in phase III trials (Prado & Qian, 2019). Developing a treatment which results in the attenuation of skeletal muscle derived inflammation through targeting tumor derived factors which induce the inflammatory phenotype in the muscle would be an interesting option. Furthermore, as our results have suggested that skeletal muscle recruits neutrophils to the muscle environment and these cells may play a role in the progression of cachexia, a treatment that aims to reduce neutrophil recruitment or attenuates the expression of neutrophil chemoattractants / activators, such as CXLC1 or TIMP1, in the muscle environment could also be explored.

Muscle damage has been shown to occur in the muscle of patients with cachexia, however the mechanism of damage is currently unknown (He et al., 2013; Talbert et al., 2014; Talbert & Guttridge, 2016). Using a creatine kinase activity assay and Evans Blue staining, our study has shown that the cancer secretome does not appear to directly damage the myotube

membrane. As we are not seeing any damage in the *in vitro* model of cachexia, this suggests that the mechanism of damage seen *in vivo* is likely due to other changes and interactions occurring in the muscle environment. It is important to note that a study found that mitochondrial creatine kinase is detectable in the conditioned medium of LNCaP cells, which is a model of prostate cancer (Pang et al., 2009). The presence of mitochondrial creatine kinase in the cancer conditioned medium would be a confounding factor and may explain the high levels of creatine kinase activity detected in the 0h sample composed of the 1:1 mix of DM and CM before being put on the myotubes. It is possible that due to the presence of mitochondrial creatine kinase in the conditioned media, the creatine kinase activity assay would not be sensitive enough to detect further changes in creatine kinase activity. Furthermore, assessment of damage *in vivo* should also be performed to confirm that damage is occurring in our model. This could be done using electron microscopy to observe changes in the myofiber membrane integrity (He et al., 2013). Alternatively, Evans Blue dye injection could also be used for *in vivo* assessment, as it would infiltrate and stain the damaged fibers (He et al., 2013). Given inflammatory pathologies can dysregulate immune cells leading to persistent activation and accumulation and eventually cause tissue damage, it is interesting to speculate that in cachexia, neutrophils are recruited to the muscle environment by myofiber-derived secreted factors and, in response to the chronic inflammatory state, become persistently activated and cytotoxic resulting in myofiber damage (Oliveira-Costa et al., 2022). Further studies should focus on the correlation between neutrophil recruitment and muscle damage and establishing a timeline for these in cachexia.

9. CONCLUSION

Our findings demonstrate that myotubes in a cachectic environment begin to express pro-inflammatory cytokines and mediators of immune response. We have also shown that myotubes exposed to different cachectic cancers upregulate different sets of inflammatory genes,

suggesting that the muscle response varies depending on the cancer secretome to which it is exposed, and that this response may also play a role in mediating the mechanism of wasting and immune recruitment to the muscle environment. Additionally, creatine kinase activity measurement and Evans Blue staining of CM treated myotubes suggests that the mechanism of muscle damage in cachexia is not a direct effect of the tumor secretome. This suggests that the myofiber damage seen in cachexia is likely mediated by other cell types and interactions occurring in the muscle environment.

10. FUTURE DIRECTIONS

Future directions for this project include assessing the *in vivo* mechanism of muscle damage occurring in cachexia. There should be a focus on establishing a timeline of damage and neutrophil recruitment to the muscle environment to determine if they are correlated. Additionally, further research on the role of muscle-derived factors such as *Cxcl1* and *Timp1* in cachexia and neutrophil recruitment and activation should be pursued. Lastly, as we have shown that different cachectic cancers result in different mechanisms of wasting, further research into both the direct and indirect mechanisms of wasting should be investigated to better understand the pathogenesis of cachexia with the objective of identifying novel therapeutic targets for its treatment and prevention.

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