The effects of amino acids on macrophage metabolism and function

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Abstract

Acute lung injury (ALI) is a life-threatening condition characterized by breakdown of the lung alveolar-capillary barrier and respiratory failure that can occur after a direct (*e.g.*, pneumonia) or indirect (*e.g.*, sepsis) inflammatory insult. Macrophages play an important role in ALI, and their activity is regulated by changes in metabolism and gene transcription required for stress responses and cytokine production. These are tightly controlled by the protein kinase 'mammalian target of rapamycin' (mTOR) and the endoplasmic reticulum (ER) stress response. mTOR nucleates two highly-conserved macromolecular complexes – mTOR complex-1 (mTORC1) and mTORC2. mTORC1 and the ER stress response, via the protein 'general control nonderepressible 2' (GCN2) are cellular sensors of essential amino acid (EAA) availability, and clinical studies demonstrate an association between protein availability and clinical outcomes in the critically ill. We therefore proposed that the availability of EAAs, particularly leucine and/or arginine, is critical for metabolic, stress, and inflammatory responses in macrophages that are related to ALI. Bone marrow-derived macrophages (BMDMs) were exposed to control media or that lacking leucine, arginine, and lysine (EAA restriction), in the presence or absence of saline or *E. coli* lipopolysaccharide and interferon-gamma (LPS and IFN-γ) for 6 h. The mTORC1 inhibitor rapamycin reduced cellular oxygen consumption related to oxidative phosphorylation in BMDMs; the effects of LPS and IFN-γ or EAA restriction on metabolism could not be assessed for technical reasons. However, the induction of interleukin-6 (IL-6) mRNA and protein by LPS and IFN-γ was inhibited by EAA restriction; IL-6 induction was restored by the addition of leucine alone. Moreover, IL-6 induction was inhibited by exposure to media lacking leucine alone. EAA restriction enhanced the induction of ER stress response genes (*i.e.*, CHOP, GADD34) by LPS and IFN-γ. As expected, EAA restriction blocked activity of the mTOR pathway (*i.e.*, phosphorylation

of 4EBP1) and enhanced that of the ER stress pathway (*i.e.*, phosphorylation of eiF2α). Our results point to ER stress and/or mTORC1 and leucine sensing as important regulators of macrophage stress and inflammatory responses. These results form the basis for future studies identifying novel clinical biomarkers or therapeutic targets in ALI.

Résumé

Le syndrome respiratoire aigu est un état clinique mortel caractérisé par la rupture de la membrane alvéolaire-capillaire et par une défaillance respiratoire. Cette condition peut se survenir après une atteinte inflammatoire directe (une pneumonie) ou indirecte (une sepsie). Les macrophages jouent un rôle important dans le contexte du syndrome respiratoire aigu. Leur activité est contrôlée par des modifications métaboliques et transcriptionnelles des gènes nécessaires pour monter une réponse suffisante au stress et pour la production de cytokines. Celles-ci sont strictement régulées par la protéine kinase connu sous le nom de “cible de la rapamycine chez les mammifères » (mTOR), qui nucléent deux complexes macromoléculaires conservés: le complexe mTOR-1 (mTORC1) et mTORC2. mTORC1 est un détecteur cellulaire de la disponibilité des acides aminés essentiels par la protéine «GCN2» (GCN2). Des études cliniques démontrent une association entre la disponibilité des acides aminés et les résultats cliniques chez les personnes gravement malades. Nous avons donc proposé que la disponibilité des acides aminés essentiels, en particulier de la leucine et / ou de l’arginine, soit vital pour les réponses métaboliques, le stress et les réactions inflammatoires des macrophages liés au syndrome respiratoire aigu. Les macrophages dérivés de la moelle osseuse ont été exposés à un milieu de croissance complet ou dépourvu de leucine, d'arginine et de lysine, en présence ou en absence de saline ou de lipopolysaccharide d'*E. Coli* et d'interféron gamma (LPS / IFN-γ) pendant 6 h. La rapamycine, l’inhibiteur de mTORC1, a réduit la consommation d'oxygène cellulaire liée à la phosphorylation oxydative dans les macrophages dérives de la moelle osseuse. Les effets de LPS / IFN-γ ou la restriction des acides aminés essentiels sur le métabolisme n'ont pas pu être évalués en raison de difficultés techniques. Cependant, l'induction de l'ARNm et de la protéine d'interleukine-6 (IL-6) par le LPS / IFN-γ était

entravé par la restriction des acides aminés essentiels. Bien au contraire, l'induction d'IL-6 a été restaurée par l'addition de leucine seul. De plus, l'induction d'IL-6 était inhibée par l'exposition à un milieu dépourvu de leucine seul. La restriction des acides aminés essentiels a augmenté l'induction des gènes de réponse au stress de la réticulum endoplasmique (CHOP, GADD34) par LPS / IFN-γ. Comme on pouvait s'y attendre, la restriction acide aminés essentiels a bloqué l'activité de la voie mTOR (c'est-à-dire la phosphorylation de 4EBP1) et a augmenté celle de la voie du stress de la réticulum endoplasmique (c'est-à-dire la phosphorylation de eiF2α). Nos résultats indiquent que mTOR et son mécanisme de détection de la leucine sont important pour la régulation du stress et des réponses inflammatoires des macrophages et constituent la base des futures études identifiant de nouveaux biomarqueurs cliniques ou cibles thérapeutiques pour le syndrome respiratoire aigu.

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Preface and Contribution of Authors

This study was conceived through the collaboration of AK and MD. AK and ME designed the experiments and ME performed the experiments with technical assistance from KBJ and EP. ME performed the data analysis. This thesis was written by ME, AK and MD.

List of Abbreviations

AA	- amino acids
ALI	- acute lung injury
AM	- alveolar macrophages
ARDS	- acute respiratory distress syndrome
Arg	- arginine
ATF4	- activating transcription factor 4
ATG13	- autophagosome-related gene 13
BMDM	- bone marrow derived macrophages
CCR2	- C-C chemokine receptor 2
CHOP	- C/EBP homologous protein
DEPTOR	- DEP-domain-containing mTOR interacting protein
EAA	- essential amino acid
ECAR	- extracellular acidification rates
EGF	- epidermal growth factor
eif2α	- Eukaryotic Initiation Factor 2- alpha
ELISA	- enzyme-linked immunosorbent assay
ER	- endoplasmic reticulum
FAO	- fatty acid oxidation
FCCP	- fluorocarbonyl cyanide phenylhydrazone
GADD34	- growth arrest and DNA damage-inducible protein-34
IFN-γ	- interferon gamma
IL	- interleukin
Leu	- leucine
LPS	- lipopolysaccharide
Lys	- lysine
mLST8	- mammalian lethal with Sec13 protein 8
mSIN1	- mammalian stress-activated protein kinase interacting protein
mTOR	- mammalian target of rapamycin

mTORC1	- mammalian target of rapamycin complex 1
NO	- nitric oxide
OCAR	- oxygen consumption rate
OXPHOS	- oxidative phosphorylation
PRAS40	- proline-rich AKT substrate 40 kDA
Proctor-1	- protein observed with Rictor-1
Raptor	- regulatory-associated protein of mTOR
Rictor	- rapamycin-insensitive companion of mTOR
SRC	- spare respiratory capacity
SREBP1	- sterol responsive element binding protein 1
STAT6	- signal transducer and activator of transcription 6
S6K1	- S6 kinase 1
TCA	- tricarboxylic acid cycle
TLR	- toll-like receptor
TSC1	- tuberous sclerosis complex 1
ULK1	- unc-51-like kinase 1
UPR	- unfolded protein response
VEGF	- vascular endothelial growth factor
4EBP1	- Eukaryotic translation initiation factor 4E-binding protein 1

Chapter I: Introduction

1.1 Acute Lung Injury: Disease and Epidemiology

Acute lung injury (ALI) and its more severe form, acute respiratory distress syndrome (ARDS), are life-threatening pulmonary diseases among critically ill patients. After being first described in 1967 by Ashbaugh and colleges[1], ALI and ARDS continue to have up to 40% mortality and account for over 10% of intensive care unit admissions worldwide[2]. The diseases can be a result of direct (pneumonia) or indirect (*e.g.*, sepsis) injury.

In ALI and ARDS, there is diffuse, aggressive inflammation of the lung tissue which leads to increased pulmonary edema, excessive transepithelial immune cell infiltration[3], and breakdown of the alveolar-capillary membrane, which ultimately leads to impaired gas exchange and respiratory failure. Clinically, ALI is defined using the Berlin Definition which has the following criteria: hypoxemia which has three exclusive degrees of severity (mild, moderate, and severe), bilateral pulmonary infiltrates determined by CT or radiography, respiratory system compliance ($\leq$40 mL/cm H_2O), positive end-expiratory pressure ($\geq$10 cm H_2O), and corrected expired volume per minute ($\geq$10 L/min)[4].

In 1977, Bachofen and Weibel were the first to pathologically define ALI as having three distinct phases known as exudative, proliferative and fibrotic[5]. The exudative phase occurs within the first 1-6 days in which there is an accumulation of interstitial and alveolar fluid accompanied by an influx of innate immune cells and red blood cells, as well as thickening of the hyaline membrane in the alveoli[6]. In addition, histological review of the exudative phase shows significant endothelial and epithelial damage[6]. The proliferative phase occurs during days 7-14 when the

resolution of pulmonary edema begins and alveolar epithelial type II cells proliferate[7]. Lastly, the fibrotic phase occurs when there is significant resolution of pulmonary immune infiltrates, repopulation of the alveoli with resident cells, and continued repair of the alveolar-epithelial barrier accompanied with fibrin and collagen deposition in the alveolar lumen[8].

Despite numerous clinical studies conducted to improve medical intervention in ALI/ARDS, treatment for lung injury remains supportive in nature. Many ALI/ARDS patients undergo mechanical ventilation as it is necessary to sustain life when faced with hypoxia due to impaired gas exchange. However, many experimental and clinical studies have shown that mechanical ventilation may, in fact, exacerbate lung injury and contribute to patient morbidity and mortality[9,10]. Despite various clinical trials, there are currently no effective pharmacological therapies for the treatment of the underlying pathophysiology of ALI[11].

1.2 Immune system of the lung

The innate immune system encompasses physical barriers, soluble humoral components and cellular components. Macrophages are quintessential innate immune cells with a high degree of plasticity to achieve various functions. Macrophages were first described by Metchnikoff in 1883 as phagocytic cells[12] and have further been defined as a heterogeneous cell population differentiated by factors such as maturation, tissue migration, phenotypes, and cellular interactions[13-15]. Macrophages are key effector cells in the immune response that influence and orchestrate the adaptive immune system, playing a significant role in tissue inflammation.

Since the lungs are exposed directly to the external environment, they have a specialized immune system to defend against microbial products and inhaled particulates. The lungs are protected by physical barriers as well as both humoral and cellular components. Physical barriers of the airways are imperative to host defence and, include branching of the respiratory tree, ciliary beat, mucus clearance and the cough response[16]. After the physical barriers, there are humoral components such as defensins and complement[17]. Finally, there is the immune environment of the alveolar space. Under homeostatic conditions resident alveolar macrophages make up approximately 95% of leukocytes in the lower lung; lymphocytes contribute 1 to 4% and only around 1% are neutrophils[17]. There are two main types of macrophages in the lower airways: long-lived resident alveolar macrophages (AMs) and, during tissue inflammation, recruited bone marrow-derived macrophages (BMDMs). AMs are the predominant population in the alveolar space during homeostasis and are located in the alveolar lumen, with a population density of approximately seven macrophages per alveolus[18]. AMs are of embryonic yolk sac origin and have self-renewal capabilities[19,20]. Normally, AMs are quiescent in nature, have an immunosuppressive

role in maintaining homeostasis in the lung, and phagocytose excess surfactant proteins. Defects in AM maturation cause the disease alveolar proteinosis, which leads to accumulation of alveolar protein and innate immune defects[13,21,22]. In contrast to AMs, BMDMs are recruited macrophages that migrate to the lungs when a stimulus occurs. BMDMs originate from hematopoietic stem cells in the bone marrow[23]. Monocytes egress from the bone marrow into the bloodstream in a CC-chemokine receptor 2 (CCR2)-dependant manner[24]. Inflammatory monocytes, which express high levels of CCR2 and Ly6C, are the major source of recruited macrophages in the lung during an infection[24].

Mature macrophages are commonly classified based on their function. There are M1-like, pro-inflammatory or M2-like, anti-inflammatory macrophages. The M1 phenotype is classically achieved *in vitro* through the stimulation of monocytes with Toll-like receptor (TLR) ligands (such as lipopolysaccharide (LPS)) and/or inflammatory cytokines, such as interferon-γ (IFN-γ)[25]. M1 macrophages secrete pro-inflammatory cytokines such as interleukin (IL)-1β, IL-8, and produce nitric oxide which is known to have antimicrobial properties[26]. In contrast, M2-like macrophages are stimulated by type II cytokines such as IL-4 or IL-13[27]. M2-like macrophages exhibit a higher phagocytic capacity than that of M1-like macrophages, produce extracellular matrix components, and secrete angiogenic and chemotactic factors[28]. Furthermore, M2 macrophages enhance the conversion of arginine to ornithine, a substrate for proliferation, and promote tissue repair[27]. Together, these functions of M2-like macrophages, and their known secretion of IL-10 have led them to be termed anti-inflammatory, pro-resolving, wound healing, tissue repair or regulatory macrophages. Therefore, under the M1-M2 paradigm, macrophages serve a dual role in the body:

surveillance, initiation of inflammation and resolution of inflammation and tissue repair after an infection or insult.

1.3.1 Innate immune response during acute lung injury

1.3.1.1 Macrophage-mediated initiation and repair in acute lung injury

Macrophages play a role in the initiation, resolution and repair phases of acute lung injury. Initial studies revealed increased macrophage cell numbers in the airways and lung tissues of animals after acute lung injury was induced by bacterial LPS, bleomycin or particulate matter[29]. A multitude of studies looking at the various effects of lung toxicants on macrophage polarization and function have shown that regardless of the specific stimulant, macrophages generate an increased cytotoxic and proinflammatory response including upregulation of IL-6, IL-1β, ROS, TNF-α, chemokines, eicosanoids, and bioactive lipids[30]. It has been previously demonstrated by Ganter and colleagues that macrophage-secreted IL-1β acts on alveolar endothelium and epithelium inducing vascular permeability in an integrin-mediated epithelial tumour growth factor-β-dependent manner[31]. Experimental models using CCR2 knockout mice which are defective in monocyte recruitment from the bone marrow, conferred protection against ALI, indicating an important role for BMDM's in lung inflammatory injury[32-34].

A primary contribution to the pathogenesis of ALI is the accumulation of pulmonary edema. During ALI, the release of pro-inflammatory mediators such as IL-1β and TNF-α inhibits apical membrane epithelial Na^+ channels that are responsible for controlling the osmotic gradient, thereby inhibiting the clearance of edema fluid from the alveolar space[35].

Lung inflammation is not simply terminated once the pathogen or stimulus is cleared during ALI. The resolution of inflammation and initiation of repair is an active and tightly regulated response that involves activation of counter-regulatory mechanisms needed to terminate the initial inflammatory response[36]. Despite strong evidence supporting a detrimental effect of macrophages in ALI, anti-inflammatory or pro-repair M2-like macrophages are also important for successful resolution and repair. M2-like macrophages express markers such as CD206, Ym-1, Arg-1, and Fizz-1[37] and populate the lung following the influx of pro-inflammatory macrophages. Such a delayed response relative to M1-like macrophages coincide with their role in resolution and repair. Following the onset of ALI, M2-like macrophages orchestrate the suppression of inflammation and the initiation of repair mechanisms through the secretion of cytokines such as IL-4, IL-10 and IL-13[38,39], signaling for the generation of extracellular matrix protein and growth factors[40]. Resident AMs also protect against ALI. In human bronchoalveolar lavage fluid of patients with ALI, an increased number of mature alveolar macrophages were associated with more favourable prognosis[41,42].

Macrophages can also actively terminate neutrophil infiltration. Upregulation of the receptor for cytokine IL-1β by macrophages downregulates the release of MIP-2 and ICAM-1 by the alveolar epithelium, effectively attenuating alveolar neutrophil recruitment[43]. Herold and colleagues have also shown that macrophage-derived IL-1 receptor α induces neutrophil apoptosis as a means to clear the inflammatory infiltrates in the lung[44]. AMs are the primary source of TNF-α during ALI[43] and during the resolution phase of ALI, higher concentrations of TNF-α are associated with increased neutrophil apoptosis and clearance[45]. Furthermore, resident alveolar macrophages and GR-1^{high}CCR2^{high} alveolar macrophages were shown to have increased

expression of the death ligand TRAIL which further contributes to neutrophil apoptosis in an LPS-induced lung injury model[46].

Finally, macrophages play a significant role in the removal of apoptotic cells through phagocytosis to limit persistent inflammation and tissue damage by initiating clearance and promoting resolution. “Eat me” signals expressed by neutrophils undergoing apoptosis trigger alveolar macrophages to engage in efferocytosis[47]. Efferocytosis, or the phagocytic clearance of apoptotic cells[48], induces a phenotypic change in macrophages leading to the secretion of growth factors such as vascular endothelial growth factor (VEGF)[49] or hepatocyte growth factor[50] which are crucial for tissue repair following injury. Furthermore, efferocytosis induces the secretion of anti-inflammatory mediators such as transforming growth factor-β, IL-10 and prostaglandin E-2[48].

1.3.1.2 The role of macrophage-derived factors in the initiation and repair of acute lung injury

Cytokines and chemokines are low molecular weight proteins that are essential for mediating intercellular communication. They are primarily produced by macrophages and lymphocytes and are responsible for orchestrating and maintaining a multitude of cellular processes including the initiation and regulation of local and systemic inflammation, cell proliferation, metabolism, chemotaxis and tissue repair. There are several macrophage-derived cytokines and chemokines that are known to be influenced by amino acid availability such as VEGF and IL-6, which are important for the initiation and repair of acute lung injury.

The angiogenetic factor VEGF is important for proper development but also contributes to many pathological states. VEGF mRNA is most highly expressed in the lungs of adults with alveolar protein levels approximately 500 times that of the plasma[51,52]. Alveolar epithelial cells are the major source of VEGF in the lung[53] although it is also expressed by smooth muscle cells, macrophages and endothelial cells[54,55]. During the initiation of ALI, VEGF stimulates endothelial cell permeability and acts as a pro-inflammatory mediator due to its effects as a chemoattractant for neutrophils, monocytes, and macrophages. VEGF secretion from activated macrophages and neutrophils have been hypothesized to contribute to the increased VEGF levels observed during early phases of ALI. A study published in 2016 used a murine two-hit model of ventilator-induced ALI and found that bone marrow-derived Ly6C^{high} monocytes secrete VEGF that contributes to its pathogenesis[56].

Interleukin-6 (IL-6) is a pleiotropic cytokine that can be produced by various cell types including macrophages, lymphocytes, endothelial cells, and epithelial cells[57]. Whereas IL-6 might appear to be detrimental in some studies, others have demonstrated a salutary effect. IL-6 traditionally acts as a significant mediator of the pro-inflammatory response during acute inflammation[58]. IL-6 is necessary for the induction of the acute phase inflammatory response and is important for systemic and local responses including fever, leukocyte recruitment and hemodynamic effects[59]. In patients with ALI, plasma and bronchoalveolar lavage (BAL) fluid IL-6 levels are significantly increased[60,61]. IL-6 is used as a prognostic biomarker for patients with ALI, such that persistently high levels of IL-6 is negatively correlated with patient prognosis[62].

Many studies have used an IL-6 knockout (IL-6 $^{-/-}$) murine model to examine the role of IL-6 in the induction and resolution of ALI. In a study using an IL-6$^{-/-}$ murine model of LPS and mechanical ventilator-induced lung injury, IL-6$^{-/-}$ mice exhibited more significant lung inflammation, lung edema and lung injury compared to wild-type mice[59]. Moreover, administration of recombinant human IL-6 could diminish the damage by LPS and mechanically ventilated IL-6$^{-/-}$ mice[59]. IL-6$^{-/-}$ mice were more susceptible to a lethal dose of Influenza A virus and had reduced repair and resolution of lung injury[63]. The authors also observed decreased macrophage recruitment to the lung and the macrophages that did end up in the lung exhibited reduced efficacy in phagocytic activities[63]. Therefore, the authors suggest that the absence of recruited and not just resident macrophages are detrimental to the repair and resolution of ALI following IAV infection[63]. For the purpose of this thesis, we will assess IL-6 expression as a representative target, and as a major macrophage-derived cytokine known to be amino acid sensitive. We propose that factors altering the expression of macrophage-derived IL-6 might modulate the evolution of acute lung injury. Here we focus on nutrient availability and its effect on mTOR and ER stress signaling as modulators of macrophage function.

1.4 Mammalian Target of Rapamycin (mTOR)

1.4.1 Structure and organization of mTOR complexes

Mechanistic target of rapamycin (mTOR) is a serine/threonine kinase that is highly conserved across species from yeast to mammals. mTOR is a 289-kDa protein belonging to the phospho-inositide 3- kinase family that has a crucial role in the integration of cellular stimuli to control and promote anabolic cellular processes while suppressing catabolic activities[64]. mTOR functions in two structurally distinct complexes termed mechanistic target of rapamycin complex 1 (mTORC1) and mTORC2.

mTORC1 is the better-characterized complex due to its sensitivity to rapamycin, which has allowed for extensive studies of its regulation and function. mTORC1 is comprised of five components: the catalytic subunit of the complex mTOR, regulatory-associated protein of mTOR (Raptor), mammalian lethal with Sec13 protein 8 (mLST8), proline-rich AKT substrate 40 kDA (PRAS40), and DEP-domain-containing mTOR interacting protein (Deptor). Raptor plays a significant role in recruitment, recognition and binding[65] of substrates containing a TOR signaling motif[66,67] and is necessary for the subcellular localization of mTORC1[68]. The role of mLST8 remains elusive, while PRAS40 and Deptor are known endogenous kinase inhibitors[64].

In contrast to mTORC1, mTORC2 is comprised of six proteins. mTORC2 shares central components mTOR and mLST8, as well as, Deptor with mTORC1. However, it differs in its defining proteins. mTORC2 is comprised of rapamycin-insensitive companion of mTOR (Rictor), protein observed with Rictor-1 (proctor-1), and mammalian stress-activated protein kinase

interacting protein (mSIN1)[64]. Together, Rictor and mSIN1 function as the structural basis of mTORC2[69]. Similar to its function in mTORC1, Deptor acts as a negative regulator of mTORC2[70].

mTOR function has been extensively studied using the bacterial macrolide rapamycin. mTORC1 and mTORC2 were primarily defined by their sensitivity to rapamycin. It was originally observed that mTORC1 was rapamycin-sensitive whereas mTORC2 was not. Jacinto and colleges observed that upon treatment, rapamycin enters the cell interacting with the mTOR subunit and actively inhibiting mTORC1 function, meanwhile having no effect on mTORC2 function[71]. However, studies looking at chronic rapamycin exposure have demonstrated inhibition of mTORC2 assembly and therefore inhibition of its activity[72,73]. mTOR's inhibition with rapamycin enhanced LPS-induced ALI in mice[67].

1.4.2 mTOR Effector Functions

mTORC1 integrates both intra- and extracellular signaling including growth factors, amino acids, oxygen, energy status, and stress in order to regulate major cellular processes such as protein synthesis, metabolism, and autophagy[74]. mTORC1 controls protein synthesis through the direct phosphorylation of eukaryotic initiation factor 4E-binding protein (4EBP1) and p70 S6 kinase 1 (S6K1)[75]. Phosphorylation of 4EBP1 prevents mTOR binding to eIF4E, thus permitting eIF4E cap-dependent translation[76]. Protein synthesis is further stimulated through the mTORC1 dependent stimulation of S6K1, which promotes an increase in mRNA biogenesis, cap-dependent translation, and regulation of downstream ribosomal proteins[75]. Additionally, mTORC1 activation has been shown to increase ribosomal biogenesis by enhancing the transcription of ribosomal RNA

genes[77]. Other anabolic functions of mTORC1 include increased nucleotide synthesis through activation of activating transcription factor 4 (ATF4), thus promoting cell proliferation[78].

mTORC1 controls cellular metabolism and ATP production through regulation of transcriptional and translational targets such as hypoxia-inducible factor 1-α[79]. mTORC1 activates sterol responsive element binding protein 1 (SREBP1) which is downstream of S6K1 and ultimately controls the expression of genes involved in lipid synthesis as well as increasing stimulation of the oxidative pentose phosphate pathway[79]. Furthermore, mTORC1 regulates metabolism by its nuclear association with PPAR-γ coactivator 1α and transcription factor Ying-Yang 1 which is responsible for regulating genes for mitochondrial biogenesis and oxidative function[37].

The role of mTORC1 as a negative regulator of autophagy has been well established. During cellular stress such as nutrient deprivation, mTORC1 is inhibited and this activates autophagy by dephosphorylation of the autophagy initiator unc-51-like kinase 1 (ULK1). When mTORC1 is activated, it inhibits autophagy at both the transcriptional and post-transcriptional level[80]. mTORC1 activation results in the phosphorylation of ULK1 and autophagosome-related gene 13 (ATG13), thereby inhibiting the formation of the autophagosome. Furthermore, mTORC1 directly regulates the transcription of p73[81] and transcription factor EB (TFEB)[82], which are both important contributors to autophagy.

Although mTORC1 has been extensively studied, mTORC2 functions remain still relatively elusive. mTORC2 is known to play a role in cell survival, growth and proliferation through its ability to phosphorylation of Akt[83]. mTORC2 has also been implicated in many studies to play a role in the structure and remodeling of the cytoskeleton[84] as well as cell migration[85-87].

1.4.3 Endogenous mTOR modulators:

mTOR is a central signaling hub that can sense a variety of signals to then orchestrate the appropriate anabolic or catabolic response. Nutrient availability is a highly conserved modulator of mTORC1 activity. In a nutrient and energy sufficient environment, mTORC1 is active and promotes anabolic responses for cell growth, proliferation, and function[88]. In contrast, energy or nutrient restriction inactivates mTORC1, thus, promoting catabolic responses[88]. mTOR can be activated by secreted growth factors, cytokines, and hormones, but the most highly conserved input is nutrient availability. Studies have shown that in yeast, availability of nutrients such as amino acids, glucose, and oxygen is imperative for proper mTOR activity[64]. For this thesis, emphasis will be upon amino acids as an input signal for the mTOR pathway.

Amino acids (AA) are most fundamentally known as the building blocks for proteins. Beyond that primary function, however, individual AA can regulate cell functions. AA are critical for regulating growth, development, immunity, and overall health. Although the majority of AA can be produced by the body or otherwise obtained through a healthy diet, AA deficiency still occurs and is a troubling concern. Most commonly, dietary protein deficiencies lead to a decrease in the concentrations of most AAs in plasma[89] and thus decrease the availability of AAs to

be utilized by cells. Without adequate amounts of amino acids, the body must initiate catabolic reactions to provide free amino acids from elsewhere in the body, or, the body risks compromising critical pathways and networks due to a lack of available resources. Essential amino acids can only be derived from dietary intake. The presence of essential amino acids promotes mTOR function through the interaction of mTORC1 with Rag GTPases at the lysosomal surface, where it is activated by the GTPase Rheb[90]. The lysosome is an integral organelle for maintaining amino acid availability as it digests proteins to release amino acids that can then be utilized by the cell[91].

In recent years, studies have shown that there are mTOR adaptor proteins which sense and bind specific amino acids in order to activate mTOR and facilitate proper responses. The first to be discovered was SLC38A9. SLC38A9 is a lysosomal transmembrane protein that acts as a positive regulator for the mTORC1 pathway with an affinity for arginine[92]. The function and signaling mechanism of SLC38A9 have yet to be fully defined. Two of the more thoroughly studied mTORC1 adaptor proteins are the CASTOR and Sestrin families which bind arginine and leucine, respectively[93-95].

1.4.4 mTOR and the endoplasmic reticulum stress response

The endoplasmic reticulum (ER) stress response is another potential sensing mechanism for amino acid availability. The ER is a membrane-enclosed organelle that is critical for the proper synthesis, folding and secretion of proteins among other biological functions such as calcium homeostasis and lipid biosynthesis. The ER is the site at which proteins are folded into their proper conformation and assembled into multi-subunit proteins. The ER is sensitive to physiological

stresses such as unfavourable metabolic conditions (*e.g.*, hypoxia, energy deprivation and oxidative stress), loss of calcium homeostasis within the ER, and pathological stresses as viruses. As a result of ER stress, the protein folding machinery within the ER lumen is disrupted resulting in the accumulation of misfolded proteins or protein aggregates [96]. The cells respond to ER stress through a mechanism termed the unfolded protein response (UPR). The UPR is a complex, interrelated network of signalling mechanisms that initially aims to reduce the amount of unfolded proteins in part by inhibiting new protein synthesis. Inhibition of protein synthesis occurs via the phosphorylation of eiF2α and its subsequent attenuation of the translational machinery. If the cell is not able to restore homeostasis, or the stress remains persistent, then the cell can switch to a pro-cell death response, inducing apoptosis, which is also initiated by ER stress response intermediates[97].

The ER stress response can also sense amino acid availability via a mechanism termed the 'integrated stress response', which is in part regulated by the general control nonderepressible 2 (GCN2) pathway. Amino acid depletion leads to uncharged tRNA, which then binds and activates the protein kinase GCN2[98]. Upon sensing the accumulation of uncharged tRNAs, GCN2 can then phosphorylate eif2α which inhibits general mRNA translational mechanisms, but preferentially enhances translation of selected stress response mRNAs, including that for activating transcription factor-4 (ATF4)[99]. ATF4 induces downstream expression of C/EBP (CHOP), as well as amino acid transporters[99]. CHOP is responsible for activating genes involved in apoptosis, as well as inducing transcription of growth arrest and DNA damage-inducible protein-34 (GADD34)[100]. GCN2 can directly inhibit mTOR by increasing levels of GADD34, in turn activating the mTORC1 repressor tuberous sclerosis complex-2[101].

There has been strong evidence accumulating suggesting that cellular stress including nutrient availability and the induction of the ER stress response can heavily influence the function of immune cells. The role of ER stress in macrophage differentiation and function has been studied. In the human monocyte cell line THP1, induction of ER stress by the LPS receptor TLR4 leads to impaired monocyte differentiation[102]. ER stress signalling through TLR4 is also important for macrophage survival during the immune response and upon LPS stimulation will downregulate CHOP expression[103]. In contrast, prolonged ER stress can lead to CHOP-mediated apoptosis through the activation of Fas, a death receptor[104]. A study published by Iwasaki et al. linked metabolism induced ER stress to IL-6 expression in macrophages through ATF4[105]. They found that ATF4 is required for IL-6 induction in response to metabolic stresses. Using an IL-6 promoter luciferase assay in RAW264 cells, they observed that stimulation of macrophages with LPS induces TLR4 activation and synergizes with ATF4 to further enhance IL-6 expression[105]. Moreover, they elucidated that the mechanism of induction is dependent on ATF4 activation of nuclear factor-κB and that ATF4 is capable of directly activating the IL-6 promotor in BMDM[105]. Thus, the ER stress response in macrophages contributes to the activation of important downstream signaling pathways related to macrophage differentiation and function.

Recent evidence shows that there is cross-talk between the two pathways when determining cell survival or cell death fates[106]. For instance, inhibition of mTOR activity by rapamycin during ER stress can increase cell viability[107]. Furthermore, there is evidence to suggest that the UPR can function upstream of mTOR as determined through pharmacological induction of the UPR which subsequently activates mTOR[106]. Therefore, we propose that amino acid restriction will modulate

macrophage function (*i.e.*, IL-6, CHOP and GADD34 expression) via the mTOR and/or ER stress pathways.

1.5 Immunometabolism

Immunometabolism is a rapidly growing field in immunology as researchers are looking towards how immune cell metabolism can alter effector function and thus, how it can be modulated to improve the outcomes of various diseases. Regulated by mTORC1, metabolic reprogramming of macrophages is a key area of interest as it has been established that M1 and M2-like macrophages utilise different metabolic pathways to assert their respective effector functions.

1.5.1 M1-like macrophage metabolism

M1-like macrophages are often the primary first responder to sites of inflammation therefore requiring a rapid source of energy. M1-like macrophages exhibit upregulated aerobic glycolytic metabolism, which is necessary for generating enough ATP to support phagocytic and microbicidal functions. Glycolysis is less efficient than oxidative phosphorylation, generating only two molecules of ATP per molecule glucose versus oxidative phosphorylation which generates thirty-six molecules of ATP per glucose. It would appear counterintuitive for M1-like macrophages to use the glycolytic pathway, however, since glycolysis can be utilized more rapidly than oxidative phosphorylation, it provides the necessary energy to efficiently produce the intermediates necessary for the cell to orchestrate many of their inflammatory functions such as cytokine production.

LPS is a potent activator of M1-like macrophages through toll-like receptor 4 (TLR4). LPS increases glucose uptake, enhances anaerobic glycolysis and impairs oxidative phosphorylation[108,109]. Impaired oxidative phosphorylation via the break-down of the tricarboxylic

acid (TCA) cycle results in an accumulation of TCA intermediates. These intermediates contribute to an enhanced pro-inflammatory and microbicidal environment through the upregulation of enzymes involved in glycolysis. For instance, succinate and pyruvate kinase-M2 accumulation leads to the stabilization of hypoxia-inducible factor-1α and the increase in the pro-inflammatory cytokines such as IL-1β[109,110]. The increased uptake of glucose observed during TLR4 stimulation results in the production of biosynthetic intermediates that are then utilized via the pentose phosphate pathway to produce NADPH. NADPH is crucial for reactive oxygen species (ROS)[111] and nitric oxide (NO) production[112] which then participate in the elimination of pathogens. Altogether, the change in metabolic phenotype enables the cell with a rapid amount of energy that is essential for bactericidal activity. For example, phagocytic BMDMs switch to glycolysis as opposed to alveolar macrophages which use oxidative phosphorylation. A study performed by Mould et al. characterized the immunometabolism of resident versus recruited macrophages during acute lung injury and showed that the two subsets differed significantly in their response to LPS. They showed that recruited macrophages had upregulated immune signaling, inflammatory pathway markers, glycolytic, and arginine metabolism; AMs exhibited increased proliferative capacity and utilized the tricarboxylic acid pathway and fatty acid metabolism[113]. This metabolic reprogramming is one mechanism that may initiate metabolic changes in cells.

Another distinct characteristic of M1-like macrophages possibly related to changes in metabolism and gene expression is their use of essential amino acids (EAA). Arginine is depleted at sites of inflammation and thus, acts as a limiting factor[114]. M1-like macrophages express inducible NO synthase (iNOS), an enzyme that metabolizes arginine into large amounts of NO and citrulline[115]. NO is cytotoxic to bacteria, and also forms toxic metabolites[116].

1.5.2 M2-like macrophage metabolism

M2-like macrophages are activated via type II cytokines such as IL-4 or 13 and are traditionally thought to play an exclusive role in tissue repair. In contrast to the M1-like macrophage metabolic phenotype, M2-like macrophages rely on oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) enabling a longer sustained energy supply[117] without having to engage aerobic glycolysis[118]. By using OXPHOS and FAO as primary sources of ATP, M2-like macrophages limit the amount of ROS and NO produced[119].

The metabolic switch from anaerobic glycolysis to OXPHOS and FAO is primarily driven by signal transducer and activator of transcription 6 (STAT6). STAT6 induces expression of mitochondrial genes such as PGC1-β, PPARγ, and PPARδ[120]. PGC1-β plays an important role in the initial metabolic switch towards OXPHOS as it is responsible for inducing mitochondrial respiration, mitochondrial biogenesis. Furthermore, PGC1-β is integral to produce key mitochondrial components such as cytochrome C and ATP synthase[119]. STAT6 transcription factor and CEBP/β promote the induction of the enzyme arginase-1 in M2-like macrophages by binding directly to the enhancer[121], effectively utilizing arginine and catalyzing the hydrolysis of ornithine and urea[114,122]. Mills and colleagues first alluded to the importance of macrophage-derived ornithine to be crucial for wound healing as it diverted the metabolic pathway away from inflammatory NO production[123,124] .

1.5.3 Role of mTOR in macrophage metabolism and function

Since macrophages are highly plastic cells whose functions and phenotypes are tightly linked to their metabolic status, many studies have attempted to elucidate the molecular mechanism by which they are linked. Due to mTOR's role as a central regulator that connects environmental signals to downstream processes, studies have been trying to uncover the connection between mTOR function and macrophage function. Studies have investigated the role of constitutive mTOR activation in macrophage polarization using tuberous sclerosis complex 1-(TSC1) deficient mice. Byles and colleagues found that TSC1-deficient macrophages exhibited the M2 phenotype through a mechanism independent of STAT6 and PPARγ signaling[125]. Furthermore, they found that constitutive mTORC1 activity induced by TSC1 deficient macrophages provides a negative feedback loop that can augment cytokine production[125]. In 2014, Zhu et al. confirmed that TSC1 deficient macrophages had increased mTORC1 activity that causes increased sensitivity to LPS leading to M1-like macrophage polarization in a RAS-ERK dependent manner[126]. The same study observed that constitutive mTOR activation reduces M2-like macrophage polarization due to the mTORC1- CEBP/β axis[126]. Another study looking at how IFN-γ effects macrophage polarization found that IFN-γ suppresses mTORC1 activation through selective inhibition of mRNAs involved in metabolism and protein synthesis[127]. The mechanism by which mTOR acts as an integrator of amino acid availability and how it subsequently affects macrophage polarization and function has yet to be elucidated. In this thesis, we investigate IL-6 induction and ER stress activation as indicators of how amino acid availability modulates mTOR-dependent signaling in macrophages exposed to LPS and IFN-γ.

1.6 Central Hypothesis and Aims

We proposed that **reduced extracellular (*in vitro*) availability of essential amino acids regulates the induction of mTOR-dependent transcriptional programs (*i.e.*, reduction in IL-6; GADD34 CHOP) in macrophages,** which, in future studies, might worsen the severity of ALI.

To investigate this hypothesis, we designed two specific aims:

1: To define the effects of amino acid restriction on mTOR-dependent transcriptional (*i.e.*, IL-6, ER Stress) and metabolic programs in bone marrow-derived macrophages.

2: To determine which amino acid is responsible for the transcriptional and metabolic phenotype, and via which molecular mechanism.

Chapter II: Materials and Methods

2.1 Mice

Six- to ten- week old C57BL/6 mice were bred in house. All experiments were conducted in accordance with the guidelines of the animal research ethics board of McGill University (AUP #5960).

2.2 Preparation of media

Media used for the generation of bone marrow-derived macrophages were made using RPMI-1640 (Sigma R8758 containing 0.04 g/L L-Lysine HCL, 0.05 g/L L-Leucine, and 0.02g/L L-Arginine) supplemented with 10% heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% essential amino acids 1% non-essential amino acids, and 100U/mL penicillin/streptomycin all from Wisent).

Amino acid-complete media used for the treatment of BMDMS was made using RPMI-1640 (Sigma R8758 containing 0.04 g/L L-Lysine HCL, 0.05 g/L L-Leucine and, 0.02g/L L-Arginine) supplemented with 10% amino acid-free (dialyzed) heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% non-essential amino acids, and 100U/mL penicillin/streptomycin all from Wisent)

Amino acid-deficient media was made using RPMI-1640 lacking leucine, lysine and arginine (Sigma R1780) supplemented with 10% amino acid-free (dialyzed) heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% non-essential amino acids, and 100U/mL penicillin/streptomycin all from Wisent).

Single amino acid add-back media or single amino acid-deficient media were made by reconstituting 0.04 g/L L-Lysine HCL (Bioshop LYS101), 0.05g/L L-Leucine (Bioshop LEU222), and/or 0.02 g/L L-Arginine (Bioshop ARG006) in RPMI 1640 lacking leucine, lysine, and arginine (Sigma 1780) supplemented with 10% amino acid-free (dialyzed) heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% non-essential amino acids and, 100U/mL penicillin/streptomycin all from Wisent).

2.3 Generation of bone-marrow-derived macrophages (BMDM)

Femurs and tibias from ten to twelve-week-old C57BL/6 mice were harvested aseptically. Bones were flushed with 10% (RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS), from Wisent) and whole bone marrow was plated in 10 mL BMDM medium (RPMI-1640 (Sigma) supplemented with 10% heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% essential amino acids 1% non-essential amino acids, and 100U/mL penicillin/streptomycin all from Wisent), containing 30% L929 cell- (ATCC) conditioned medium at the density of 5-10x10^6 cells per 10cm petri dish and cultured at 37°C, 5% CO_2. After 4 days, 9 mL of fresh BMDM medium containing 30% L929 medium was added to each dish. On day 6, non-adherent cells were aspirated, and adherent macrophages were washed once with PBS prior to using Cell Stripper (Corning).

2.4 *In vitro* stimulation of BMDMs

After isolation, BMDM were plated at 1x10^6/well in a 6-well plate in BMDM medium and allowed to adhere for 24 hours prior to stimulation with 100 ng/mL *E. coli* lipopolysaccharide (Sigma) and

150 U/mL IFN-γ (Sigma), 100 ng/mL rapamycin in either BMDM treatment media (RPMI-1640 (Sigma) supplemented with 10% heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% non-essential amino acids, and 100U/mL penicillin/streptomycin all from Wisent) or media deficient in arginine, leucine and/or lysine (RPMI-1640 -3aa (Sigma) supplemented with 10% heat-inactivated FBS, 2% HEPES, 1mM sodium pyruvate, 1% essential amino acids 1% non-essential amino acids and, 100U/mL penicillin/streptomycin all from Wisent).

2.5 Quantitative reverse transcription PCR (RT-qPCR)

Total RNA was isolated from macrophages and purified using the RNeasy mini kit (Ambion). 500ng of RNA was reverse transcribed to cDNA using Superscript IV Reverse Transcriptase (ThermoFisher Scientific) as per the manufacturer's instructions. cDNA was subsequently used for the qPCR reaction with SYBR power up mastermix (ThermoFisher) and the following primers: *GAPDH*-forward: 5'-ACCACAGTCCATGCCATCAC-3'; *GAPDH* reverse 5'-TCCACCACCCTGTTGCTGTA- '3, *IL-6*-: 5'-CATGTTCTCTGGGAAATCGTG -3'; *IL-6* reverse 5'-TTCTGCAAGTGCATCATCG- '3, *CHOP*-: 5'- AACAGAGGTCACACGCACAT -3'; *CHOP* reverse 5'- ACTTTCCGCTCGTTCTCCTG- '3, *GADD34*-: 5'-AGCCTAAGCAGGAGGGAGAA -3'; *GADD34* reverse 5'- CAGCATTCCGACAAGGGTGA- '3 . Ct values were obtained and analysed using the $^{\Delta\Delta}$Ct method. Expression of all mRNA was normalized to GAPDH and depicted as a fold-change.

2.6 Seahorse Extracellular Flux Assay

Real-time oxygen consumption rates (OCR) and extracellular acidification rates (ECAR) of BMDMs was measured using a Seahorse XFe 96 Analyzer (Agilent Technologies). After differentiation into macrophages, BMDMs were plated at $1x10^5$/well in a 96-well plate in XF media (non-buffered DMEM containing 2mM L-glutamine, 25mM glucose, and 1mM sodium pyruvate). Three basal measurements were taken, followed by the injection of oligomycin (1uM, Sigma) and then 2 consecutive measurements. Next, fluorocarbonyl cyanide phenylhydrazone (FCCP, 1.5uM, Sigma) was injected followed by another 2 consecutive measurements. Prior to another 2 consecutive measurements, antimycin A (0.5uM, Sigma) and rotenone (0.5uM, Sigma) were injected in unison. Basal respiration was calculated using the last basal measurement minus the non-mitochondrial respiration rate (as determined by the last measurement taken following antimycin A/rotenone injection). ATP production was determined as the decrease in OCR following oligomycin injection. Finally, spare respiratory capacity was determined as the absolute increase in OCR proceeding the injection of FCCP compared to basal respiration. All measurements were normalized to the viable cell number as determined by a crystal violet dye extraction assay. OCR curves and calculates were generating using Wave Desktop 2.3 (Agilent Technologies).

2.7 High-performance liquid chromatography (HPLC)

High-performance liquid chromatography (HPLC) experiments were performed by the lab of Dr. Linda Wykes (School of Human Nutrition, McGill University, Canada). The concentrations of the following 20 plasma AAs were measured by HPLC: isoleucine, leucine, valine, lysine, methionine,

phenylalanine, threonine, tryptophan, alanine, arginine, asparagine, citrulline, glutamate, glutamine, glycine, histidine, ornithine, proline, serine, and tyrosine. Supernatant samples were de-proteinized by the addition of 300 ul of methanol to 100 ul of supernatant followed by filtration with 0.2-uM filters. All samples were analyzed, in duplicate, using an Agilent 1290 UHPLC (Agilent Technologies, Mississauga, Canada) on an Agilent Poroshell 120 EC-C18 4.6 150 mm 2.7 uM column. Supernatant AAs were analyzed by UHPLC. The AA's were detected after pre-column derivatization with o-phthalaldehyde (OPA) and 9-fluorenyl-methyl chloroformate (FMOC). Separation was carried out at a flow rate of 1.5 ml/min under gradient conditions. Mobile phase A was 10 mM Na_2HPO_4, 10 mM $Na_2B_4O_7$, pH 8.2, and mobile phase B was acetonitrile: methanol: water (45:45:10). Initial gradient was 2% B for 0.5 min, then 2% to 57% B in 20 min followed by 5 min re-equilibration before the next injection. Derivatized AAs were measured by fluorescence with excitation and emission wavelengths of 230 nm and 450 nm, respectively. Data from standards and samples were analyzed using MassHunter B.05 software.

2.8 ELISA

The production of IL-6 protein was measured using commercial enzyme-linked immunosorbent assay kits (R&D systems). After subjecting BMDMs to *in vitro* stimulation for six hours, cell media was collected and centrifuged at 4^{o}C, 1,500 rpm for 10 minutes to pellet the debris. The soluble portion was then collected and stored at -80^{o}C overnight. 100uL of cell supernatant sample was used per well of a 96-well plate. The assay was performed as per the manufactures instructions and the fluorescence was read at OD450.

2.9 Western Blot

Protein expression analysis of BMDMs were performed by Western blot. Cells were lysed using RIPA buffer (AbCam) for 10 minutes on ice followed by 10-minute centrifugation at 4°C and 13,000 rpm to pellet debris. The supernatant was collected and quantified using BCA protein quantification. The OD595 of each sample was then measured. 15ug of cell protein per sample was loaded and run through a 12% SDS-PAGE gel before transferring electrophoretically at 300mA for 2 hours onto nitrocellulose membrane (BioRad). The membrane was then blocked for 1 hour in 5% BSA dissolved in 1x Tris Buffered Saline and 0.1% Tween (TBS-T) for 1 hour at room temperature and probed overnight at 4°C with the desired antibody. The following day, membranes were washed three times in TBS-T followed by a one-hour incubation at room temperature in Dylelight800 anti-rabbit secondary antibody (Invitrogen).

The results were developed using a LI-COR Odyssey infrared imaging system (LI-COR Biosciences). For quantification, the density of the bands was determined using ImageJ. The density of the phosphorylated band was compared to the density of the total band for eif2α for 3 independent experiments. All calculations were normalized to the 0h control to determine the fold change induction of each treatment. The density of the phosphorylated bands for 4EBP1 were quantified for one experiment as a representation.

2.10 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 7.02 (GraphPad Software, San Diego, California, USA). Figure 1 shows the mean of 3-5 technical replicates. The error bars represent the SEM of those technical replicates. Although between the two experiments the trends

and significance were the same, the absolute values between the two experiments varied. Thus, the results were not pooled, and representative figure was shown. Figures 3-6 show the mean value of 'n' experiments where the averages of technical replicates from each individual experiment were used (i.e., biological replicates). The error bars represent ± SEM and difference were considered signification if $P<0.05$. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparisons.

Chapter III: Results

3.1. Effects of amino acid restriction on oxidative metabolism

To evaluate the effects of mTOR activity and amino acid restriction on the oxidative phosphorylation capacity of bone marrow-derived macrophages, we performed extracellular flux analysis. Extracellular acidification of the cell media is linked to the glycolytic rate of the cell. Since anaerobic glycolysis generates lactate and free hydrogen ions, it decreases the pH of the media. Since oxidative phosphorylation (OXPHOS) consumes O_2, the amount of oxygen in the media can be measured as an indication of the amount of energy the cells generate *via* OXPHOS. We measured the OXPHOS generated in BMDMs at baseline (untreated), and those exposed to rapamycin (positive control for inhibition of mTORC1), stimulated with LPS and IFN-γ, all in the presence or absence of arginine, leucine and lysine. Measurements were taken at baseline, and after the sequential injection of mitochondrial inhibitors oligomycin, FCCP and both antimycin A and rotenone. There was no effect of AA restriction on basal respiration. Inhibition of mTORC1 with rapamycin blocked basal respiration. (Fig. 1A). Following baseline measurements, Oligomycin was injected to inhibit ATP synthase. This permits measurement of the component of OXPHOS related ATP synthesis. There was no difference in ATP production between amino acid replete or deplete conditions except when stimulated LPS and IFN-γ, where we observed that amino acid deplete conditions were not able to generate as much ATP *via* OXPHOS (Fig. 1D). Next, FCCP was injected into the media of the cells. FCCP is an uncoupling agent which disrupts the proton gradients and induces maximum oxygen consumption. This measurement allows us to calculate the maximum respiration of the cells, as well as the spare respiratory capacity. Again, the same trend was observed where amino acid restriction reduced maximum respiration (Fig. 1B) and spare respiratory capacity (Fig. 1C) in LPS and IFN-γ-exposed macrophages. Unfortunately,

control experiments to ensure that the proper levels of amino acids were added back to experimental media showed inconsistencies in the final amino acid concentrations in the control media, and those containing no leucine, arginine, and lysine due to an error in the making of the media by the author of this thesis (Fig. 2). Results showed that there was twice the amount of leucine, arginine, and lysine as intended in our control media (Fig. 2A). Furthermore, there was also twice the concentration of amino acids that were not of interest for this study (*e.g.*, tyrosine, methionine) compared to our amino acid-deficient media (Fig. 2B). Therefore, we cannot attribute these results to the depletion or repletion of only leucine, arginine, and lysine. However, we can conclude that inhibition of the mTORC1 with rapamycin strongly attenuates oxygen consumption in macrophages related to ATP synthesis and maximal respiration.

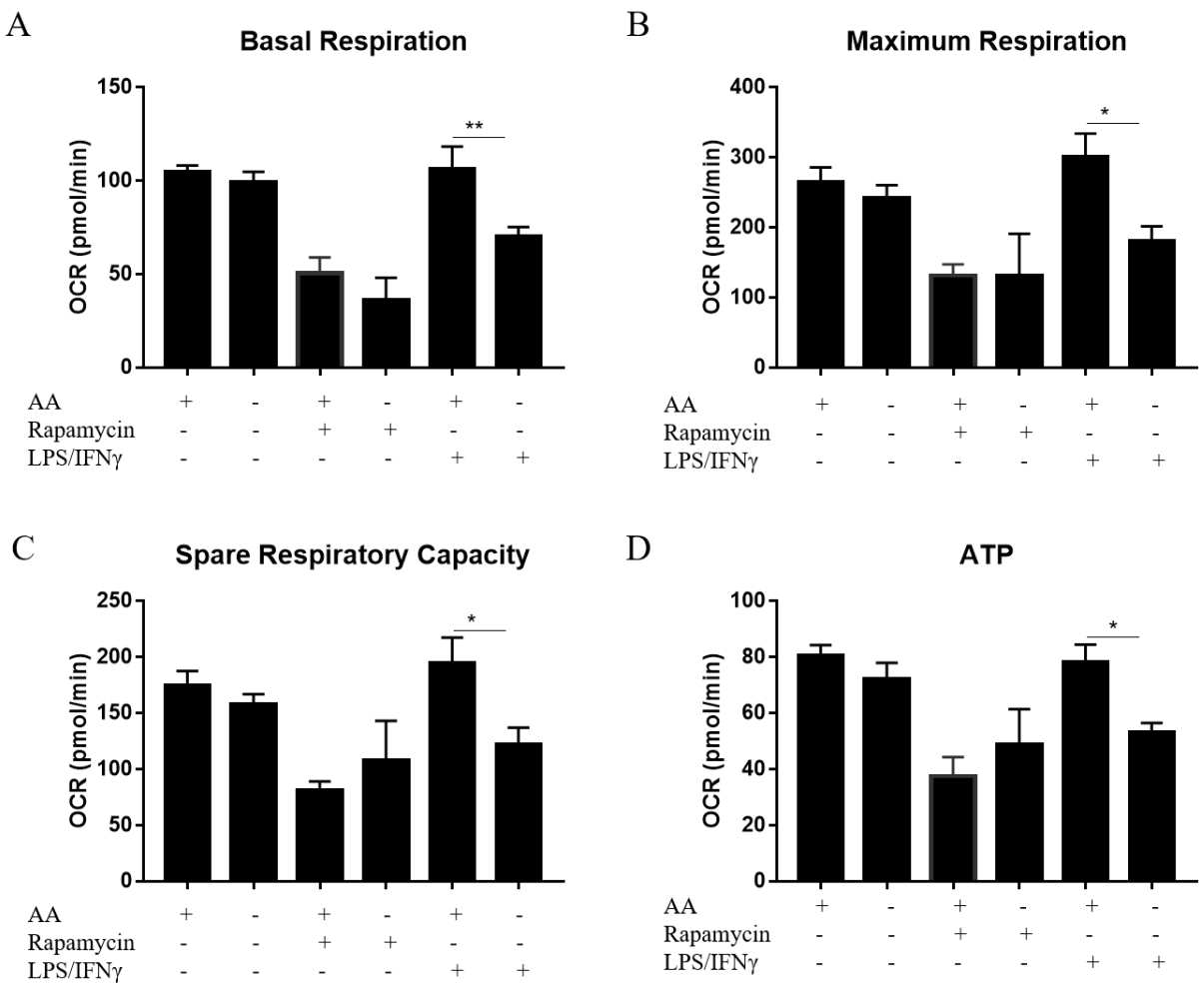

Figure 1: Amino acid restriction may alter the oxidative phosphorylation capacity of BMDMs during stimulation with LPS and IFN-γ. BMDMs were treated with RPMI media without or with leucine, arginine, and lysine, and treated with 100 ng LPS and 100U/ml IFN-γ or 100ng/ml of rapamycin for 6 hours prior to being subjected to a Seahorse assay to measure cellular metabolism. Shown are (A) Basal respiration, (B) Maximum respiration, (C) Spare Respiratory Capacity, (D) ATP production. This graph represents one experiment in which 5 technical replicates for each condition were analyzed and the data are given as the mean ± SEM. The results are representative of 2 independent experiments. *$p<0.05$. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparisons.

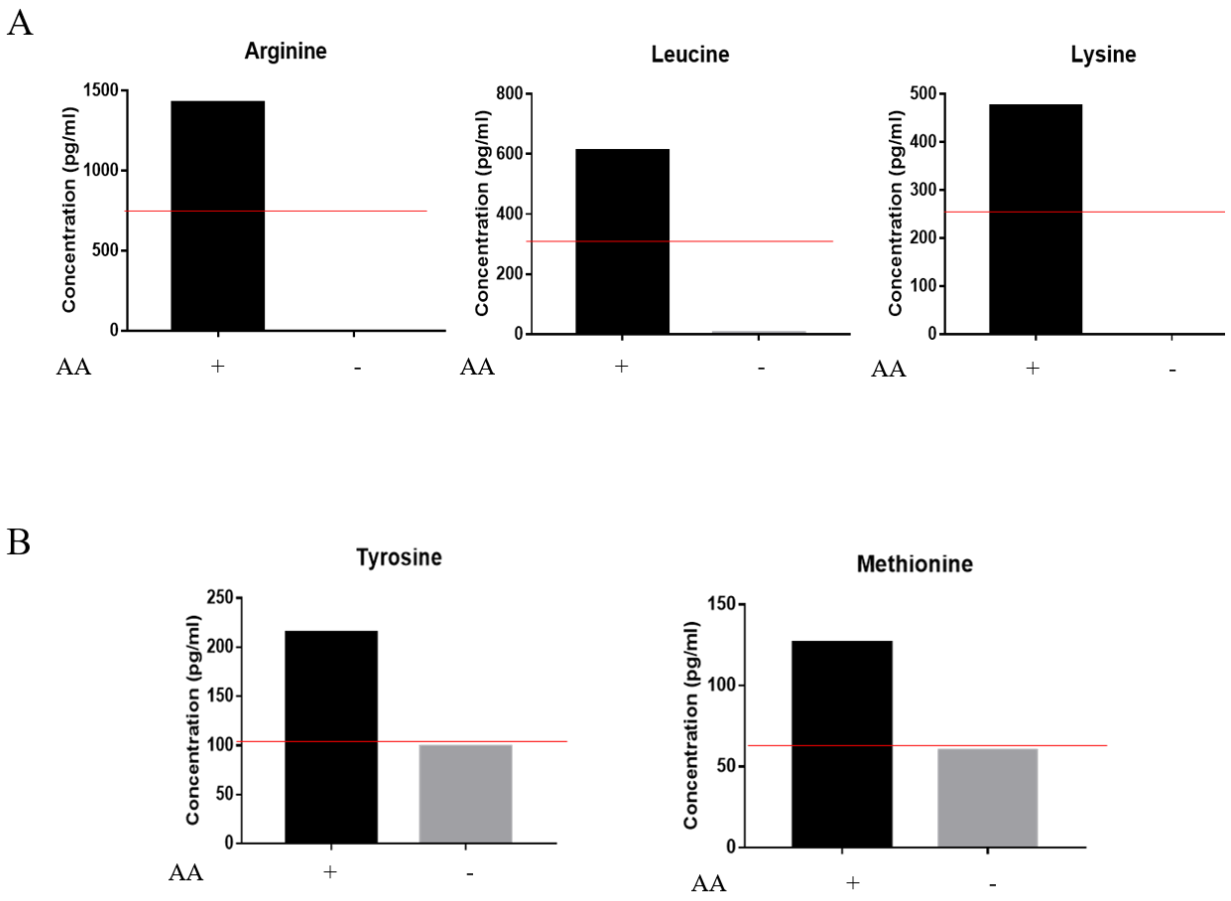

Figure 2: HPLC analysis of media used for Seahorse experiments showed inconsistencies in amino acid concentrations between treatment media. (A) Amino acid concentrations (leucine, arginine, and lysine) in amino acid full and amino acid deplete medium. (B) Amino acid concentrations of off-target amino acids in amino acid replete and amino acid deplete medium. The red line represents the amount of amino acid that should be in the media. 2 sample replicates were individually analyzed.

3.2. Effects of amino acid restriction on IL-6 mRNA and protein

Next, we corrected the previous technical errors and assessed the effect of amino acid restriction (*i.e.*, media lacking leucine, arginine, and lysine) on the expression of known mTOR dependent genes. First, we assessed the levels of IL-6 mRNA, as well as protein secretion during amino acid restriction of *in vitro* bone marrow-derived macrophages treated with LPS and IFN-γ (Fig. 3). Amino acid restriction alone did not alter the transcription or secretion of IL-6. Stimulation of BMDMs with LPS and IFN-γ induced IL-6 transcription and protein secretion. Induction of IL-6 was diminished by about 50% by removal of arginine, leucine, and lysine from the media when BMDMs were stimulated with LPS and IFN-γ. Therefore, arginine, leucine, and/or lysine are necessary for induction of IL-6 expression in macrophages exposed to LPS and IFN-γ.

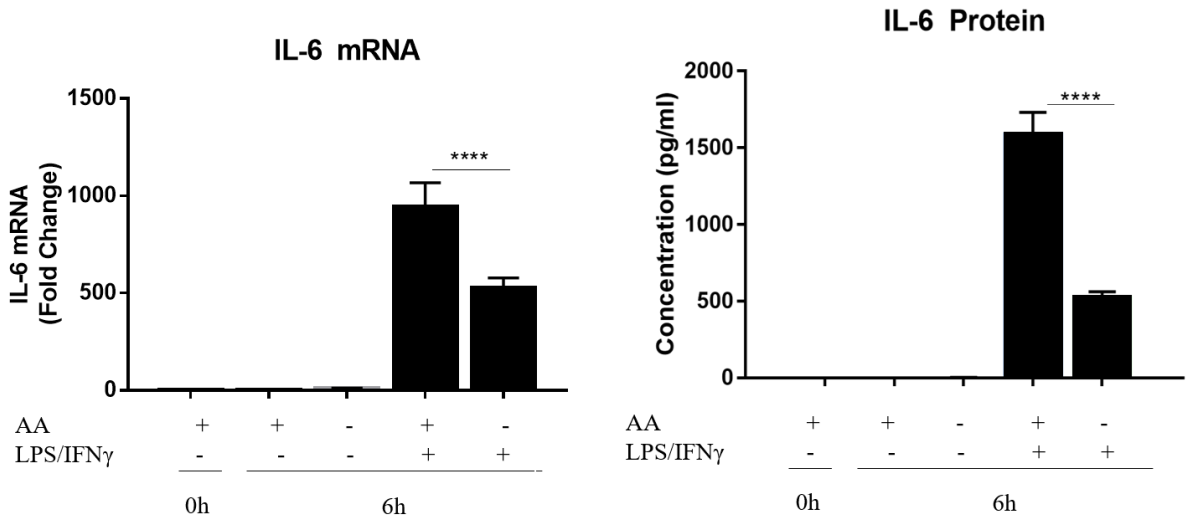

Figure 3: Amino acid restriction blocks induction of IL-6 mRNA and IL-6 protein by LPS and IFN-γ. BMDMs were exposed to RPMI media or RPMI media lacking leucine, arginine, and lysine and treated, with or without 100 ng LPS and 150U/ml IFN-γ for 6 hours. mRNA transcription was measured by qPCR and samples were standardized to GAPDH. IL-6 protein was measured in the culture supernatant by ELISA. Data are shown as the mean ± SEM. **** $p<0.001$. The results for IL-6 mRNA are representative of 6 independent experiments. The results for IL-6 protein are representative of 3 independent experiments. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparison.

3.3. Effects of amino acid restriction on Endoplasmic Reticulum Stress genes

We next assessed the effect of amino acid restriction on known mTOR-dependent genes that indicate endoplasmic reticulum (ER) stress (Fig. 4). We observed that amino acid restriction alone did not alter the expression of the ER-stress gene CHOP. However, amino acid restriction enhanced the expression of CHOP in cells exposed to LPS and IFN-γ in contrast to the significant reduction in CHOP during amino acid replete conditions. For the ER-stress gene GADD34, amino acid restriction alone significantly increased mRNA levels and was further enhanced when stimulated with LPS and IFN-γ. Therefore, amino acid restriction increases ER stress gene induction, potentially via the activation of eiF2α.

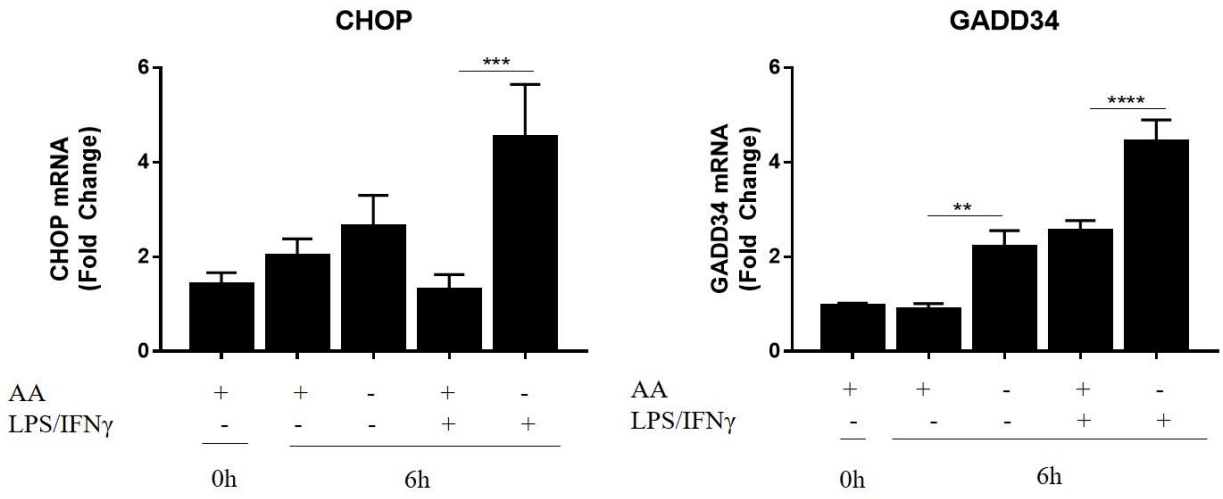

Figure 4: Amino acid restriction of BMDMS enhances the induction of ER stress genes by CHOP and GADD34. BMDMs were treated with or without leucine, arginine, and lysine, or without 100 ng LPS and with 150U/ml IFN-γ for 6 hours. mRNA levels were measured by qPCR and samples were standardized to GAPDH. Data are shown as the mean ± SEM. **p<0.01, ****p<0.001, **** p<0.001. The results are representative of 3-4 independent experiments. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparisons.

3.4. Effects of single amino acid restriction or repletion on IL-6 transcription

Since the induction of IL-6 transcription by LPS and IFN-γ was inhibited by amino acid restriction, we next evaluated whether the effect could be attributed to a single amino acid. Of the three amino acids, restriction of leucine alone was required to block IL-6 induction (Fig. 5A). Addition of leucine alone to AA-deficient medium reversed the effect of AA restriction on IL-6 induction. The addition of arginine or lysine alone did not restore IL-6 induction (Fig. 5B). Together, these results demonstrate that leucine is an independent modulator of IL-6 induction by LPS and IFN-γ in macrophages.

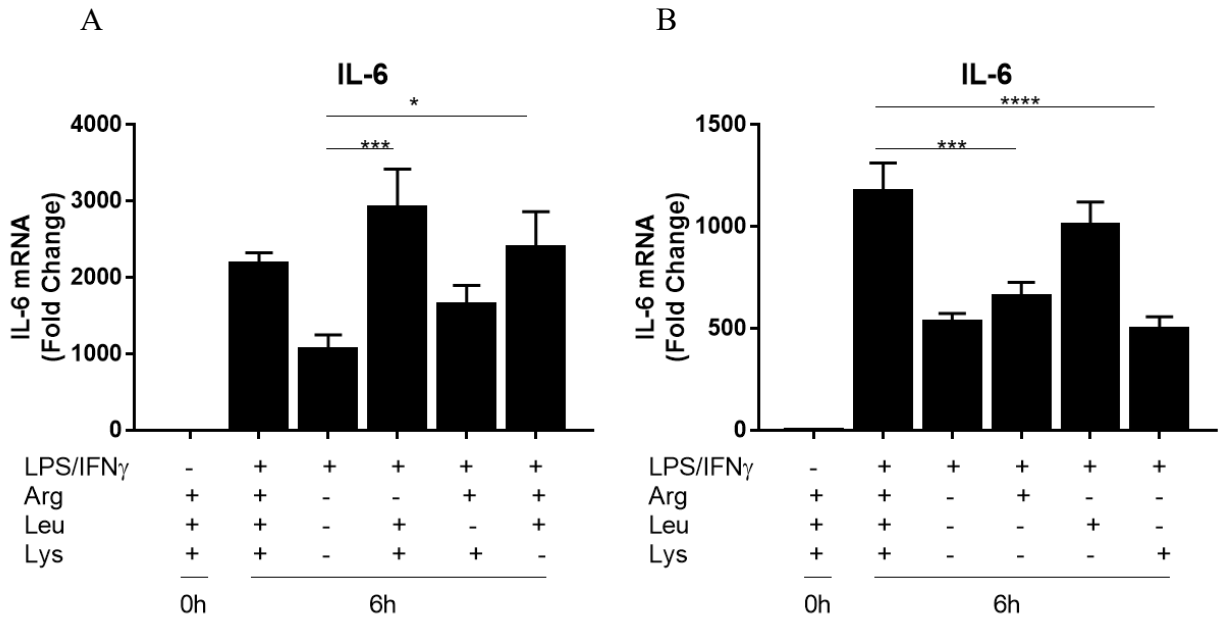

Figure 5: Leucine restriction in BMDMs controls transcriptional induction of IL-6. (A) BMDMs were exposed with media deprived of either leucine, arginine, or lysine, 100ng LPS and 150U/ml IFN-γ for 6 hours. (B) BMDMs were exposed with amino acid-deficient media replenished with leucine, arginine, or lysine, 100ng LPS and 150U/ml IFN-γ for 6 hours. mRNA levels were measured by qPCR and samples were standardized to GAPDH. Data are shown as the mean ± SEM. *p<0.05, **p<0.01, ****p<0.001, **** p<0.001. The results are representative of 3-5 independent experiments that have been pooled. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparisons.

3.5. Effects of amino acid restriction on mTORC1 and GCN2 activity

To evaluate the effects of amino acid restriction on mTORC1 and GCN2 activity, western blots were performed to assess the phosphorylation of their respective targets 4EBP1 and eif2α (Fig.6). For mTORC1-dependent phosphorylation of 4EBP1, we used an anti-phospho-4EBP1 or anti-total 4EBP1 antibody. The intensity of the bands detected by the former represents the degree of phosphorylation at Thr37 or Thr46. Bands using the anti-total 4EBP1 antibody (γ, β and α subunits) represent the gel mobility of the isoforms, which migrate less when phosphorylated. Therefore, a shift in detection to lower molecular weight bands represents de-phosphorylation. Amino acid restriction, in the absence or presence of LPS and IFN-γ, reduced phosphorylation of 4EBP1 (Fig. 6A, C; lanes 3 *vs.* 2, and 5 *vs.* 4), indicating a reduction in mTORC1 activity. Band shifts for total 4E-BP1 are difficult to reliably quantify and were not analysed by densitometry. Consistent with the literature[128], during LPS and IFN-γ stimulation, there was an increase in 4EBP1 phosphorylation when compared to control conditions (lane 4 *vs.* 2).

The integrated stress response was also interrogated. LPS and IFN-γ inhibited phosphorylation of eif2α (p-eif2α) in BMDMs under amino acid replete conditions (Fig. 6A, B; lane 4 *vs.* 2); amino acid restriction reversed the dephosphorylation of eif2α in cells exposed to LPS and IFN-γ (lane 5 *vs.* 4). Therefore, both the mTORC1 pathway and GCN2 pathway are regulated by the deprivation of leucine, arginine and lysine in BMDMs. The mTORC1 inhibitor rapamycin during amino acid replete conditions was used as a positive control to show that there is a reduction in 4EBP1 phosphorylation, and consistent with the literature, a significant increase in eif2α phosphorylation (Fig. 6A, B; lane 6).

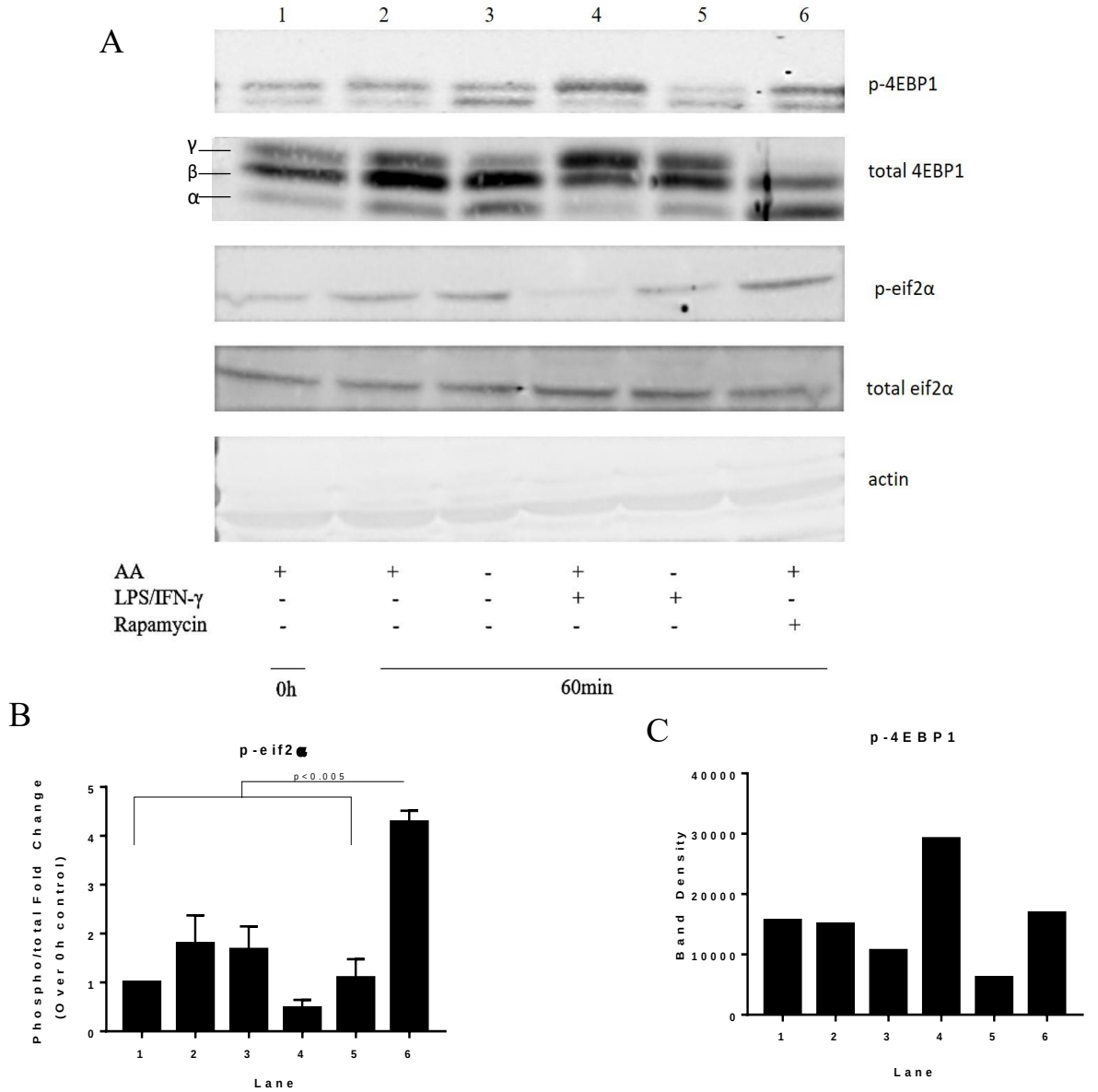

Figure 6: Amino acid restriction inhibits mTORC1 activity and increases p-eif2α. BMDMs were treated with RPMI media or RPMI media deprived of leucine, arginine, and lysine and treated with 100ng LPS and 150U/ml IFN-γ, or 100ng/ml of rapamycin for 60 minutes. Shown are Western blots for the indicated targets, which are representative of 2-3 individual experiments (A). The density of the bands was quantified and the ratio of phospho/total eif2-α was determined for 3 individual experiments. Data are shown as the mean ± SEM. Significance was determined by a 1-way ANOVA followed by Sidak's multiple comparisons. (B). The density of the bands for p-4EBP1 was determined for one blot.

Chapter IV: Discussion

Acute lung injury (ALI) remains a highly fatal disease with mortality rates as high as 30-40%. Macrophages play an important role in ALI and their activity is regulated by changes in metabolism, and gene transcription required for stress responses and cytokine production. These are tightly regulated by the protein kinase 'mammalian target of rapamycin' (mTOR), which nucleates two highly conserved macromolecular complexes – mTOR complex-1 (mTORC1) and mTORC2. Our results point to a leucine sensing mechanism, possibly via mTORC1, as an important regulator of macrophage stress and inflammatory responses and form the basis for future studies identifying novel clinical biomarkers or therapeutic targets in ALI.

Due to a lack of properly controlled media and inconsistencies in amino acid concentrations between treatments groups due to an error in the making of the medium by the author of this thesis, we are unable to interpret the impact of amino acid restriction on macrophage metabolism from our extracellular flux analysis. For Figure 1, the error resulted in double the intended concentration of amino acids (Fig. 2; red line) in the control condition due to failure to change the growth medium to a new control medium. In addition, concentrations of amino acids other than leucine, arginine, and lysine (*e.g.,* tyrosine, methionine; Fig. 2) were correct in the AA-deficient, indicating that there was no error on the part of the manufacturer, and that the HPLC AA assay was accurate. Nonetheless, rapamycin-treated macrophages consistently showed reduced mitochondrial function as indicated by consistent decreases in OCR for all measurements (Fig. 1). These results corroborate with those of Ramantha et. al where rapamycin-treated leukemic cells display reduced mitochondrial function[129]. They showed that when oxidative phosphorylation function in leukemic cells was inhibited, the cells resorted to energy production via enhanced aerobic glycolysis[129].

Since macrophage function heavily relies on their metabolic status, if a similar mechanism persists in macrophages, inhibition of oxidative phosphorylation by either rapamycin or amino acid restriction could inherently force macrophages to polarize towards a pro-inflammatory state. Therefore, repetition of these experiments to determine how extracellular amino acid restriction alters macrophage function will provide valuable insight. After the error in the making of the media was identified, all subsequent experiments (*i.e.,* figures 3-6) were performed using properly controlled media.

The most robust findings in our model was that IL-6 mRNA induction and protein secretion were inhibited in BMDM when subjected to essential amino acid (EAA) restriction and stimulated with LPS and IFN-γ. Although IL-6 was formerly thought to be an exclusively pro-inflammatory cytokine, in different models of ALI, IL-6 has shown to exhibit anti-inflammatory properties. For instance, in a model of influenza A, IL-6 induction contributes to the resolution and recovery phases by exerting anti-inflammatory properties[59]. Therefore, it would be important to investigate how the IL-6 induction we see *in vitro* impacts the overall disease progression in an *in vivo* amino acid restricted, LPS-induced ALI model. This might, for instance, be achieved using a macrophage-specific IL-6 knockout mouse. Furthermore, there are many other cytokines and growth factors that contribute to the various phases of ALI that were not evaluated in this study such as TNF-α, VEGF, and IL-1β. Investigating a broader range of cytokines that contribute to ALI would provide a more global view of the lung microenvironment during amino acid restriction and LPS-induced ALI.

Since IL-6 transcription and protein were so robustly modulated in our model, it would be of interest to look at other macrophage-derived factors that are known to play a role in ALI and are known to be secreted by macrophages. For instance, VEGF is a macrophage-derived factor that would be of particular interest due to its participation not only in the induction but also the resolution phases of ALI. Studies show that increases in VEGF resulted in an increase in vascular permeability and the accumulation of pulmonary edema[130]. Li et al. also demonstrated that an increase in VEGF mRNA and protein was associated with increased microvascular permeability, as well as neutrophil influx and epithelial cell apoptosis[131]. Karmpaliotis et al. also provided evidence using immunostaining of VEGF in the lung to show that VEGF is associated with the increased innate immune cells in the alveolar compartment seen in LPS models of ALI[132].

In contrast, during the repair phase of ALI, the angiogenic effect of VEGF is essential for the appropriate vascular remodelling during tissue repair after inflammation or injury[133]. In an LPS-induced ALI murine model, exogenous VEGF significantly decreased vascular leakage and edema in the lungs. The authors attributed this observation to the ability of VEGF to suppress apoptosis by reducing the activity of pro-apoptotic active caspase-3[134]. Furthermore, studies have looked at the response of VEGF to amino acid availability and found that VEGF levels can be increased during amino acid deprivation (*i.e.*, glutamine), which Abcouwer et al. attribute to regulation by the ER stress-dependent response [135]. Therefore, given a potential role for VEGF in induction, resolution or repair during ALI, as well as its potential regulation by amino acid availability, it would be of interest to investigate VEGF mRNA and protein levels in our model.

Our second major finding pertaining to IL-6 was that the transcription of IL-6 mRNA and protein secretion was primarily driven by the presence or absence of leucine. There is currently very little literature on the role of leucine in IL-6 production. One study investigating exogenous leucine availability and myocyte function in a human exercise model, found that transient regulation of NF-κB signaling was induced by leucine availability[136]. They suggested that this mechanism is governed by NF-κB causing downstream nuclear regulation of cyclin D1 ultimately inhibiting expression of pro-inflammatory cytokines IL-6, TNF-α and IL-1[136]. Early work by Weichhart et al. showed that mTORC1 limits proinflammatory cytokine production, including IL-6, by inhibiting the activation of NF-kB[137]. Although these findings oppose those of this thesis, the experiments performed by Weichhart et. al were conducted using rapamycin to inhibit mTOR as well as using human monocytes[137]. It would be of interest to investigate the regulation of the NF-κB signaling by mTOR in macrophages in response to extracellular leucine availability in our model using BMDMs. To do so, transfection of luciferase reporter plamids in murine BMDMs might be employed to investigate the relationship between NF-κB and IL-6 during leucine restriction. Overall, it is essential to not only understand the mechanism, but also how the observed increase in IL-6 during leucine deprivation impacts overall disease progression. The lack of knowledge pertaining to the role of leucine in IL-6 induction provides the opportunity to delineate the mechanism using *in vitro* lentiviral knockdown of mTOR adaptor proteins, such as Sestrin2[94] and Castor1[93], or known leucine transporters such as L-type amino acid transporter 1[138].

Assessing the role of Sestrin2 in our model of leucine restriction would be an important aspect to investigate, as it is a known leucine-binding adaptor protein for the mTORC1 pathway[139]. Endogenous Sestrins have been associated with metabolic pathologies such as mitochondrial

dysfunction and oxidative damage[140]. During amino acid deprivation, specifically leucine deprivation, Sestrin2 is upregulated in an ATF4-dependent manner[141]. Recent studies have highlighted a role of Sestrin2 in macrophage polarization during different disease states. For instance, during myocardial infarction, Yang et al. showed that Sestrin2 overexpression in cardiac-derived macrophages suppressed the inflammatory response of M1 macrophages both *in vitro* and *in vivo*[142]. Another study using RAW264.7 cells stimulated with LPS showed inhibition of pro-inflammatory cytokines including TNFα, IL-1β, and IL-6 release and expression were inhibited in Sestrin2 expressing cells[143]. Therefore, investigating the role of leucine in the regulation of Sestrin2 during M1-polarization of macrophages remains an important target to be investigated as it could explain the decrease in IL-6 mRNA and protein in our study. Furthermore, knockout murine models could be employed to investigate the aforementioned mechanisms during amino acid deprivation and lung injury *in vivo*.

Evidence suggests that endoplasmic reticulum (ER) stress may play a role in LPS-induced lung inflammation[144,145]. We, therefore, wanted to assess the effect of EAA restriction in our macrophage model using surrogate markers of ER stress, CHOP and GADD34. We observed that EAA restriction enhanced CHOP and GADD34 induction when stimulated with LPS and IFN-γ. These findings are consistent with those of Li et al. who found that LPS activation of TLR4 suppressed CHOP expression as a method to increase macrophage survival during immune activation[103]. Since we established a critical role for leucine in the induction and secretion of IL-6, it would be of interest to investigate the effects of leucine *per se* on the induction of ER stress genes such as CHOP and GADD34. Previous work has established that GCN2 participates in the regulation of mTORC1 activity by leucine in hepatic cells[146,147]. These two studies found that in

mice receiving a leucine-free diet, GCN2 was required for mTORC1 signaling. The first study showed that in GCN2 knock-out ($GCN2^{-/-}$) mice on leucine deprived diets, there was reduced dephosphorylation of 4E-BP1 and S6K1 [146]. The second study also used an *in vitro* model using HepG2 cells knocked-down for GCN2 and starved of leucine for 24 hours. They found that leucine starvation did not affect mTOR signaling in the knock-down cells[147]. This suggests that during leucine deprivation, GCN2 acts upstream of mTORC1. These studies provide evidence for a potential connection between the increased activation of GCN2 pathway and our observed increase in ER stress genes CHOP and GADD44 during EAA restriction. Whether the increase in CHOP and GADD34 observed during restriction of arginine, leucine and lysine can be attributed specifically to leucine awaits future single amino acid depletion and repletion experiments. Additionally, since ATF4 is a critical link between GCN2 and CHOP and GADD34 induction during ER stress, exploring ATF4 function and expression could provide greater insight and establish a more complete understanding of the role of leucine in macrophage ER stress during EAA restriction.

Finally, we demonstrated that amino acid restriction in macrophages alters mTOR and ER stress activation as determined by their downstream targets 4EBP1 and eif2α, respectively. Amino acid restriction alone, regardless of the absence or presence of LPS and IFN-γ, reduced phosphorylation of 4EBP1. During amino acid-replete conditions 4EBP1 was more phosphorylated than during amino acid-deficient conditions. These findings are in line with the literature, which has established that under homeostatic conditions, mTORC1 phosphorylates 4EBP1 which permits its dissociation from the translation factor eif4e and subsequent translation[148]. We observed phosphorylation of 4EBP1 during amino acid-replete conditions when

the macrophages were stimulated with LPS and IFN-γ, but not during amino acid restricted conditions.

When looking at the effects of amino acid availability on the ER stress signaling pathway, we saw that LPS and IFN-γ inhibited phosphorylation of eif2α (p-eif2α) in BMDMs under amino acid replete conditions in contrast to the amino acid-restricted BMDMs, which exhibited a reversal in the dephosphorylation of eif2α when exposed to LPS and IFN-γ. Therefore, both the mTORC1 pathway and GCN2 pathway are regulated by the deprivation of leucine, arginine and lysine in BMDMs. The mTORC1 inhibitor rapamycin during amino acid replete conditions was used as a positive control to show that there is a reduction in 4EBP1 phosphorylation, and consistent with the literature, a significant increase in eif2α phosphorylation mTORC1 signaling has been shown to be critical for appropriate macrophage polarization and function[125,149]. Further experiments need to be conducted to evaluate if a single or combination of amino acids is responsible for the alteration in phosphorylation patterns. This could be investigated using a single amino acid add-back or single amino acid-deprived method. Additionally, to define the amino acid sensor that is being utilized in this signaling pathway, shRNA experiments could be employed to knock down known adaptor proteins such as Sestrin2 or Castor1.

mTORC1 activity is essential for producing a pro-inflammatory or M1 like phenotype, while impairing M2-like macrophage polarization. It has been shown that LPS and IFN-γ classically activate M1- like macrophages[30], therefore, it would make sense that in our LPS and IFN-γ treated macrophages in the amino acid replete environment are able to properly phosphorylate 4EBP1 (Fig. 6). However, the attenuation of LPS and IFN-γ-induced 4EBP1

phosphorylation by amino acid restriction provides evidence that there is an impaired response. Further immunophenotyping of these cells will be necessary to determine if these cells are acting in an M1 or M2-like manner. Our results for our rapamycin treated control samples were concurrent with the literature[150] in which rapamycin inhibits 4EBP1 function as determined by the shift of γ-form (Fig. 6, lane 6).

Overall, our results indicate that reduced extracellular amino acid availability alters BMDM function when stimulated with LPS and IFN-γ (summarized in Fig. 7). We studied how LPS and IFN-γ induce polarization of macrophages towards M1-like inflammation. From our study, we have established that restriction of leucine alone is required for the attenuated induction of IL-6. This result may be due to a mechanism involving the decrease in leucine being sensed by amino acid adaptor protein Sestrin2. It is established in the literature that Sestrin2 is upregulated when there are deficient levels of leucine, subsequently deactivating mTOR activity[143]. Yang et al. also showed that an increase in Sestrin2 expression resulted in a decrease in proinflammatory cytokine (*i.e.*, IL-6) mRNA and protein secretion[143], therefore providing rational for proposing that the observed decrease in IL-6 in our study may be due to an upregulation of Sestrin2 as a result of leucine restriction in our model. Another potential mechanism which may be involved with our observed decrease in IL-6 may involve NF-κB. Since it is established in the literature that mTOR activity is dominant for activating NF-κB[151], perhaps amino acid restriction in combination with LPS and IFN-γ treatment inhibits the mTOR pathway and results in a decrease in IL-6. To investigate this hypothesis, chromatin immunoprecipitation (ChIP) or more specifically ChIP-seq can be employed to determine any NF-κB-dependent interactions between mTOR and genes involved in tissue injury or regeneration, including IL-6. We have also shown that eif2α is

regulated by arginine, leucine, and lysine. Subsequently, the increased phosphorylation of eif2α results in an increase of ER stress genes CHOP and GADD34. In the future, we wish to evaluate how extracellular amino acid availability alters BMDM function when stimulated with M2-like macrophage polarizing signals, such as IL-4 or IL-13. Furthermore, the role of M2 activation and the influence of EAA can be explored in an *in vivo* model or ALI. We would also evaluate the resident *vs.* recruited macrophages and how amino acid deficiency affects their function and metabolism. This could provide evidence regarding which macrophage subsets are most affected by EAA restriction during ALI. Although macrophages comprise a majority of the inflammatory cells in the lung population at steady state, during disease states there is a significant influx of neutrophils, followed by lymphocyte recruitment and activation. In fact, mTOR activity has been implicated in controlling the function of other immune cells. For instance, mTOR is required for B-cell proliferation[152] and inhibition of mTOR via rapamycin increased B-cell sensitivity to LPS, leading to increased cytokine production and apoptosis[153]. In the case of natural killer cells, mTOR is essential for proliferation, as Wai et al. demonstrated that rapamycin blocks progression of their cell cycle from G1 to S phase[154]. Finally, studies elucidating the role of mTOR signaling in neutrophils revealed that inhibition of mTOR suppresses neutrophil chemotaxis[155]. In an LPS murine model of ALI, administration of rapamycin attenuates neutrophil recruitment to the lung[67].

Exploring the role of ER stress and the GCN2 pathway is also an important avenue to investigate during LPS induced ALI in other immune cells. The ER stress signaling pathway is integral for proper immune responses. For example, GCN2 is required for effective activation and antigen presentation in dendritic cells[156]. $CD8^+$ T cells lacking GCN2 are unable to properly integrate signals during amino acid starvation[157,158]. Van de Velde et al. found that GCN2 in $CD8^+$

T cells is required for efficient proliferation during amino acid restricted conditions[158]. Although other studies have elucidated important roles of GCN2 in immune function, the role of GCN2 in the context of ALI has yet to be investigated.

Finally, using an *in vivo* model of LPS induced ALI would be an essential tool for understanding not only the overall impact of amino acid deprivation during the progression and resolution of ALI but would provide a more robust environment for exploring different pathways. The use of appropriate tissue-specific (*i.e.*, lungs) knockout mice such as Sestrin2, GCN2 or 4EBP1 could be utilized to better understand how macrophage sensing mechanisms regulate ALI in vivo. Investigation and immunophenotyping of the lungs and airways during amino acid restriction and LPS-induced ALI will be essential to identify other potential leukocytes that may be malfunctioning and contributing to disease pathogenesis. Together, *in vitro* and *in vivo* models of elucidating the roles and mechanisms of amino acid deficiency in macrophage function could identify potential biomarkers or pharmacological targets for future therapies for ALI.

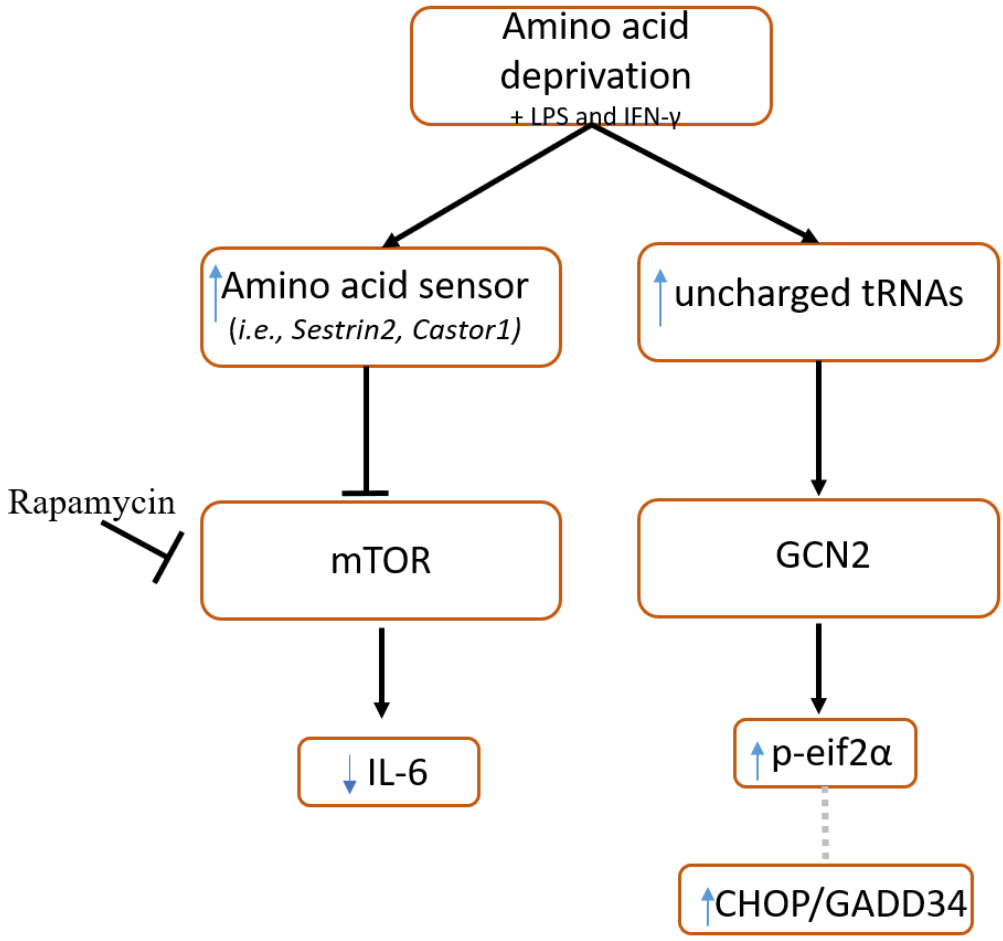

Figure 7: Proposed working model of the effects of amino acids on macrophages. The absence of amino acids is sensed via two pathways. The lack of amino acids, specifically leucine causes an increase in Sestrin2 expression or activity, and subsequent deactivation of mTOR. Ultimately, IL-6 is downregulated which may be attributed to the attenuation of rate-limiting transcription factors for the induction of IL-6 (*e.g.*, NF-κB). The chemical inhibitor rapamycin also deactivates mTOR and has been shown to attenuate NF-κB and proinflammatory cytokine production. The other pathway involves the cell sensing the uncharged tRNAs resulting from insufficient amino acids which activate GCN2. This pathway results in the increase in phosphorylation of eif2α which cause an increase in transcription of CHOP and GADD34. Cross-talk between the mTOR and ER stress pathways may exist, as we and others have shown that rapamycin can increase phosphorylation of eiF2α. In combination, regulation of these adaptive signaling events (*i.e.*, IL-6 induction, ER stress) by leucine restriction may inhibit the metabolic and regenerative responses that promote repair after acute lung injury.

Chapter V: Conclusion

Clinical studies demonstrate an association between protein availability and clinical outcomes in the critically ill and mTORC1 is a cellular sensor of essential amino acid (EAA) availability. Acute lung injury (ALI) is a common co-morbidity of critically ill patients and is associated with a high mortality rate of 30-40%. Unfortunately, there is currently no effective pharmacological treatment for patients with ALI, nor are there efficient clinical biomarkers to predict those who are at higher risk for the development or detrimental progression of this disease. Our studies provide evidence indicating that mTOR and/or GCN2, and particularly their leucine sensing mechanism, are important regulators of macrophage stress and inflammatory responses. Therefore, further understanding of the role of macrophages and their mechanisms during disease progression of ALI offers insight into potential targets or biomarkers that may be used to establish future therapies.

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