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TIMP3 Regulation of Macrophage Activation and Apoptosis

Michael S. Brock
The University of Western Ontario

Supervisor
Dr. Sean Gill
The University of Western Ontario

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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TIMP3 REGULATION OF MACROPHAGE ACTIVATION AND APOPTOSIS

(Thesis format: Monograph)

By

Michael Brock

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment

of the requirements for the degree of

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The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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Abstract

Acute respiratory distress syndrome (ARDS) is a lung disease involving profound inflammation. Origins of persistent inflammation in select cases of ARDS are poorly understood, and we propose persistent inflammatory macrophages may be one of its mechanisms. Macrophages polarize to either promote inflammation, or suppress inflammation. Tissue inhibitor of metalloproteinases 3 (TIMP3) reduces the pro-inflammatory polarization in macrophages. Additionally, studies have shown TIMP3 promotes apoptosis, and its absence delays recovery from bleomycin-induced lung injury.

We hypothesize that TIMP3 promotes apoptosis of murine macrophages through inhibition of metalloproteinase activity and stabilization of FAS on the cell surface. Pro-inflammatory $\text{Timp}^3^{-/-}$ bone marrow-derived macrophages (BMDMs) have significantly higher metalloproteinase activity, and significantly lower sFASL-induced apoptosis compared to WT BMDMs measured with FLICA and Annexin V. rTIMP3 treatment rescued both metalloproteinase activity and apoptosis.

In conclusion, excessive metalloproteinase activity in $\text{Timp}^3^{-/-}$ BMDMs is associated with sFASL-induced apoptosis potentially due to metalloproteinase dependent death receptor processing.

Keywords
TIMP3, macrophage, inflammation, BMDM, metalloproteinase, apoptosis, FAS, sFASL, Annexin V, FLICA, TUNEL, ARDS, lung, MMP, ADAM
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## Table of Contents

Table of Contents ................................................................. iv

List of Figures ................................................................. vii

List of Abbreviations ............................................................. ix

Chapter 1. Introduction .......................................................... 1

  1.1 Overview .............................................................................. 1

  1.2 Acute Respiratory Distress Syndrome ..................................... 2
    1.2.1 ARDS and inflammation ............................................... 3

  1.3 Macrophages ........................................................................ 5
    1.3.1 Macrophage polarization ............................................. 6

  1.4 Metalloproteinases ............................................................ 11
    1.4.1 Metalloproteinases in ARDS ....................................... 12
    1.4.2 Metalloproteinases in Macrophage Function .................... 13
    1.4.3 Metalloproteinases and Apoptosis ............................... 14

  1.5 Tissue inhibitors of metalloproteinases .................................. 15
    1.5.1 TIMP3 and ARDS .................................................... 16
    1.5.2 TIMP3 and Macrophage function ................................ 17
    1.5.3 TIMP3 and Apoptosis ............................................... 18

  1.6 Rationale: ............................................................................. 21

  1.7 Hypothesis: ........................................................................ 22

  1.8 Objectives: .......................................................................... 22

Chapter 2. Methods .............................................................. 23

  2.1 BMDM Isolation and Culture ............................................... 23
    2.1.1 L929 cell culture ....................................................... 24
2.1.2 BMDM Polarization.............................................................................................. 25

2.2 Conditioned media and protein/RNA isolation...................................................... 26
  2.2.1 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR).............. 27
  2.2.2 OmniMMP assay ........................................................................................... 27

2.3 Assessment of macrophage apoptosis.................................................................... 28
  2.3.1 FLICA Staining............................................................................................... 28
  2.3.2 TUNEL Staining ............................................................................................ 29
  2.3.3 Annexin V Staining....................................................................................... 30

2.4 Western Blot ......................................................................................................... 31

2.5 Statistical Analysis............................................................................................... 32

Chapter 3. Results .................................................................................................. 33

3.1 BMDM culture establishment............................................................................... 33
  3.1.1 BMDM-derived TIMP3 regulates Il12b and Nos2 expression in M1-polarized macrophages................................................................. 33
  3.1.2 Quantifying metalloproteinase activity in WT and Timp3/- unpolarized and M1-polarized BMDMs................................................................. 36

3.2 BMDM apoptosis................................................................................................. 41
  3.2.1 Effect of sFASL treatment on caspase activity in unpolarized BMDMs.. 43
  3.2.2 M1-polarized WT BMDMs show induction of caspase activity following sFASL stimulation................................................................................. 43
  3.2.3 Timp3/- BMDMs have suppressed sFASL induced caspase activation compared to WT BMDMs ................................................................. 46
  3.2.4 TUNEL positivity induced by sFASL in M1-polarized WT BMDMs.... 49
  3.2.5 sFASL stimulation induces less Annexin V positivity in Timp3/- BMDMs compared to WT BMDMs ................................................................. 49

3.3 Measurements of BMDM FAS......................................................................... 53
  3.3.1 Assessing Fas mRNA levels and FAS protein abundance in unpolarized and M1-polarized WT BMDMs................................................................. 57
3.3.2 Measuring *Fas* mRNA levels and FAS protein abundance differences in WT and *Timp3*−/− BMDMs ........................................................................ 57

3.3.3 rTIMP3 rescues reduced caspase activity in M1-polarized *Timp3*−/− BMDMs following sFASL stimulation .................................................... 61

Chapter 4. Discussion ....................................................................................................... 64

4.1 Summary of findings .................................................................................................. 64

4.2 Contributions of research to current state of knowledge ......................................... 65

4.2.1 Macrophage Polarization and Metalloproteinase Activity ............................... 65

4.2.2 Macrophage Apoptosis ...................................................................................... 67

4.2.3 Regulation of FAS-sFASL Mediated Apoptosis ............................................ 70

4.3 Limitations and future directions .............................................................................. 73

4.3.1 Study limitations .............................................................................................. 73

4.3.2 Future directions .............................................................................................. 75

4.4 Summary and conclusions ...................................................................................... 76

Chapter 5. References ................................................................................................... 78

Curriculum Vitae .............................................................................................................. 91
List of Figures

Figure 1-1: sFASL/FAS ligand-receptor induced apoptosis .................................................. 10

Figure 1-2: Proposed model of TIMP3 mediated macrophage apoptosis .............................. 19

Figure 3-1: BMDM-derived TIMP3 regulates Il12b expression in M1-polarized macrophages. .......................................................................................................................... 34

Figure 3-2: M1-polarized Timp3−/− BMDMs have elevated Nos2 expression compared to M1-polarized WT BMDMs. ........................................................................................................ 37

Figure 3-3: Metalloproteinase activity levels in CM of unpolarized and M1-polarized WT and Timp3−/− BMDMs ........................................................................................................... 39

Figure 3-4: M1-polarized Timp3−/− BMDMs have elevated metalloproteinase activity in conditioned media ..................................................................................................................... 40

Figure 3-5: Recombinant TIMP3 treatment reduces peak metalloproteinase activity in M1-polarized Timp3−/− BMDMs ........................................................................................................ 42

Figure 3-6: WT unpolarized BMDMs do not elevate caspase activity in response to sFASL stimulation ............................................................................................................................... 44

Figure 3-7: M1-polarized WT BMDMs show induction of caspase activity following sFASL stimulation ............................................................................................................................... 45

Figure 3-8: Baseline Timp3−/− M1-polarized BMDM caspase activity is not significantly different from WT BMDMs ........................................................................................................... 47

Figure 3-9: Timp3−/− BMDMs have attenuated sFASL induced caspase activation compared to WT BMDMs ........................................................................................................................... 48

Figure 3-10: sFASL induces increased TUNEL positivity in WT M1-polarized BMDMs. ... 50

Figure 3-11: PBS-stimulated Timp3−/− BMDM TUNEL positivity is not significantly different from WT BMDMs ................................................................................................................ 51
Figure 3-12: sFASL-stimulated $Timp3^{-/-}$ BMDM TUNEL positivity is not significantly different from WT BMDMs. .......................................................................................................................... 52

Figure 3-13: M1-polarized WT BMDMs show increased Annexin V fluorescence following sFASL stimulation. ................................................................................................................. 54

Figure 3-14: PBS stimulation of WT and $Timp3^{-/-}$ BMDMs yields no significant difference in Annexin V positivity. .............................................................................................................. 55

Figure 3-15: M1-polarized $Timp3^{-/-}$ BMDMs have less Annexin V positivity following sFASL stimulation. ......................................................................................................................... 56

Figure 3-16: WT M1-polarized BMDMs have increased $Fas$ expression. ........................................... 58

Figure 3-17: FAS protein abundance is not significantly different between WT unpolarized and M1-polarized BMDMs. ........................................................................................................ 59

Figure 3-18: Steady state $Fas$ mRNA levels are not significantly different between WT and $Timp3^{-/-}$ M1-polarized BMDMs. ..................................................................................................... 60

Figure 3-19: There are no significant differences between WT and $Timp3^{-/-}$ M1-polarized BMDM FAS protein levels. ........................................................................................................... 62

Figure 3-20: rTIMP3 rescues depressed caspase activity in M1-polarized $Timp3^{-/-}$ BMDMs following sFASL stimulation. ......................................................................................................... 63
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADAMs</td>
<td>A disintegrin and metalloproteinase</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>ARDS</td>
<td>Acute respiratory distress syndrome</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
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<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
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<tr>
<td>BMDMs</td>
<td>Bone marrow-derived macrophages</td>
</tr>
<tr>
<td>CM</td>
<td>Conditioned media</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl-sulphoxide</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>FADD</td>
<td>FAS activated death domain</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FLICA</td>
<td>Fluorescent labeled inhibitor of caspases</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IL1ra</td>
<td>Interleukin 1 receptor antagoninst</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M1</td>
<td>Classically activated macrophage</td>
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<tr>
<td>M2</td>
<td>Alternatively activated macrophages</td>
</tr>
<tr>
<td>M0</td>
<td>Naïve unpolarized macrophage</td>
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<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor</td>
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<tr>
<td>MMPs</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of arterial oxygen</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PI</td>
<td>Propidium iodide</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real time-polymerase chain reaction</td>
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<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell park memorial institute</td>
</tr>
<tr>
<td>rTIMP3</td>
<td>Recombinant TIMP3</td>
</tr>
<tr>
<td>sFASL</td>
<td>Soluble FAS ligand</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor or metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TNFR</td>
<td>Tumor necrosis factor receptor</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase dUTP nick end labeling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
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Chapter 1. Introduction

1.1 Overview

The healthy human lung is comprised of a multitude of cell types working to promote oxygenation of the bloodstream from external atmospheric sources. During inhalation the thoracic cavity expands generating a decrease in pleural pressures and resulting in an influx of atmospheric gases into the lungs to equalize the resulting pressure gradient [1]. The lungs consist of an intricate system of ever smaller branching tubes leading to the smallest functional structure within the lungs, the alveolus [2]. An alveolus is a spherical structure whose walls are a single cell thick and comprised of epithelial cells [2]. These alveoli are enveloped by endothelial microvasculature comprised of a meshwork of capillaries [2]. Capillaries support gas exchange between the bloodstream and alveolus by way of bringing contained blood into close proximity of the alveolar structure [3]. Oxygenation of the bloodstream requires oxygen molecules to diffuse across two monolayers (epithelial and endothelial layers) in order to exit the alveolus and enter bloodstream [2]. The binding of oxygen molecules to hemoglobin (within red blood cells) allows for the transportation of oxygen within the bloodstream [4]. Damage to this delicate microstructure, which occurs during acute respiratory distress syndrome (ARDS), reduces the ability for adequate bloodstream oxygenation.
1.2 Acute Respiratory Distress Syndrome

ARDS is characterized by lung inflammation, tissue damage, vascular dysfunction and pulmonary edema, which collectively lead to impaired gas exchange and hypoxemia [5]. The partial pressure of arterial oxygen ($\text{PaO}_2$) divided by the fraction of inspired oxygen ($\text{FiO}_2$), which is referred to as the $\text{PaO}_2/\text{FiO}_2$ ratio, is indicative of the efficiency of pulmonary gas exchange. For a patient to be diagnosed with ARDS, bilateral opacities must be present on the chest radiograph in the absence of heart failure and the $\text{PaO}_2/\text{FiO}_2$ ratio must be below 300 mmHg (lower $\text{PaO}_2/\text{FiO}_2$ ratios are considered more severe cases of ARDS) [6]. ARDS can be caused by direct lung injury from sources such as, bacterial infection, acid aspiration or near drowning experiences [7]. Similarly, ARDS can have indirect causes, as seen following sepsis; the mechanism of disease onset depends on the initial insult to the lung [7]. Ultimately, the varied etiology and complex pathophysiology of ARDS, which are associated with a high mortality rate, make designing preventative care therapies for ARDS patients difficult resulting [8]. However, one commonality within ARDS pathophysiology, is the generation of severe inflammation within the lungs following the initial injury [8].

The release of pro-inflammatory cytokines and chemokines from cells within damaged pulmonary tissues leads to the recruitment of inflammatory cells, such as neutrophils and macrophages to aid in the clearance of deleterious substances [9]. Acute inflammation, however, can also cause loss of endothelial and epithelial barrier integrity, resulting in an influx of proteinaceous edema fluid into the alveolar spaces [8]. In the
most serious cases of ARDS the inflammation progresses to the point of respiratory failure, leading to severe hypoxemia and death [6]. The overall goal of the current treatment strategy is to support patient oxygenation with mechanical ventilation while allowing them to recover from the pulmonary injury [10].

Importantly, in some patients the severe inflammation persists beyond the normal recovery timeline resulting in lack of resolution of symptoms and clinical features of ARDS [11, 12]. Many studies have shown persistent inflammation can lead to worse outcomes in ARDS [11–13], and that exhaled nitric oxide, which can be produced by many inflammatory cells including macrophages, is a reasonable marker for persistent lung inflammation [14]. The mechanism of onset for this persistent inflammation in ARDS, however, is currently unknown. Regulation of inflammation within the lung tissues following injury is paramount, and through garnering a better understanding as to how inflammation, particularly chronic inflammation is resolved, we will yield new approaches to directly treat ARDS rather than simply providing supportive care.

1.2.1 ARDS and inflammation

Inflammation is a localized physical reaction to a sustained injury or infection that causes fluid infiltration, cellular influx and swelling within a damaged tissue [15]. Inflammation within the lung environment is initiated by cells within the alveolus in an attempt to rid the lung environment of deleterious substances or pathogens. Following an insult to the lungs, an immune response is initiated, which is primarily driven by macrophages within the lung [16]. Macrophages, which are resident to the alveolus,
become activated and release multiple different cytokines and chemokines in order to recruit additional inflammatory cells toward the site of injury [9]. Activation of resident macrophages leads to the release of a multitude of cytokines including: interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and interleukin 1 beta (IL1β) [5]. The release of these cytokines drives activation of nearby macrophages and epithelial cells in a similar manner and promotes further influx of inflammatory cells, such as macrophages and neutrophils to the site of injury [17]. These recruited macrophages will further promote the inflammatory state to ensure all of the materials/pathogens that are injurious to the tissue are engulfed and subsequently removed [17]. Following removal of the hazardous materials or pathogens, resolution of inflammation occurs, which is required to allow repair of the injured tissue [18].

Resolution of inflammation is generally associated with apoptosis and clearance of infiltrated inflammatory cells, whose persistence could lead to prolonged inflammatory symptoms [19]. Furthermore, failure to resolve inflammation despite removal or resolution of the initial mechanism of injury could lead to enhanced tissue injury, as is observed in a subset of patients following ARDS [11]. Importantly, this concept of persistent non-resolving inflammation following lung injury can lead to increased tissue damage, and one aim of this thesis is to uncover what underlying physiological processes could achieve persistent non-resolving inflammation.
1.3 Macrophages

Macrophages are immune system cells that specialize in the phagocytosis of pathogens and deleterious substances, produce immune effector molecules, and typically associate with sites of infection [20]. The macrophage is a terminally differentiated cell type derived from monocyte precursors [21]. Monocytes becomes classified as macrophages upon extravasation into target tissues; extravasation can be in response to inflammatory signals, known as chemokines, or to generate resident macrophages within the tissue [22]. Macrophages exist primarily as phagocytic cells engulfing particulate matter in their nearby environment and relaying changes in their microenvironments to other immune cells [23].

Macrophage subsets within the lungs include both resident and recruited macrophages. The cells that permanently reside within the alveolar spaces (even in healthy lungs) are termed resident macrophages, whereas macrophages drawn into the lungs following injury are termed recruited macrophages [9]. These two populations differ in their longevity as well as their intended function [24]. Resident macrophages are long-lived cells necessary for relaying information to the immune system about their surroundings, and play a key role in surfactant recycling [25]. The resident macrophage is also involved in adaptive immunity through antigen presentation to helper T cells such that appropriately targeted immune responses can be mounted within the lungs [26]. Resident macrophage activation causes release of many pro-inflammatory cytokines that
recruit additional neutrophils and macrophages to the site of injury in order to subdue the threat by engulfment and eventual clearance [17, 27].

Monocytes from the bone marrow travel toward the site of injury through the vasculature and upon reaching the site of the injury, will extravasate into the tissue at the area of increased cytokine and chemokine levels [21]. Within the lungs, after monocytes enter the pulmonary capillary bed, they will migrate through the endothelial monolayer, pass through the interstitial space, and migrate across the epithelial barrier [9]. It is at this point, once contained with the injured tissues, that monocytes become classified as recruited macrophages. Recruited macrophages are best described as cells derived from monocytes that have translocated into a tissue in order to carry out an immunomodulatory function at a specific location where infection and/or tissue injury has occurred [23]. Once the macrophage arrives at the site of injury, a drastic alteration to their gene expression occurs allowing for the appropriate response to the local tissue microenvironment – a process termed macrophage polarization [28].

1.3.1 Macrophage polarization

Macrophage polarization is a macrophage-specific cellular activation process that causes a shift in the expression of multiple genes in response to the presence of inflammatory signaling molecules within their local tissue microenvironment [29]. Environmental cues, such as bacterial products, or pro- and anti-inflammatory cytokines drive this change in gene expression thereby enabling the macrophage to carry out specific immune functions [28]. Two distinct and opposite ‘polarizations’ exist in
macrophages, including the pro-inflammatory, classically activated (M1-polarized)
macrophage, and the anti-inflammatory, alternatively activated (M2-polarized)
macrophage [28].

One of the primary functions of M1-polarized (pro-inflammatory) macrophages is
to promote the immune response in order to sufficiently deal with an injury/infection
within a specific tissue. In the overall disease process, however, the inflammatory
response must subside in order to promote the recovery of damaged tissues back to a
healthy state. The cessation of inflammation is in-part due to a second role of
macrophages. The function of M2-polarized macrophages is to support the resolution of
inflammation, primarily through secretion of multiple anti-inflammatory cytokines such
as, interleukin 1 receptor antagonist (IL1ra), interleukin 10 (IL10), and transforming
growth factor beta (TGFβ) [9, 30]. Though macrophages directly clear foreign materials,
pathogens, and debris by phagocytosis, they are also carry out efferocytosis, the act of
engulfment of apoptotic cells [31]. By engulfing apoptotic cells, macrophages protect
their environment from the pro-inflammatory internal contents of neutrophils and other
macrophages, which would be released following disruption of cell membranes [32].

Ultimately, macrophage activity within an injured tissue reflects the injury repair
process. The initial role of the macrophage is to promote inflammation in order to clear
the pathogen and prevent systemic inflammation [9]. Once the initial insult/injury is
cleared, there is a shift in the activity of macrophages towards promoting the resolution
of the inflammation and repair of the damaged tissue [33]. Importantly, if the M1-
polarized (pro-inflammatory) macrophage persists, this may result in an aberrant pro-inflammatory environment and subsequently lead toward chronic tissue damage [11]. Based on this concept, work within this thesis specifically focuses upon the M1-polarized state of macrophages, as the main focus is to identify if ARDS-associated persistent inflammation is brought about by persistent, pro-inflammatory macrophages.

1.3.2 Macrophage Apoptosis

Apoptosis, or programmed cell death, is a process utilized within living systems in order to clear or remove cells that are no longer required, or are damaged beyond repair, and to ensure they do not spill their contents into their environment causing damage to surrounding cells [34]. One mechanism for removal of apoptotic cells is efferocytosis, the engulfment of apoptotic bodies by phagocytic cells [34]. Efferocytosis relies on the presence of cell surface proteins, or “eat-me” signals to identify appropriate targets [35]. One example of an eat-me signal is phosphatidylserine, which is found on the inner leaflet of the cell membrane of healthy cells but accumulates on the surface of apoptotic cells [35, 36]. Apoptosis is a highly controlled and regulated cascade that begins with an event, such as damage to the cell or a signalling molecule, which triggers the initiation of apoptotic machinery.

One major group of surface proteins that initiate apoptosis following interaction with an extracellular ligand are the ‘tumor necrosis factor (TNF) receptor superfamily’ [37]. Members of this family include the receptors TNFR1, TNFR2 and FAS, with prominent ligands including, TNFα, soluble FAS ligand (sFASL), and TRAIL
sFASL binding to FAS leads to trimerization of similarly bound receptor-ligand complexes (Figure 1-1)[37]. Following trimerization, FAS receptors associate with the FAS activated death-domain (FADD), which in turn binds to and activates pro-caspase-8 (Figure 1-1)[39].

Caspases, which are cysteine-dependent aspartate-directed proteases, are internal cell signalling proteins with many known functions, including the proteolytic processing of proteins associated with the apoptotic cascade [40]. Twelve caspases exist in humans and most can be divided into two primary groups. These groups include the initiator caspases (2, 8, 9 and 10), which start the apoptotic cascade, and the executioner caspases (3, 6 and 7), which once activated, lead to irreversible apoptosis [41, 42]. Within the FAS apoptotic cascade, caspase 8 (an initiator caspase) will activate caspase 3 (an executioner caspase) to cause apoptosis [43]. Apoptosis is thought to reduce inflammation within the lungs via clearance of pro-inflammatory macrophages. This concept is supported by
Figure 1-1: sFASL/FAS ligand-receptor induced apoptosis.

(1) sFASL binds to its receptor FAS at the cell surface (2). This interaction leads to trimerization of bound receptor-ligand complexes (3), which promotes the recruitment of the FAS activated death domain (FADD) [38]. The FADD-FAS timer complex recruits Procaspase 8 (4) and subsequently complexes with it (5) [44]. The pro-domain on Procaspase 8 is cleaved (6) and active Caspase 8 is released within the cell to activate downstream executioner caspase 3 (7) and ultimately lead to apoptosis [38].
pro-inflammatory macrophage apoptosis being associated with decreased neutrophil recruitment in pneumonia, coupled to a decrease in the probability of mice becoming bacteremic [18]. An inability of apoptotic machinery to function properly could lead to persistence of M1-polarized (pro-inflammatory) macrophages, ultimately promoting a persistent inflammatory environment within tissues. Apoptosis and subsequent clearance of macrophages is a mechanism capable of invoking inflammatory suppression, and if unbalanced, could be the root of a persistent inflammatory phenotype in some patients [19]. This thesis aims to study the repair of lung injury, and as macrophages are key mediators of inflammation, and its resolution, they will be a key focal point within this study.

1.4 Metalloproteinases

Metalloproteinases are a group of enzymes whose function is to degrade and process biologically relevant proteins within the extracellular environment [45]. While there are many different families of metalloproteinases, this study focused on two of those families. First, the matrix metalloproteinases (MMPs) are a family of 24 zinc-dependent endopeptidases with the capability of degrading many extracellular and membrane bound proteins [46]. MMPs were classically thought to primarily degrade the extracellular matrix (ECM); however, a more thorough understanding of the function of these complex enzymes has led to a refined definition relating their activity to processing biologically active components within the extracellular environment [47]. This
processing includes but is not limited to, growth factor activation, chemokine processing, cell surface protein cleavage, and remodeling of the ECM [45].

The second metalloproteinase family of interest are the ‘a disintegrin and metalloproteinases’ (ADAMs) family, which differ from most MMPs by way of a transmembrane region and a disintegrin domain [48]. ADAMs differ functionally from MMPs as ADAMs are predominantly thought to be “sheddases” and are involved in ectodomain shedding of cell surface proteins and receptors [49]. One well categorized metalloproteinase with sheddase activity is ADAM17, which has been shown to cleave TNFα and its receptor from the cellular surface [50]. Most MMPs and ADAMs are produced as zymogens, and only become active once released from their pro-domain [51]. Metalloproteinases, as their name implies, require interaction of a metal ion (typically zinc) with the catalytic domain for functional activity [45]. Importantly, metalloproteinase activity is regulated at multiple levels including regulation at the level of expression as well as by tissue specific inhibitors [52].

1.4.1 Metalloproteinases in ARDS

Metalloproteinases, based on their ability to process pro-inflammatory cytokines, are known to mediate inflammatory processes within ARDS [47]. Previous publications have categorized the most prevalent metalloproteinases in ARDS [53]. Lavage fluids from subjects with ARDS have elevated levels of MMP1, -2, -3, -8, -9, and -13 [54]. It has been proposed that MMP8 and MMP13 possess anti-inflammatory properties in ARDS, mediated through inactivation of macrophage inflammatory protein-1α and
monocyte chemoattractant protein-1, respectively [55, 56]. Conversely, lavage fluid levels of MMP1 and MMP3 have been correlated with elevated mortality rates in ARDS, suggesting that they play a more deleterious role in ARDS [57]. A previous publication has shown ADAM17 to be capable of ectodomain shedding of TNFα and its receptors, which is a key inflammatory signalling molecule and thus associated with ARDS [50]. Based on their regulatory control of inflammation within ARDS, metalloproteinases will be a primary interest for our study of inflammation resolution. Macrophage function relies upon the ability of the macrophage to respond to external stimuli within the tissue microenvironment, and previously acquired data suggests that metalloproteinases are capable of regulating macrophage response to external signaling molecules [29].

1.4.2 Metalloproteinases in Macrophage Function

MMPs and ADAMs can produce pro- and anti-inflammatory effects and are produced by macrophages in both polarized states; however, these metalloproteinases are differentially expressed depending on the activated state of the macrophage [58]. For example, the expression of MMP1, -3, -7, -10, -12, and -14 is elevated in M1-polarized macrophages, whereas expression of MMP2, -8 and -19 is decreased and MMP11, -12, and -25 is increased in M2-polarized macrophages [58]. Furthermore, ADAM9, -15, and -17 have also been shown to be expressed by macrophages in pro-inflammatory settings of atherosclerotic plaques [59, 60]. Metalloproteinases can also directly modulate macrophage function. For example, MMP2 has been shown to cleave CC-chemokine ligand 7, a chemotactic protein known to induce macrophage infiltration, rendering it
inactive, and potentially decreasing the levels of pro-inflammatory macrophages recruited to the site of injury [61]. Second, MMP28 has been shown to have high expression within the mouse lung, where it is capable of reducing macrophage recruitment and polarizing macrophages towards an M2-polarization within a pulmonary fibrosis injury model [62, 63]. Thus, macrophages can promote or restrict inflammation based on their polarization and this polarization is regulated by environmental cues. These cues, namely cytokines, can be processed by metalloproteinases suggesting that metalloproteinases, which are expressed by macrophages, may have an important role in regulating macrophage response to environmental cues and consequently, the inflammatory environment following injury.

1.4.3 Metalloproteinases and Apoptosis

Persistent non-resolving inflammation in select cases of ARDS could be due aberrant apoptosis within the pro-inflammatory macrophage. Metalloproteinases have been shown to play a role in the processing of cellular surface receptors, and importantly, some of these receptors are involved in apoptotic signaling cascades [50, 64]. FAS, one such receptor, can cause apoptosis in macrophages [65]. Previous studies on hepatocytes has led to a direct correlation between apoptosis of hepatocytes and the shedding of the FAS ectodomain [66]. Furthermore, in hepatocytes ADAM17 has been shown to cleave members of the TNF superfamily restricting the activation of cell death signaling pathways [67]. ADAM17 activity is regulated by tissue inhibitor of metalloproteinases 3 (TIMP3) [68]. In the absence of TIMP3, ADAM17 activity is elevated, FAS ectodomain
shedding is increased, and hepatocytes are protected from undergoing cell death [67]. Unabated metalloproteinase-associated receptor shedding might render cells unable to properly respond to extracellular signaling that typically would induce an apoptotic cascade [50].

1.5 Tissue inhibitors of metalloproteinases

The TIMP family of metalloproteinase inhibitors is comprised of four distinct family members, TIMP1, -2, -3, and -4 [45]. Each TIMP can inhibit metalloproteinases, though their specificity and ability to act in certain environments varies [52]. TIMPs have been studied thoroughly within the context of wound healing, and are thought to have a key role in regulating the ability of metalloproteinases to enable tissue repair and remodeling, as well as inflammatory processes [45]. TIMPs inhibit metalloproteinases through binding to the active site on the metalloproteinase; this association inhibits the catalytic activity of metalloproteinases [69].

Past studies have shown that TIMP3 is the only TIMP to associate with the ECM [70]. Specifically, TIMP3 is known to be localized to sulphated proteoglycans within the ECM [71]. TIMP3 can be produced by multiple different cell types within the lungs, including endothelial cells, fibroblasts and macrophages [72, 73]. TIMP3 regulates multiple metalloproteinases and without sufficient TIMP3, the activity of these proteases, such as ADAM17, becomes increased, which in-turn leads to increased shedding of multiple cell surface receptors [67].
1.5.1 TIMP3 and ARDS

*Timp3<sup>[−/−]</sup>* mouse lung morphology is altered from wild type (WT) animals; *Timp3<sup>[−/−]</sup>* mice develop enlarged alveolar spaces, and die prematurely beginning at 13 months of age implicating TIMP3 as an important protein within the lung [74]. Additionally, following both bleomycin-induced and lipopolysaccharide (LPS)-induced lung injury, mice lacking TIMP3 recovered more slowly than the WT animals [72, 75]. Intensified *Timp3<sup>[−/−]</sup>* lung inflammation and hindered recovery were demonstrated by greater inflammatory cell presence within bronchoalveolar lavage (BAL), and through decreased markers of inflammatory resolution within BAL fluids from *Timp3<sup>[−/−]</sup>* mice vs. WT mice [72, 75]. Furthermore, BAL fluid from the *Timp3<sup>[−/−]</sup>* lungs induced greater neutrophil chemotaxis compared to WT lavage fluids in culture suggesting that the persistent neutrophil accumulation in *Timp3<sup>[−/−]</sup>* mice was due to ongoing neutrophil influx [75]. In addition, *Timp3<sup>[−/−]</sup>* mice have enhanced lung compliance at baseline, which is made worse following sepsis [76, 77] As well, these alterations following sepsis appear to be due to macrophages, as when macrophages were depleted, mice were protected from alterations in lung mechanics brought about by sepsis [77]. These examples of lung dysfunction following injury in *Timp3<sup>[−/−]</sup>* lungs mimic the issues of persistent inflammation following ARDS, and also suggest that TIMP3 plays an important role in lung injury repair. Thus, TIMP3 regulates inflammation and repair through control of metalloproteinase activity, and based on this role, it will be a principal protein of interest in regards to this thesis.
1.5.2 TIMP3 and Macrophage function

Loss of TIMP3 from the lung results in dysfunctional repair and resolution of inflammation following injury. Macrophages, which are known to express TIMP3, are known to be key mediators of both the initiation and resolution of inflammation as well as tissue repair following lung injury [9, 78]. \( Timp3^{-/-} \) bone marrow-derived macrophages (BMDMs) appear to be genetically and functionally different than WT BMDMs [72]. Following LPS treatment (an M1 polarizing stimulus), BMDMs from \( Timp3^{-/-} \) mice exhibited increased pro-inflammatory gene expression compared to similarly polarized WT BMDMs. This elevated pro-inflammatory gene expression levels in \( Timp3^{-/-} \) BMDMs was rescued when BMDMs were treated with recombinant TIMP3 (rTIMP3) [72]. Furthermore, \( Timp3^{-/-} \) BMDMs also induced significantly more neutrophil chemotaxis \textit{in vitro} and appeared resistant to apoptosis compared to WT BMDMs [72].

\( Timp3^{-/-} \) macrophages were also found to release more TNF\(\alpha\) than their WT counterparts due to increased ADAM17 activity, which promoted a more pro-inflammatory environment suggested by increased IL6 levels in serum [78]. Interestingly, overexpression of TIMP3 in macrophages had the opposite effect; a decrease in the mRNA levels of multiple pro-inflammatory genes was observed within fatty tissue in mice on a high fat diet [79]. Together, this data suggests that TIMP3 has an important role regulating macrophage function and response to environmental cues. As ADAM17 is known to target members of the TNF superfamily, lack of ADAM17 inhibition by TIMP3 could modify macrophage function leading to macrophage persistence and subsequent non-resolving lung inflammation.
1.5.3 TIMP3 and Apoptosis

There are many studies that lend support toward TIMP3 acting as a pro-apoptotic protein. For example, overexpression of TIMP3 causes increased levels of apoptosis within multiple different cells lines [80]. Adenoviral expression of TIMP3 in melanoma cells, leads to a marked increase in the levels of FAS, TNFR1 and Caspase 3 detected via immunostaining (Figure 1-2) [81]. TIMP3 overexpression has also shown to be able to induce elevated levels of apoptosis within smooth muscles cells from rat aortic tissues [82]. Additionally, macrophages lacking TIMP3 appear to be resistant to both staurosporine and sFASL-induced apoptosis, which further supports a role for TIMP3 in the control of apoptosis [72]. Importantly, use of a mutant TIMP3 with abolished MMP inhibitory function no longer had a pro-apoptotic function, linking the ability for TIMP3 to promote apoptosis to an MMP-dependent mechanism [83]. Based on previous studies TIMP3 has been shown to promote apoptosis in multiple different cell models, and coupled to its anti-inflammatory role in the lungs, it is a key focus for study within this thesis.
Figure 1-2: Proposed model of TIMP3 mediated macrophage apoptosis.

(A) TIMP3 has been shown in previous studies to stabilize pro-apoptotic receptors, known as death receptors, on the cell surface through its ability to inhibit metalloproteinase activity [67, 80, 81, 83]. Stabilized death receptors on the cell surface allow for ligand-receptor interaction at the cell surface and promote activation of a pro-apoptotic signaling cascade [84]. (B) Deregulated metalloproteinase activity (i.e. in the absence of TIMP3) leads to enhanced shedding of the death receptors (e.g. FAS) leading to desensitization to apoptotic stimuli [72]. Apoptosis within the pro-inflammatory macrophage is a major contributing factor to the overall cessation of inflammation in wound healing [19]. Thus, TIMP3 may have a key role in regulating the ability of macrophages to undergo apoptosis (A), and a loss of TIMP3 may result in macrophages that are resistant to undergoing apoptosis (B). Importantly, failure of M1-polarized macrophages to undergo apoptosis could allow these macrophages to persist within the damaged tissue following injury, ultimately resulting in ongoing, chronic inflammation.
**Wild Type**

- **Metalloproteinase**
- **TIMP3**
- **FADD**
- **Internal signalling**
- **Procaspe 8**
- **Caspase 8**
- **Apoptosis**

**Timp3-/-**

- **Metalloproteinase**
- **Shedding**
- **Hindered Internal signalling**
- **Reduced Apoptosis**
1.6 Rationale:

1) Persistent or chronic inflammation following lung injury can lead to increased tissue damage.

2) Macrophages are key mediators of both the initiation and resolution of inflammation.

3) TIMP3 has a role in regulating inflammation through control of metalloproteinase activity.

4) TIMP3 is expressed in the lung by multiple cells types, including macrophages.

5) Mice lacking TIMP3 have impaired resolution of inflammation following lung injury, possibly due to altered macrophage polarization and apoptosis.

It is known that mice lacking TIMP3 have a more inflammatory lung environment following lung injury [75]. The macrophages within the Timp3⁻/⁻ lungs likely are promoting greater inflammation as supported by the increased pro-inflammatory gene expression observed in Timp3⁻/⁻ BMDMs [72]. Timp3⁻/⁻ murine lungs have increased metalloproteinase activity levels [74, 75, 85] and TIMP3 is known to stabilize death receptors at the cell membrane [67, 80, 81, 83]. Together, this data suggests a potential mechanism of apoptotic resistance for the M1 macrophage population within the lungs of the Timp3⁻/⁻ mice. Specifically, persistent inflammation in Timp3⁻/⁻ lungs following injury would be the result of the sustained presence of inflammatory macrophages in the alveolar spaces due to increased metalloproteinase activity leading to shedding and
desensitization of death receptors on the cell surface of M1-polarized Timp3^+/− macrophages. However, if shedding of death receptors occurs within the alveolar macrophage and whether this shedding of death receptors from macrophages would drive the persistent inflammation following ARDS is not yet known. This thesis proposes to study a potential mechanism for the persistent inflammation associated with ARDS, and potentially answer how this poorly understood complication could arise.

1.7 Hypothesis

We hypothesize that TIMP3 promotes apoptosis of murine macrophages through inhibition of metalloproteinase activity and the stabilization of FAS on the cell surface.

1.8 Objectives:

1) Establish and characterize WT and Timp3^+/− bone marrow-derived macrophage (BMDM) cultures.

2) Identify the mechanism through which TIMP3 regulates macrophage apoptosis.
Chapter 2. Methods

2.1 BMDM Isolation and Culture

BMDMs were isolated and cultured from the bone marrow of WT and Timp3−/− mice using techniques previously found to produce a pure population of macrophages [57, 67]. Mice between the ages of 8 and 12 weeks were euthanized via pentobarbital sodium overdose (Euthanyl forte, Bimedia-MTC; ethics approval #Mehta2011-026). Following sacrifice, the tibia and femur from the hind legs of the animal were isolated by blunt dissection and placed into cold 15 mM EDTA/PBS (pH 7.4). The proximal and distal ends of the bones were removed and the bones placed into separate 500 µL Eppendorf tubes that had been punctured through the bottom with an 18.5 gauge needle. The 500 µL tubes were nested within 1.5 mL Eppendorf tubes and the tubes spun at 2000 RCF for 1 minute at 4 °C. The isolated marrow within the 1.5 mL Eppendorf tube was then resuspended in 1 mL of 15 mM EDTA/PBS and marrow from the same animal was combined in a 15 mL falcon tube. Cells were centrifuged at 400 RCF for 10 minutes at 4 °C and the supernatant discarded. The cell pellet was resuspended in 1 mL of red blood cell (RBC) lysis buffer (eBioscience, Catalogue #00-4333-57) and placed on ice for 4 minutes, after which, 5 mL of PBS was added to the cell suspension. The suspension was then centrifuged at 400 RCF for 10 minutes at 4 °C and the supernatant removed. This cell pellet was then resuspended in 8 mL macrophage media (RPMI 1640, 30% L929 cell supernatant [refer to 2.1.1], 10% FBS, 2mM L-glutamine, 100 I.U. /mL penicillin, and
100µg/mL streptomycin), plated onto a 100 mm tissue culture treated plate, and placed at 37 °C for 24 h.

After 24 h, the conditioned media from each of the 100 mm plates was collected and each plate was rinsed with 5 mL sterile PBS to collect non-adherent and loosely adherent cells. The conditioned media and PBS rinse were placed into a sterile 15 mL falcon tube and centrifuged at 400 RCF for 10 minutes at 4 °C. The supernatant was removed, the cell pellet resuspended in 8 mL macrophage media, and the cells plated on a 150 mm sterile non-tissue culture treated plates containing an additional 12 mL macrophage media. These 150 mm plates were then cultured at 37 °C for 6 additional days.

2.1.1 L929 cell culture

In order to differentiate bone marrow isolate into BMDMs, culture media requires the presence of macrophage-colony stimulating factor (M-CSF). Conditioned media from L-929 cell cultures (ATCC, Catalogue # CCL-1), which contains high concentrations of M-CSF, is well documented as an inexpensive source of M-CSF for use in BMDM cell culture [86]. To produce adequate concentrations of M-CSF, 4.4 X 10⁶ L-929 cells were plated on 150 mm tissue culture treated plates and grown in 30 mL of IMDM media containing 10% FBS, 100 I.U./mL penicillin and 100 µg/mL streptomycin. Cells were grown for 14 days after which the conditioned media (i.e. L-929 cell supernatant) was collected, passed through a sterile 20 µm filter and stored at -80 °C until use.
2.1.2 BMDM Polarization

After 1 week of growth, the conditioned media was collected, used to rinse the plate once, and then placed in a 50 mL falcon tube on ice. 20 mL of cold PBS was then added to the plate and the plate placed at 4 °C for 30 minutes. Cells were then lifted by gently washing the plate with the chilled PBS. This suspension was then added into the 50 mL falcon tube with the conditioned media and the sample was spun at 400 RCF for 10 minutes at 4 °C. The supernatant was aspirated and the macrophages resuspended in 1 mL macrophage media. The number of macrophages was then determined via haemocytometer and the macrophage concentration diluted to 2 x 10^6 cells/mL.

Macrophages were then plated into 6- or 24-well plates at 2 x 10^6 and 4 x 10^5, respectively, and allowed to grow for 24 h at 37 °C. After 24h, media was removed from the cells and replaced with new media lacking M-CSF (i.e. without L-929 cell supernatant), referred to as stimulation media (RPMI 1640, 10% FBS, 2mM L-glutamine, 100 I.U./mL penicillin, and 100µg/mL streptomycin). Stimulation media also included either LPS (E.Coli-0111LB4; 100 ng/mL; Sigma-Aldrich, Catalogue #L-2630-100mg) or PBS to drive the cells toward an M1-polarized or unpolarized (M0) phenotype respectively. BMDMs were stimulated with stimulation media over 24 h to allow for macrophage polarization to occur, a method supported by previous studies [72]. A subset of macrophages was also incubated in the presence/absence of human recombinant TIMP3 (rTIMP3; 1µg/mL) during the 24h polarization.
2.2 Conditioned media and protein/RNA isolation

BMDMs (2 x 10^6 cells per well) were plated onto tissue culture treated 6-well plates and polarized as described above [2.1.2]. Following polarization, the conditioned media was removed and aliquoted into 1.5 mL Eppendorf tubes before being stored at -80 °C for later use. Cells were then rinsed with PBS prior to protein isolation. To isolate protein, 200µL of Sigma CelLytic™ M Cell Lysis Regent was added to each well (Sigma-Aldrich, Oakville, ON; C2978-50ML) and the cells left on ice for 20 minutes. After 20 minutes, wells were scraped and cell lysate collected and placed in Eppendorf tubes on ice. Samples were further sonicated for 20 seconds at 60% amplitude and spun down at 20 000 RCF for 15 minutes to ensure insoluble debris was not collected in our protein samples. Following sonication, samples were stored at -80°C.

RNA was isolated using the RNeasy kit (Qiagen; Cat. #74104). Briefly, 200 µL RLT buffer containing 2 µL beta-mercaptoethanol was added to each well. Wells were then scraped, and cell lysate collected and placed in Qiashredder tubes (Qiagen; Cat. #79656). Samples were spun at 10 000 x g for 30 seconds. Flow through was collected from the Qiashredder tubes, placed into RNeasy spin columns, washed with a series of buffers, and the RNA eluted from the columns by water, as per the manufacturer instructions.
2.2.1 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Purity (230/260 and 260/280 ratios) and concentration of isolated RNA was determined by analysis with the NanoDrop 1000 spectrophotometer (Thermo, Waltham, MA, USA). Isolated RNA (2 ug) was then reverse transcribed by using a High capacity cDNA reverse transcription kit (Invitrogen 43368814 ABI) according to manufacturer’s instructions. *Il12b* and *Nos2* gene expression was then analyzed using TaqMan Gene Expression Assays from Applied Biosystems (Invitrogen, Grand Island, NY, USA; *Il12*, Cat. # 4331182; *Nos2*, Cat. # Mm00440502_m1) and the CFX96 Real Time System (BioRad, Philadelphia, PA, USA). Hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), a housekeeping gene, was used to normalize expression of genes of interest. qRT-PCR generated the cycle threshold (Ct) value for each gene and this value was then used to determine gene expression relative to WT unpolarized BMDMs. For this analysis, ΔCt was the normalization of a given gene to *Hprt* within a specific sample (i.e. WT M1-polarized BMDMs), ΔΔCt was the normalization of a specific sample (i.e. WT M1-polarized BMDMs) to the control sample (WT unpolarized BMDMs), and the relative quantity (RQ) was the fold change in expression of a specific sample (i.e. WT M1-polarized BMDMs) relative to the control sample (WT unpolarized BMDMs). RQ was determined by the following equation: $RQ = 2^{-\Delta\Delta Ct}$.

2.2.2 OmniMMP assay

Total metalloproteinase activity in conditioned media and cell lysates was determined through use of the OmniMMP Assay, a broad-spectrum, fluorescent assay.
Lysate and conditioned media analyses were conducted with the use of a Victor 3 plate reader (PerkinElmer, Woodbridge, ON). Equal volumes of sample were loaded in each well and prior to adding the active fluorescent substrate (P126), baseline levels of fluorescence were measured for each sample. Following the addition of P126 (Excitation/Emission – 328 nm/393 nm), samples were compared to values of positive controls, wells containing P127 (the fluorescent conjugate generated from cleavage of P126; Excitation/Emission – 328 nm/393). Measures of activity in a given sample were taken after 1, 3, 5, 10, 15, 30 and 60 minutes. Following the initial hour of the experiment, further measurements were taken after 2, 3, 4, 8, 22, 24, 32, 46 and 48 h. Data was subsequently analyzed through overall time course plots, area under the curve calculations, as well as single time point assessments.

2.3 Assessment of macrophage apoptosis

2.3.1 FLICA Staining

Differentiated BMDMs were plated onto 48-well tissue culture plates (2 x 10^5 cells per well) and after 24 h, were polarized toward an M1-polarization or left unpolarized as described above (2.1.1 and 2.1.3). BMDMs were then stimulated with sFASL (100ng/mL; SuperFasLigand, Enzo Life Sciences, Address; ALX-522-020-3005) for 2, 4, and 8 h. Following induction of apoptosis with sFASL, caspase activation was assessed with the FLICA caspase assay as per the manufacturer’s instructions (ImmuoChemistry Technologies). For these studies, FLICA is resuspended in DMSO
(50 µL DMSO/vial) and this DMSO/FLICA mixture is then further diluted in PBS (1:4 FLICA/DMSO to PBS). For the last hour of the sFASL stimulation period, the diluted FLICA reagent or DMSO/PBS alone was added directly into the well (3.3µL/100µL media), the plates gently agitated to ensure even dispersal of the FLICA reagent, and the plates placed back at 37°C. Following stimulation, the plates were removed from the incubator, the media aspirated, and the wells washed with PBS before 200 µL of 10% formalin was added to each well to fix the cells. After 45 min of fixation at room temperature, the formalin was aspirated and wells were once again washed with PBS. Hoechst’s stain (Hoechst 33342; Invitrogen, Catalogue #H3570) was used in order to fluorescently label the nuclei of the BMDMs. Plates were then imaged using fluorescent microscopy (FLICA excitation/emission: 550 nm/590-600 nm; Hoechst excitation/emission: 361 nm/486 nm) the number of Hoechst positive cells per field of view were counted through the use of a macro in ImageJ. Automated counts of the number of FLICA positive cells were confirmed by two blinded reviewers.

### 2.3.2 TUNEL Staining

Differentiated BMDMs were plated onto glass coverslips contained within 24-well plates (4 x 10⁵ cells per well) and after 24h, were polarized toward the M1-polarization or left unpolarized as described above (2.1.1 and 2.1.3). Apoptosis was induced via stimulation with sFASL for 2, 4 and 8h after which, cells were fixed in 10% formalin for 30 minutes at room temperature, and then washed repeatedly with PBS. Fixed BMDMs were then permeabilized with a 1% Na⁺ citrate and 0.1% triton X-100
solution for 150 seconds at 4°C and the endogenous peroxidase activity was quenched by treatment with 3% H$_2$O$_2$ in 50% methanol for 30 minutes at room temperature. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was then performed according to the manufacturer’s instructions to assess DNA fragmentation (Roche, Catalogue #11-684-817-910). Briefly, diluted TUNEL enzyme was added into each well and incubated in darkness for 1 hour at 37°C. Wells containing glass coverslips were washed with PBS and 50 µL of POD converter solution was added to each well for 30 minutes at 37°C. Prior to imaging the slides, 50 µL of DAB chromagen solution was added to each well. The DAB solution was incubated for between 1-5 minutes, during which the reaction was monitored under the microscope to ensure development was not excessive. The developer was then quenched by dilution with PBS and slides were viewed under a visible light microscope. The total number of cells and the number of TUNEL positive cells per field of view were counted by blinded reviewers due to automation errors.

2.3.3 Annexin V Staining

BMDMs were differentiated, replated at 2 x 10$^6$ cells per well in a 6-well non-tissue culture treated plate, and after 24 h, left unpolarized or driven toward M1-polarization as described above (2.1.1 and 2.1.3). Following polarization BMDMs were stimulated with either PBS or sFASL (100 ng/mL) for 1 and 2 h to induce apoptosis. At the end of stimulation the 6-well plates were centrifuged at 200 RCF for 5 minutes at 4°C, media was removed, and BMDMs were washed with PBS. BMDMs were then
treated with 0.5 mM EDTA/PBS at 37°C for 5 minutes to gently lift from the plate after which, collected BMDMs were pipetted into a U-bottomed 96 well plate and centrifuged at 200 RCF for 5 minutes at 4°C. EDTA/PBS was aspirated from the wells and replaced with 100 µL of Annexin V binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM CaCl$_2$). Annexin V and propidium iodide (PI; 2 uL of each) were added into the wells for 10 minutes in darkness at room temperature. Once stained, an additional 150 µL of Annexin V binding buffer was added to each well. The presence of Annexin V and PI in each of the cell populations was then analyzed by flow cytometry in the Guava-12HT flow cytometer (Millipore, Billerica, MA, USA). Annexin V$^+$/PI$^-$ cells were considered apoptotic cells, whereas Annexin V$^+$/PI$^+$ cells were considered dead cells and Annexin V$^-$ /PI$^-$ cells were considered live cells.

2.4 Western Blot

Total protein concentration of BMDM cell lysates was determined by Bradford assay. Once concentrations were calculated, 40 µg of total protein from each sample was heated to 100°C in the presence of β-Mercaptoethanol, loaded on a 4-15 % gradient mini gel (Biorad, Catalogue #J00094) with PageRuler Plus protein ladder loaded (Thermo Fisher Scientific, Catalogue #G00203) in Tris-Glycine SDS Running buffer (25mM Tris, 192 mM Glycine, 0.1 % SDS, pH 8.3), and run at 100 V for 60 minutes. Separated proteins were then transferred to a PVDF membrane over the course of 60 minutes at 100 V in transfer buffer containing 20 % methanol. Blots were then rinsed in PBS, blocked in PBS with 3 % BSA for 1 h, and probed with rabbit-anti-mouse FAS antibodies (1:1000;
Enzo Life Sciences, Catalogue #ADI-AAP-221-F) overnight at 4°C. After overnight incubation, blots were washed in TBS-T (137 mM NaCl, 2.7 mM KCl, 19 mM Tris Base, 1% Tween, pH 7.4) and then probed with goat anti-rabbit IgG antibodies conjugated to horseradish peroxidase (1:10 000) for 1 h at room temperature. Blots were once again washed in PBS with 1% BSA and then treated with a Bio-Rad Clarity Western ECL substrate kit (Bio-Rad 1705061). Blots were then imaged using the Froggabio camera system to determine the levels of antibody binding. Following imaging blots were washed in TBS-T and reprobed with an antibody against the housekeeping protein, GAPDH (1:5000; Abcam, Catalogue # ab9485), before being reimaged through the same protocol in order to normalize FAS levels to GAPDH though densitometry using ImageJ software.

2.5 Statistical Analysis

N values represent separate animal tissue harvests coupled to multiple repetitions of intra-experimental trials. Collected data was analyzed through Microsoft Excel 2010 to calculate standard deviation, and generate figures. Raw data was subsequently imported into GraphPad Prism 5 in order to carry out various statistical analyses depending upon the number of variables associated with the given experiment. T-tests were utilized within data collected with only one measured variable, and Two-way ANOVA was conducted on subject matter concerning 2 variables coupled to bonferonni post-hoc tests. Significance threshold was set to be $\alpha = 0.05$, to account for only 5% chance of observing the given trend by chance alone.
Chapter 3. Results

3.1 BMDM culture establishment

The initial focus of this study was to establish the WT and $\text{Timp}^{3/-}$ BMDM cultures and to characterize these cell lines to ensure responses to stimulation were similar to previously published studies [72]. Achieving these goals required establishing cell cultures to support the growth of BMDMs (i.e. L929 cells, a source of M-CSF, which is required for the differentiation of pluripotent hematopoietic stem cells to BMDMs), and optimization of culture materials and animal colonies [86]. The goal of culture establishment was to generate functionally similar BMDMs to those described previously within the literature [72]. The following results are associated with BMDM culture establishment and characterization.

3.1.1 BMDM-derived TIMP3 regulates $\text{Il12b}$ and $\text{Nos2}$ expression in M1-polarized macrophages

$\text{IL12b}$ and $\text{Nos2}$ are genes associated with the M1-polarized macrophages. In past studies $\text{Timp}^{3/-}$ BMDMs expressed greater levels of these two genes and similar trends were expected from our cultured BMDMs [72]. Steady state mRNA levels of the M1 specific genes $\text{Il12b}$ and $\text{Nos2}$ were analyzed through qRT-PCR in unpolarized (M0) and M1-polarized WT and $\text{Timp}^{3/-}$ BMDMs. Expression of $\text{Il12b}$ was significantly elevated in the M1-polarized $\text{Timp}^{3/-}$ BMDMs as compared to M1-polarized WT BMDMs (Figure 3-1). Furthermore, treatment of $\text{Timp}^{3/-}$ BMDMs with rTIMP3 resulted in a significant decrease in $\text{Il12b}$ expression (Figure 3-1). $\text{Il12b}$ mRNA expression in $\text{Timp}^{3/-}$
Figure 3-1: BMDM-derived TIMP3 regulates \textit{Il12b} expression in M1-polarized macrophages.

Use of qRT-PCR revealed that M1-polarized WT and \textit{Timp3}\textsuperscript{-/-} BMDMs have significantly increased \textit{Il12b} mRNA levels compared to unpolarized BMDMs of a similar genotype (A). Unpolarized BMDM \textit{Il12b} expression is not significantly different between WT and \textit{Timp3}\textsuperscript{-/-} BMDMs; however, M1-polarized \textit{Timp3}\textsuperscript{-/-} BMDMs have significantly increased \textit{Il12b} mRNA levels compared to WT M1-polarized BMDMs (A; mean ± SEM, N = 4-6; * indicates P < 0.05 vs. all other experimental groups, # indicates P < 0.05 vs. all other experimental groups, Two-way ANOVA with post-hoc Bonferroni correction). Importantly, the elevated \textit{Il12b} expression in \textit{Timp3}\textsuperscript{-/-} BMDMs is significantly reduced following the addition of rTIMP3 for the duration of the polarizing stimulus (B; mean ± SEM, N = 6; * indicates P < 0.05 vs. WT M1-polarized PBS, # indicates P < 0.05 vs. \textit{Timp3}\textsuperscript{-/-} M1-polarized PBS, Two-way ANOVA with post-hoc Bonferroni correction).
rTIMP3 treated cells was not significantly different from untreated M1-polarized WT BMDMs (Figure 3-1). Additionally, expression of Nos2 was significantly elevated in the M1-polarized Timp3+/− BMDMs vs. M1-polarized WT BMDMs (Figure 3-2). Nos2 expression following rTIMP3 treatment of Timp3+/− BMDMs was not significantly different from the WT BMDMs or the untreated Timp3+/− BMDMs (Figure 3-2). Importantly, while treatment of Timp3+/− BMDMs with rTIMP3 did not result in a significant decrease in Nos2 expression compared to untreated Timp3+/− BMDMs, expression of Nos2 in rTIMP3-treated Timp3+/− BMDMs was no longer significantly different vs. WT BMDMs (Figure 3-2) suggesting partial effect of the treatment.

3.1.2 Quantifying metalloproteinase activity in WT and Timp3+/− unpolarized and M1-polarized BMDMs

Our characterization of the levels of IL12b and Nos2 showed similar trends to past publications, allowing us to now expand on previous studies and further characterize these cells by examining the role of TIMP3 in regulating metalloproteinase activity. Following 24 h of BMDM culture, elevated metalloproteinase activity in conditioned media (CM) of Timp3+/− BMDMs was detected (Figure 3-3). The OmniMMP activity assay was performed over a 48 h time course (Figure 3-3). Data was analysed by area under the curve (AUC) for each respective polarization and treatment, which allowed for quantification of total metalloproteinase activity over time. The results from the AUC analysis show no differences in metalloproteinase activity between WT and Timp3+/− unpolarized BMDMs (Figure 3-4). WT M1-polarized BMDMs have reduced
Figure 3-2: M1-polarized *Timp3<sup>−/−</sup>* BMDMs have elevated *Nos2* expression compared to M1-polarized WT BMDMs.

Analysis with qRT-PCR revealed M1-polarized WT and *Timp3<sup>−/−</sup>* BMDMs have significantly increased *Nos2* mRNA levels compared to unpolarized BMDMs of a similar genotype (A). Furthermore, *Timp3<sup>−/−</sup>* M1-polarized BMDMs have significantly elevated *Nos2* expression compared to similarly polarized WT BMDMs (A; mean ± SEM, N = 4-6, * indicates P < 0.05 vs. all other treatment groups, # indicates P < 0.05 vs. all other treatment groups, Two-way ANOVA with post-hoc Bonferroni correction). There is no significant difference in *Nos2* expression between M1-polarized *Timp3<sup>−/−</sup>* BMDMs treated with supplemental rTIMP3 and any other treatment group (B; mean ± SEM, N = 6, * indicates P < 0.05 vs WT M1-polarized PBS, Two-way ANOVA with post-hoc Bonferroni correction).
Figure 3-3: Metalloproteinase activity levels in CM of unpolarized and M1-polarized WT and Timp3$^{-/-}$ BMDMs

WT and Timp3$^{-/-}$ BMDM metalloproteinase activity, as measured by the OmniMMP assay. Samples from the CM of unpolarized WT and Timp3$^{-/-}$ BMDMs are elevated when compared to respective M1-polarized samples. (mean ± SEM, N = 4).
Figure 3-4: M1-polarized \textit{Timp3}^{−/−} BMDMs have elevated metalloproteinase activity in conditioned media.

Area under the curve analysis of OmniMMP data for WT and \textit{Timp3}^{−/−} BMDM CM from 8 to 48 h of measurement represents overall metalloproteinase activity in measured BMDM CM. Metalloproteinase activity was significantly decreased in M1-polarized WT BMDMs vs. unpolarized (M0) WT BMDMs. Furthermore, significantly greater metalloproteinase activity was detected in M1-polarized \textit{Timp3}^{−/−} BMDMs vs. M1-polarized WT BMDMs (mean ± SEM, N = 4, * indicates P < 0.05 vs WT M0, # indicates P < 0.05 vs WT M1-polarized, according to two-way ANOVA, with post-hoc Bonferroni correction).
metalloproteinase activity levels in conditioned media samples when compared to their WT unpolarized counterparts (Figure 3-4). M1-polarized Timp3<sup>−/−</sup> BMDMs, however, have significantly elevated metalloproteinase activity compared to M1-polarized WT BMDMs (Figure 3-4). The 46 h measurement marked peak metalloproteinase activity within the 48 h time course. Treatment of M1-polarized WT BMDMs with rTIMP3 did not appear to affect peak metalloproteinase activity. Supplemental rTIMP3 within culture media, however, did significantly reduce metalloproteinase activity from M1-polarized Timp3<sup>−/−</sup> BMDMs (Figure 3-5). Furthermore, following treatment with rTIMP3, no differences in metalloproteinase activity were detected between M1-polarized Timp3<sup>−/−</sup> and WT BMDMs (Figure 3-5).

### 3.2 BMDM apoptosis

Previous studies suggested that TIMP3 appears to have a role in regulating apoptosis [80]. Thus, our next focus in testing our hypothesis was to test the ability of Timp3<sup>−/−</sup> macrophages to undergo apoptosis. Importantly, we utilized three complimentary methods to detect apoptosis: 1) Caspase activation (FLICA staining); 2) DNA fragmentation (TUNEL staining); and 3) loss of membrane polarity (Annexin V staining). These methods were used together to confirm that we were specifically assessing macrophage apoptosis as it has previously been shown that use of individual markers of apoptosis, such as caspase activation, yields false positives in some cell types (i.e. macrophages) [87].
Figure 3-5: Recombinant TIMP3 treatment reduces peak metalloproteinase activity in M1-polarized $\text{Timp3}^{-/-}$ BMDMs.

M1-polarized $\text{Timp3}^{-/-}$ BMDMs in the presence of rTIMP3 have significantly reduced peak metalloproteinase activity (at the 46 h time point) compared with untreated $\text{Timp3}^{-/-}$ BMDMs. rTIMP3 treated $\text{Timp3}^{-/-}$ BMDMs are not significantly different when compared to WT M1-polarized BMDMs (mean ± SEM, N = 4, # indicates P < 0.05 vs $\text{Timp3}^{-/-}$ PBS treated cells, according to two-way ANOVA with post-hoc Bonferroni correction).
3.2.1 Effect of sFASL treatment on caspase activity in unpolarized BMDMs

As sFASL is a known apoptotic stimulus for macrophages, baseline levels of unpolarized (M0) BMDM apoptosis were first determined [65]. Unpolarized BMDMs treated with sFASL for 2, 4 or 8 h showed no significant changes in caspase activity (Figure 3-6). In fact, caspase activity levels decreased throughout the 8 h stimulation time course (Figure 3-6). Baseline FLICA measurements revealed that 1 – 2 % of all unpolarised BMDMs have active caspases regardless of the presence of pro-apoptotic stimulation. Thus, as this data suggests that unpolarized BMDMs resist sFASL-induced apoptosis, further apoptosis studies on unpolarized cells were not conducted.

3.2.2 M1-polarized WT BMDMs show induction of caspase activity following sFASL stimulation

WT M1-polarized BMDMs were stimulated with either PBS or sFASL for 2, 4 or 8 h and stained with FLICA to measure intracellular caspase activity in order to measure sFASL induced apoptosis. The baseline readings at the 2 h mark showed a relatively high baseline of caspase activity in the M1-polarized BMDM with no significant differences at 2 h between PBS and sFASL treated WT BMDMs (Figure 3-7). At the 4 h time point, baseline PBS caspase activity diminished toward levels similar to those seen in the unpolarized WT BMDMs (Figure 3-6). Furthermore, a significant increase in caspase activity was detected at the 4 h time point in WT BMDMs treated with sFASL (Figure 3-7). By 8 h, caspase activity in PBS treated M1-polarized WT BMDMs decreased to very low levels, though a similar length stimulation of these cells with sFASL did significantly increase the level of caspase activity (Figure 3-7). Thus, WT M1-polarized BMDMs
Stimulation of WT BMDMs with sFASL did not yield any notable increase in caspase activity in unpolarized BMDMs. Measurements were taken at 2, 4 and 8 h of sFASL stimulation, and no caspase induction occurred at any point along the time course. By the end of the 8 h stimulation, levels of caspase activation had diminished beyond the 2 and 4 h measurements (mean ± SEM, N = 5-8, P = ns, according to t-test).
Measurements of FLICA from 2 - 8 h in WT M1-polarized BMDMs show significant induction of caspase activity at 4 and 8 h time points with sFASL treatment. The amount of caspase activity diminished during the 8 h time course, at the end of which the signal was minimal (mean ± SEM, N = 5-8, * indicates P < 0.05 vs PBS treatment, according to t-test). (B) Representative images at the 4 h time point FLICA stained WT M1-polarized BMDMs show elevated caspase activity in sFASL treated M1-polarized BMDMs.
demonstrated a response to sFASL by increasing caspase activation, though this activity was only apparent at the 4 and 8 h time points. Baseline caspase activity measurements were not significantly different between WT and Timp3−/− BMDMs at 2, 4 or 8 h following treatment with PBS (Figure 3-8). For these studies, results were normalized to WT BMDMs for each experiment to display absolute changes between genotypes. As previously observed in WT BMDMs at baseline (Figure 3-7), the percentage of positive cells decreased in both genotypes over the course of the 8 h PBS stimulation.

3.2.3 Timp3−/− BMDMs have suppressed sFASL induced caspase activation compared to WT BMDMs

Stimulation with sFASL was previously shown to induce an increase in M1-polarized WT BMDM caspase activity at the 4 and 8 h time points (Figure 3-7). Repeating those previous experiments with M1-polarized Timp3−/− BMDMs revealed a marked attenuation in the sFASL-induced caspase activity in M1-polarized Timp3−/− BMDMs compared to WT BMDMs (Figure 3-9). At 2 h, there is no significant difference detected between the WT and Timp3−/− BMDMs (Figure 3-9). However, the difference between WT and Timp3−/− BMDMs was significant at the 4 h time point (Figure 3-9). By 8 h post-stimulation, absolute caspase activity levels had decreased in both WT and Timp3−/− BMDMs with an equivalent level of caspase activity between the two genotypes. Overall these results support Timp3−/− BMDMs having decreased caspase activity in response to sFASL compared to WT BMDMs.
Figure 3-8: Baseline $Timp3^{-/-}$ M1-polarized BMDM caspase activity is not significantly different from WT BMDMs.

FLICA assay measurements of M1-polarized WT and $Timp3^{-/-}$ BMDMs treated with PBS for 2, 4 and 8 h show no significant differences between the two genotypes. The baseline values depicted in this figure are normalized to M1-polarized WT PBS stimulated BMDMs for each experiment to account for day-to-day variability within samples (mean ± SEM, $N = 5-8$, $P = ns$, according to two-way ANOVA with post-hoc Bonferroni correction).
Figure 3-9: *Timp3<sup>−/−</sup>* BMDMs have attenuated sFASL induced caspase activation compared to WT BMDMs.

FLICA assay measurements on M1-polarized WT and *Timp3<sup>−/−</sup>* BMDMs stimulated with sFASL for 2, 4 and 8 h. A significant decrease in caspase activity was measured in *Timp3<sup>−/−</sup>* BMDMs compared to WT BMDMs with 4 h sFASL stimulation. Data is normalized to M1-polarized WT sFASL stimulated BMDMs for each experiment to account for day-to-day variability within samples (mean ± SEM, N = 5-8, * indicates P < 0.05 vs WT cells, according to t-test).
3.2.4 TUNEL positivity induced by sFASL in M1-polarized WT BMDMs

To further test that our caspase findings were reflective of altered apoptosis, DNA fragmentation in WT BMDMs was assessed following 2, 4, or 8 h of stimulation with PBS or sFASL by TUNEL staining. While the percentage of TUNEL positive cells significantly increased at 4 h post-stimulation with sFASL compared to PBS, no significant differences were detected at any of the other time points examined (Figure 3-10). *Timp3*^-/-^ BMDMs had similar baseline (PBS-treated) levels of TUNEL positivity compared to WT BMDMs with no significant differences observed between genotypes over the 8 h time course with PBS treatment (Figure 3-11). We next examined TUNEL staining in WT and *Timp3*^-/-^ BMDMs stimulated with sFASL to determine if there were differences in the response to sFASL between WT and *Timp3*^-/-^ BMDMs. After, stimulation of 2, 4 or 8 h with sFASL, no significant differences were detected between WT and *Timp3*^-/-^ BMDMs in the number of TUNEL positive cells (Figure 3-12). Data was again normalized to WT BMDMs to control for day-to-day variability in data collection.

3.2.5 sFASL stimulation induces less Annexin V positivity in *Timp3*^-/-^ BMDMs compared to WT BMDMs

To finalize our analysis of apoptosis, we next assessed the presence of phosphatidylserine on the cell membrane of BMDMs through the detection of Fluorescein isothiocyanate (FITC)-labelled Annexin V by flow cytometry. For these studies, M1-polarized WT BMDMs were stimulated with sFASL for either 1 or 2 h. No
Figure 3-10: sFASL induces increased TUNEL positivity in WT M1-polarized BMDMs.

WT M1-polarized BMDMs were stimulated with either PBS or sFASL for 2, 4 or 8 h. Following stimulation cells were stained with TUNEL reagent to highlight cells undergoing apoptosis. No significant differences were seen between sFASL and PBS stimulated cells at 2 or 8 h. There was a significant increase in WT BMDM TUNEL staining at 4 h following sFASL stimulation, when compared to PBS controls (mean ± SEM, N = 6, P < 0.05, according to t-test).
Figure 3-11: PBS-stimulated $\textit{Timp3}^{-/-}$ BMDM TUNEL positivity is not significantly different from WT BMDMs.

WT and $\textit{Timp3}^{-/-}$ BMDMs were treated with PBS over the course of 2, 4 and 8 h and subsequently stained with TUNEL reagent. There were no significant differences between WT and $\textit{Timp3}^{-/-}$ BMDMs in relation to TUNEL positive cells at any time point along the time course. Data is relative to the WT PBS stimulated BMDMs so as to account for any day-to-day variability in samples (mean ± SEM, N = 6, P = ns, according to t-test).
Figure 3-12: sFASL-stimulated *Timp3<sup>−/−</sup>* BMDM TUNEL positivity is not significantly different from WT BMDMs.

WT and *Timp3<sup>−/−</sup>* BMDMs were stimulated with sFASL over the course of 2, 4 and 8 h and subsequently stained with TUNEL reagent. There were no significant differences between WT and *Timp3<sup>−/−</sup>* BMDMs in relation to TUNEL positive cells at any time point along the time course. Data is relative to the WT sFASL stimulated BMDMs so as to account for any day-to-day variability in samples (mean ± SEM, N = 6, P = ns, according to t-test).
significant differences in Annexin V positivity were detected between PBS and sFASL stimulated BMDMs after 1h of stimulation (Figure 3-13). However, a significant increase in the proportion of cells staining positive for Annexin V was observed following sFASL stimulation for 2 h in WT BMDMs (Figure 3-13). Thus, based on this data, the 2 h time point was utilized in subsequent analyses. Similar to our analysis of caspase activity and DNA fragmentation, no significant differences were detected in Annexin V staining between WT and Timp3−/− BMDMs following 2 h of stimulation with PBS (Figure 3-14). This suggests that baseline apoptosis is similar between WT and Timp3−/− BMDMs. Analysis of Annexin V staining revealed that following treatment with sFASL for 2 h, however, significantly fewer Timp3−/− BMDMs were Annexin V positive compared to WT BMDMs (Figure 3-15). Data is represented as a fold change from WT sFASL treated cells so as to account for variability from day-to-day measurements. Furthermore, 14% of WT BMDMs were Annexin V positive following sFASL treatment and 8.4% of the population was non-viable based on propidium iodide staining. Timp3−/− BMDMs has significantly less Annexin V positivity (11.6%) with only 6.3% of the population being non-viable.

### 3.3 Measurements of BMDM FAS

Following apoptotic assessment of BMDMs, subsequent experiments aimed to elucidate how this attenuated response was mediated. For these studies, FAS levels, both the gene expression as well as protein abundance, were analyzed to determine if there were any baseline differences in FAS between WT and Timp3−/− BMDMs. Second, a
Figure 3-13: M1-polarized WT BMDMs show increased Annexin V fluorescence following sFASL stimulation.

Data depicts flow cytometric analysis of WT M1-polarized BMDMs stained with FITC-Annexin V and PI after 1 and 2 h stimulations with either PBS or sFASL. The data supports a significant increase in the level of Annexin V+/PI- cells after 2 h of sFASL stimulation in M1-polarized WT BMDMs. The data at the 1 h time point is consistent with this data, though no significant differences were observed (mean ± SEM, N = 3, 10; * indicates P < 0.05 vs PBS treatment, according to t-test).
Figure 3-14: PBS stimulation of WT and *Timp3*<sup>−/−</sup> BMDMs yields no significant difference in Annexin V positivity.

Data depicts flow cytometric analysis of WT and *Timp3*<sup>−/−</sup> M1-polarized BMDMs (Annexin V<sup>+</sup>/PI) after 2 h of PBS stimulation. No significant differences were found (mean ± SEM, N = 10; P = ns, according to t-test).
Figure 3-15: M1-polarized $Timp3^{-/-}$ BMDMs have less Annexin V positivity following sFASL stimulation.

Data depicts flow cytometric analysis for proportion of Annexin V$^+$/PI$^-$ WT and $Timp3^{-/-}$ M1-polarized BMDMs following 2 h of sFASL stimulation. Compared to WT BMDMs, there is a significant reduction in the percentage of Annexin V positive $Timp3^{-/-}$ BMDMs (mean $\pm$ SEM, N = 10; P < 0.05, according to t-test).
rescue study utilizing rTIMP3 to enhance metalloproteinase inhibition and increase the response to sFASL in \emph{Timp3}^{-/-} BMDMs was performed.

### 3.3.1 Assessing \textit{Fas} mRNA levels and FAS protein abundance in unpolarized and M1-polarized WT BMDMs

Microarray data of WT unpolarized (M0) and M1-polarized BMDMs from a previous study was mined to examine steady state levels of mRNA encoding \textit{Fas} \cite{72}. Analysis of the microarray data revealed that \textit{Fas} expression in M1-polarized WT BMDMs was significantly elevated compared to expression in unpolarized cells (Figure 3-16). Lower expression of Fas in the unpolarized BMDMs is consistent with our earlier data demonstrating that caspase activity was unchanged in unpolarized BMDMs in response to sFASL stimulation (Figure 3-6). Western blotting with antibodies against FAS was used to assess FAS abundance in the cell lysate from unpolarized and M1-polarized WT BMDMs with no significant differences detected (Figure 3-17). Data are represented as the relative abundance of FAS (normalized to GAPDH) as a percentage of the WT unpolarized BMDM FAS levels.

### 3.3.2 Measuring \textit{Fas} mRNA levels and FAS protein abundance differences in WT and \textit{Timp3}^{-/-} BMDMs

Comparisons of steady state mRNA levels as measured by microarray analysis of WT and \textit{Timp3}^{-/-} M1-polarized BMDMs was further mined to determine the steady state levels of \textit{Fas} mRNA \cite{72}. Expression of \textit{Fas} by M1-polarized \textit{Timp3}^{-/-} BMDMs was not significantly different from the expression observed in M1-polarized WT BMDMs (Figure 3-18). Western blot analysis for levels of FAS in cell lysate from M1-polarized
**Figure 3-16: WT M1-polarized BMDMs have increased Fas expression.**

These data were mined from previous microarray analysis of unpolarized (M0) and M1-polarized BMDMs [72]. Data is expressed as fold change in Fas expression from WT unpolarized BMDMs. Levels of Fas mRNA are significantly elevated in M1-polarized BMDMs as compared unpolarized BMDMs (mean ± SEM, N = 4; *** indicates P < 0.001, according to t-test).
Figure 3-17: FAS protein abundance is not significantly different between WT unpolarized and M1-polarized BMDMs.

This figure depicts western blot data for FAS protein abundance in unpolarized and M1-polarized BMDMs. (A) No significant differences are observed between unpolarized and M1-polarized WT BMDMs in terms of FAS protein levels in lysates. FAS abundance was normalized to GAPDH for each lane. (mean ± SEM, N = 4; P = ns, according to t-test). (B) Representative image of the western blot data.
Figure 3-18: Steady state *Fas* mRNA levels are not significantly different between WT and *Timp3*–/– M1-polarized BMDMs.

This data mined from microarray analysis of WT and *Timp3*–/– M1-polarized BMDMs represents the level of steady state *Fas* mRNA. Data is expressed as *Fas* expression relative to WT M1-polarized BMDMs. Levels of *Fas* mRNA are not significantly different between genotypes (mean ± SEM, N = 4; P = ns, according to t-test).
WT and $Timp3^{-/-}$ BMDMs revealed no significant differences between WT and $Timp3^{-/-}$ M1-polarized FAS protein levels densitometric analysis (Figure 3-19). Data are represented as a relative abundance of FAS (after normalization to GAPDH) as a percentage of the WT M1-polarized lysate FAS levels.

### 3.3.3 rTIMP3 rescues reduced caspase activity in M1-polarized $Timp3^{-/-}$ BMDMs following sFASL stimulation

WT and $Timp3^{-/-}$ BMDMs were treated with rTIMP3 to determine if the loss of TIMP3 was responsible for reduced sFASL-induced apoptosis in $Timp3^{-/-}$ BMDMs. BMDMs were stimulated with sFASL or PBS over the course of 2 h in the presence of rTIMP3. Control treated $Timp3^{-/-}$ BMDMs stimulated with sFASL had significantly lower caspase activity compared to sFASL-stimulated WT BMDMs (Figure 3-20). Caspase activity in $Timp3^{-/-}$ BMDMs treated with rTIMP3, however, was not significantly different compared to similarly treated WT BMDMs (Figure 3-20).
M1-polarized WT and Timp3\(^{-/-}\) BMDM cell lysates probed for FAS protein content. (A) Analysis of western blots for FAS protein content (normalized to GAPDH) in BMDM lysate shows no significant differences between genotypes (mean ± SEM, N = 6, 5; P = 0.0854, t-test). (B) Representative image of the western blot data.

Figure 3-19: There are no significant differences between WT and Timp3\(^{-/-}\) M1-polarized BMDM FAS protein levels.
Figure 3-20: rTIMP3 rescues depressed caspase activity in M1-polarized \textit{Timp3}\textsuperscript{+/−} BMDMs following sFASL stimulation.

Data depicting caspase activity following treatment with rTIMP3 or DMSO control, coupled to 2 h stimulation with either PBS or sFASL. Treatment with rTIMP3 during the 2 h sFASL stimulation abolishes the difference between WT and \textit{Timp3}\textsuperscript{+/−} BMDM caspase activity levels. \textit{Timp3}\textsuperscript{+/−} M1-polarized BMDMs have significantly increased caspase activity levels following rTIMP3 and sFASL treatments when compared to all other \textit{Timp3}\textsuperscript{+/−} BMDM treatment groups (P < 0.05, N = 6). rTIMP3 treated \textit{Timp3}\textsuperscript{+/−} M1-polarized BMDMs stimulated with sFASL are not significantly different from the similarly treated WT BMDMs (mean ± SEM, N = 6, * indicates P < 0.05 vs. all other \textit{Timp3}\textsuperscript{+/−} treatments, ## indicates P < 0.01 vs. WT with same treatment, according to multiple 2-way ANOVA analyses with post-hoc Bonferroni tests).
Chapter 4. Discussion

4.1 Summary of findings

Macrophages have been found to both promote and resolve inflammation in ARDS; however, the mechanisms controlling the ability of macrophages to mediate these different functions remain to be determined [9]. The focus of our study was to examine a potential mechanism enabling TIMP3 to promote apoptosis of murine macrophages. The suggested model proposes that in the absence of TIMP3 decreased metalloproteinase inhibition will result in the stabilization of FAS on the cell surface, and ultimately promote apoptosis.

My first objective was to establish and characterize WT and Timp3\(^{-/-}\) BMDM cultures. Previous studies revealed that Timp3\(^{-/-}\) BMDMs express elevated levels of pro-inflammatory genes in response to LPS treatment compared to WT BMDMs [72]. Our initial studies established all of the techniques and cultures required to generate BMDMs. Furthermore, characterization of WT and Timp3\(^{-/-}\) BMDMs revealed similar differences in gene expression as previously observed [72]. Futhermore, we show for the first time that Timp3\(^{-/-}\) BMDMs have greater metalloproteinase activity compared to WT BMDMs, and the resultant increases in metalloproteinase activity were rescued by treatment with rTIMP3.

The second objective of my study was to identify the mechanism through which TIMP3 regulates macrophage apoptosis. Timp3\(^{-/-}\) BMDMs had reduced levels of
apoptosis in response to sFASL as assessed by FLICA (caspase activity) and Annexin V staining. Additionally, the resistance to sFASL-induced apoptosis in $Timp3^{-/-}$ BMDMs was rescued by treatment with rTIMP3. Lastly, assessment of FAS mRNA and protein levels revealed no significant differences between WT and $Timp3^{-/-}$ BMDMs.

I have shown that BMDMs lacking TIMP3 have increased expression of pro-inflammatorty genes following stimulation with LPS, and thereby could promote a more pro-inflammatory milieu within their local microenvironment (Figure 3-1 and 3-2). If these inflammatory macrophages lacking TIMP3, which promote inflammatory signals above similarly activated WT macrophages, were to resist pro-apoptotic signals they would persist, and possibly generate a chronic, non-resolving inflammation within the lung.

4.2 Contributions of research to current state of knowledge

4.2.1 Macrophage Polarization and Metalloproteinase Activity

The first objective of this thesis was to establish BMDM culture method and to confirm the role of TIMP3 in the regulation of macrophage polarization and metalloproteinase activity. BMDMs were utilized within this thesis as a substitute cell type for recruited alveolar macrophages. Use of BMDMs has one major advantage over alveolar macrophages; materials harvested from one animal can produce approximately 200 fold the number of macrophages that can be extracted from the lungs of a mouse. This greatly increased yield of macrophages enables the use of multiple concurrent techniques that require large numbers of cells, and allows those techniques to utilize cells
from the same animal. However, the major issue of utilizing BMDMs is the lack of physiological translation as to what a BMDM represents in a living system [88]. BMDMs are a cell culture generated macrophage, and as such direct comparisons to tissue macrophages, specifically alveolar macrophages, are difficult. Previous works from the Center for Lung Biology (University of Washington, Seattle, Washington) had demonstrated that \textit{Timp3}\textsuperscript{-/-} BMDMs had increased expression of genes associated with M1-polarization compared to WT BMDMs following LPS stimulation [72]. We were able to reproduce elevated \textit{IL12b} and \textit{Nos2} expression in \textit{Timp3}\textsuperscript{-/-} BMDMs and confirm the importance of macrophage-derived TIMP3 to pro-inflammatory gene expression.

Macrophages, have been found to differentially express multiple metalloproteinases and TIMPs depending on macrophage polarization [58]. Furthermore, metalloproteinases have been shown to govern macrophage recruitment and function suggesting that the control of their activity is essential during the acute phase of inflammation [61, 63]. Interestingly, the specific role of metalloproteinase activity in macrophage polarization has not been addressed. In order to characterize the role of metalloproteinases in macrophage polarization, as well as the role of macrophage-derived TIMP3 in the inhibition of metalloproteinases, WT and \textit{Timp3}\textsuperscript{-/-} BMDMs were assessed for metalloproteinase activity. Total metalloproteinase activity was significantly decreased in the conditioned media from M1-polarized WT BMDMs compared to unpolarized WT BMDMs (\textbf{Figure 3-4}). Furthermore, M1-polarized \textit{Timp3}\textsuperscript{-/-} macrophages were found to have significantly increased metalloproteinase activity vs. M1-polarized WT BMDMs, which was rescued by the addition of rTIMP3 (\textbf{Figure 3-4})
and 3-5). Previous work found that *Timp3* expression increases when BMDMs are M1-polarized, compared to unpolarized, suggesting a role for TIMP3 in the M1-polarized macrophage [72]. My data supports this finding by highlighting the importance of macrophage-derived TIMP3 as an inhibitor for metalloproteinases in the M1-polarized BMDM.

While TIMP3 is primarily thought to function as an inhibitor of metalloproteinases, it has been shown to have metalloproteinase independent functions. For example, TIMP3 has been shown to inhibit angiogenesis by binding to vascular endothelial growth factor receptor (VEGFR) and blocking VEGF/VEGFR2 interaction [89, 90]. Macrophage-derived TIMP3 has been previously found to regulate macrophage polarization; however, the mechanism through which TIMP3 regulates this process is unknown [72]. With our data suggesting that *Timp3*−/− BMDMs have increased metalloproteinase activity and that treatment with rTIMP3 rescues this effect, we can conclude that TIMP3 likely regulates macrophage polarization through the inhibition of metalloproteinase activity, and that TIMP3 reduces extracellular metalloproteinase activity in BMDMs.

4.2.2 Macrophage Apoptosis

Pro-inflammatory macrophages that are resistant to apoptotic stimuli could be responsible for persistent inflammation following ARDS [88]. Regarding BMDMs, the unpolarized BMDM most closely represents *in vivo* naive tissue-macrophages within their target tissue; this is based on their differentiation into macrophages following M-
CSF stimulation coupled the lack of polarizing stimulus present in their surrounding 
environment [91]. Hence, we initially examined apoptosis within unpolarized BMDMs to 
determine the baseline levels of responsiveness to apoptotic stimuli within macrophages. 
FLICA assessment showed no differences in caspase activity levels between unpolarized 
BMDMs stimulated with sFASL or PBS, which suggests that unpolarized macrophages 
are resistant to sFASL-mediated apoptosis. The finding that naïve macrophages do not 
respond to sFASL suggests they resist apoptotic clearance from the site of injury, 
allowing first for their polarization in order to carry out immunomodulatory effects. The 
ability for unpolarized macrophages to resist apoptosis would aid in the pro-inflammatory 
or anti-inflammatory process, as dictated by the local tissue microenvironment, and the 
various signaling molecules there-in [29].

Macrophages, specifically M1-polarized macrophages, are one of the primary 
mediators of inflammation during ARDS [17]. The method of macrophage clearance 
from the inflamed tissue (e.g. apoptosis and phagocytosis vs. migration to lymph nodes), 
however, remains the focus of some debate [92, 93]. Recent work in a model of 
peritoneal inflammation, however, revealed that apoptosis and phagocytosis might be the 
primary means of macrophage clearance from inflamed tissues [19]. While this evidence 
is within peritoneal tissue, it still alludes to mechanisms that are carried out in vivo and 
may reflect the processes within the lungs. Our data suggests that M1-polarized BMDMs 
are susceptible to sFASL-induced apoptosis, which supports the concept that apoptosis 
may be a key mechanism mediating the specific clearance of pro-inflammatory 
macrophages. Furthermore, the susceptibility of M1-polarized (and not unpolarized)
macrophages to sFASL-induced apoptosis would allow for clearance of apoptotic pro-inflammatory macrophages, likely mediated through activation of death-receptors from external signaling molecules (i.e. sFASL), while at the same time allowing for protection of incoming naïve macrophages that could promote recovery from injury [19, 65, 72]. Resistance to sFASL-induced apoptosis (i.e. in the absence of TIMP3) would potentially restrict clearance of pro-inflammatory macrophages thereby limiting resolution of inflammation. Importantly, these findings support the works cited as rationale, which show hindered recovery of Timp3−/− lung inflammation by BAL fluid analysis showing greater inflammatory cell presence and decreased markers of inflammatory resolution vs. WT mice [72, 75].

Macrophage apoptosis is mediated by proteins such as TNFα and sFASL; however, resident and recruited macrophages appear to have different susceptibility to these proteins [92, 94–96]. Following bleomycin injury in the lung, resident macrophages have been found to undergo apoptosis in response to TNFα [94]. Alternatively, work by Janssen and colleagues found that specifically recruited macrophages possess high levels of Fas expression following lung injury, and that the majority of these recruited macrophages were cleared in vivo through apoptosis and phagocytic clearance [92]. Within the same study, use of FAS-blocking antibodies, which blocked FAS activation, resulted in decreased macrophage clearance following injury [92]. Our finding that sFASL stimulates apoptosis in M1-polarized macrophages and that Timp3−/− BMDMs are resistant to sFASL-induced apoptosis demonstrate that TIMP3 is a potential mediator of FAS-induced apoptosis in macrophages. Additionally, in Timp3−/− mouse lungs with
reduced clearance of macrophages based on sFASL resistance, the inherent greater M1-polarization in those macrophages could exacerbate inflammatory complications present within ARDS, thus further supporting the proposed model within this study.

Previous publications have shown that apoptosis can be mediated by TIMP3, specifically by the N-terminus of TIMP3 [80–82, 97]. For example, overexpression of TIMP3 in a number of cell types leads to increased apoptosis primarily through stabilization of the death receptor on the cell surface and increased receptor activation [80–82]. Interestingly, while studies have found that the ability of TIMP3 to promote apoptosis is dependent on the inhibition of metalloproteinase activity [81, 83, 98], a recent study found that overexpression of TIMP3 in endothelial cells promotes apoptosis through a metalloproteinase-independent mechanism [97]. In this study, we have shown that in the absence of TIMP3, macrophages are resistant to sFASL-induced apoptosis and this resistance is associated with increased metalloproteinase activity, which provides strong support for previous studies on TIMP3 regulation of apoptosis. Furthermore, we found that treatment of $\text{Timp}3^{-/-}$ BMDMs with rTIMP3, which decreased metalloproteinase activity, rescued the observed resistance to apoptosis. Therefore, from our data we conclude that TIMP3 mediates BMDM apoptosis through inhibition of metalloproteinase activity.

4.2.3 Regulation of FAS-sFASL Mediated Apoptosis

FAS interaction with sFASL leads to trimerization of the receptor-ligand complex, recruitment FADD, activation of caspase 8 and ultimately, apoptosis (Figure 1-
1) [37–39]. The final goal of these studies was to elucidate a possible mechanism regulating M1-polarized macrophage resistance to sFASL mediated apoptosis. These experiments were based on the original proposed mechanistic model, in which excessive metalloproteinase activity leads to excessive processing of FAS and subsequently, a loss of FAS activation (Figure 1-2). Our finding that Timp3−/− BMDMs are resistant to apoptosis and that this resistance is associated with increased metalloproteinase activity, as well as decreased caspase activity, which is downstream of FAS activation, supports our proposed model (Figures 3-4 and 3-9). However, our specific analyses of FAS levels within the unpolarized and M1-polarized WT and Timp3−/− BMDMs examining alterations to FAS levels did not support our hypothesis.

The ability of FAS to induce macrophage apoptosis appears to be limited to specific subsets of macrophages. In a model of LPS-induced lung infection, recruited macrophages, and not resident macrophages, were found to express high levels of FAS and undergo apoptosis in response to FAS-activating antibodies [92]. Additionally, a study by Park and colleagues showed in vitro analysis of human monocyte and macrophage FAS mediated apoptosis and discovered that monocytes but not macrophages underwent apoptosis when stimulated with FAS [99]. Importantly, the previous study utilized non-polarized monocyte-derived macrophages (i.e. unpolarized macrophages) [99]. Our data that Fas expression is increased in M1-polarized murine BMDMs and that only M1-polarized BMDMs appear to be susceptible to FAS-induced apoptosis supports these previous studies.
TIMP3 has previously been found to stabilize FAS on the cell surface, thereby promoting apoptosis [67, 81]. Furthermore, ADAM17, which is inhibited primarily by TIMP3, is known to cleave multiple members of the TNFR superfamily, including FAS [50, 100]. We found that in the absence of TIMP3, M1-polarized BMDMs were resistant to undergoing apoptosis in response to sFASL. Comparison of Fas expression between WT and Timp3−/− BMDMs revealed similar levels of expression between genotypes (Figure 3-18) suggesting that the differential response to sFASL was likely due to translational or post-translational mechanisms (i.e. shedding). Though protein levels of FAS within Timp3−/− BMDMs were decreased compared to WT BMDMs, these differences did not reach significance (P = 0.0854; Figure 3-19). The lack of a significant decrease in FAS suggests that in macrophages, the ability of TIMP3 to regulate FAS-induced apoptosis is not due to inhibition of FAS shedding. However, it is important to note that apoptosis in response to sFASL/FAS interaction requires trimerization of the ligand/receptor complexes. MMP7 has been found to cleave FAS at the N-terminus releasing a short peptide (<10kDa) and loss of this peptide impairs trimerization leading to decreased activation [101]. Furthermore, MMP7 activity has been found to be increased in mice lacking TIMP3 [77]. Hence, it is plausible that increased MMP7 activity in the absence of TIMP3 may lead to altered processing of FAS and decreased FAS activation in the absence of significantly altered FAS levels on a western blot.

Alternatively, alterations to sFASL may have mediated some of the observed survival effects present in the Timp3−/− BMDMs. In addition to FAS processing, MMP7 has also been shown to cleave sFASL, which is associated with increased cell survival [102].
Thus, increased metalloproteinase activity in the conditioned media of $Timp3^{-/-}$ BMDMs may have resulted in increased cleavage of the sFASL and the generation of altered sFASL that is ineffective in binding or signaling FAS, which would further restrict apoptosis. This could also account for $Timp3^{-/-}$ BMDMs being resistant to FAS-induced apoptosis in the absence of detectable differences in overall FAS abundance; however, this possibility was not examined in the current study.

4.3 Limitations and future directions

4.3.1 Study limitations

In order to assess BMDM apoptosis three separate techniques were utilized. Technical difficulties arose within each method and the final conclusions drawn were not supported by TUNEL results. TUNEL, which by definition labels nick ends of the DNA during the fragmentation process, has also been shown to be non-specific for apoptosis as it can also stain nuclei in cells undergoing necrotic and autolytic forms of death [103]. Furthermore, TUNEL is a late stage marker of apoptosis, at which point many cell surface associated efferocytosis targets will be present on the apoptotic cells promoting its clearance by neighboring phagocytic cells [32]. This potential clearance of neighboring apoptotic macrophages may have accounted for why we did not observe a strong induction of TUNEL staining in response to sFASL. Therefore, TUNEL staining within a homogeneous population of macrophages may not be the best indicator of
apoptosis. Additionally, TUNEL staining protocols required us to grow BMDMs on glass coverslips. This meant altering culture conditions by growing our BMDMs on untreated glass. Analysis of the numbers of BMDMs in a given field of view when cultured on glass compared with tissue culture plastic suggested that culture on glass resulted in decreased BMDMs per field of view.

FLICA analysis utilizes a fluorescent marker that binds to active intracellular caspases in order to highlight cells with internal caspase activity. Caspases, though generally associated with apoptosis, do not solely function as apoptotic markers, and thus not all cells positive for caspase activity are apoptotic [104]. Caspases have been known to become active for multiple different cellular processes including: cell proliferation [105], differentiation [106], and cell cycle regulation [107]. With this in mind FLICA positive cells are not assuredly undergoing apoptotic processes, and thus further analyses were conducted to reinforce the results.

The last maker of apoptosis was assessment of Annexin V/PI staining by flow cytometry. The majority of difficulties in this technique were associated with obtaining cells following stimulation. Following M1-polarization BMDMs adhere further to plastic culture plates, methods requiring mechanical lifting of cells are known to cause disruption of cell membranes that result in false positives for apoptosis [108]. Thus in order to safely lift the cells 37°C 0.5 mM EDTA/PBS was utilized. The lifting of the cells, coupled to the need for live and unfixed cells in annexin V staining may have altered outcomes compared to the other two fixed, and still plated, methods of apoptotic measurements.
Finally, western blotting for FAS utilizing cell lysate samples was not specific to shed FAS, or to cell-surface FAS. To remedy this shortcoming we attempted preliminary studies probing conditioned media samples for FAS, however analysis revealed non-specific smears over the region of the expected bands. A more direct technique to measure surface FAS such as flow cytometry could be used in the future to test this possibility.

4.3.2 Future directions

Inflammation following ARDS is a difficult topic, as there are many factors in involved in the induction and resolution of this complex syndrome. Our data, which found that TIMP3 potentially mediates clearance of pro-inflammatory macrophages, focused on only one possible mechanism involved in prolonging the inflammatory environment within the lungs following injury. One shortcoming of this study was the sole utilization of sFASL. Future studies should include other TNF superfamily ligands, such as TNFα in order to fully understand how apoptotic signaling is altered in a variety of pathways. Coupled to these studies, the identification of specific metalloproteinases actively involved in shedding events could be ascertained with an immunoprecipitation assay targeting TIMP3 and ADAM17 or even MMP7 in WT BMDMs to determine if TIMP3 is regulating ADAM17 or MMP7 activity and thereby, restricting shedding or cleavage of the proposed targets.

With the completion of these preliminary studies on BMDMs, future key experiments can be conducted on alveolar macrophages to better understand whether our
proposed model of macrophage persistence represents the \textit{in vivo} situation accurately. In order to support this concept, \textit{in vivo} experiments are needed. These studies would involve use of a model of ARDS in WT and \textit{Timp3}^{-/-} mice (i.e. intratracheal instillation of LPS), and subsequently treating the mice with rTIMP3 to rescue the impaired resolution of lung inflammation previously observed in \textit{Timp3}^{-/-} mice [75]. Macrophages could then be isolated from the lungs at different timepoints following injury and could be examined by flow cytometry for markers of apoptosis. Additional markers could be used to examine macrophage polarization \textit{in vivo}.

4.4 Summary and conclusions

We have shown support for a model that proposes TIMP3 regulating inflammatory cell apoptosis in order to control the level of inflammation present at any given time along tissue repair processes. In the absence of \textit{Timp3}, M1-polarized macrophages have increased metalloproteinase activity, are resistant to typical apoptotic stimuli promoting their longevity, and maintain greater pro-inflammatory gene expression. Importantly, we have rescued each of these phenotypes by rTIMP3 treatment, suggesting that rTIMP3 may be a potential therapeutic treatment. These findings further suggest persistent inflammation following an initial injury within the lung may be due to lack of clearance (via apoptosis) of pro-inflammatory macrophages. These findings also suggest that a cell specific target (i.e. macrophage specific) could alleviate the persistent inflammation in order to aid patients and allow for a more rapid resolution of inflammation following ALI.
Major in vivo studies, however, must be conducted before conclusions can be drawn regarding the application of findings within this thesis to the in vivo setting. In vitro experiments are much less complex as they utilize a highly selected population of cells, standardized culture environment, and the ability to directly visualize the cells of interest. The current study, however, has begun to provide a greater understanding of a complex problem (i.e. persistent inflammation following ARDS). It is possible that rTIMP3 could aid in the resolution of inflammation with ARDS; though it’s clear that more research is needed before definite in vivo and human conclusions can be drawn.
Chapter 5. References


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Curriculum Vitae

Name: Michael Brock

Post-secondary Education and Degrees:
The University of Western Ontario, London, Ontario, Canada
2008 - 2012 BMSc

Honors and Awards:
Graduated with Distinction
Dean’s Honor List (2008-2012)
Sexual Medicine Society of North America - Summer research Grant (2008 and 2009)

Related Work Experience:
Teaching Assistant
The University of Western Ontario 2012-2014

London health research day
Poster presentation 2013

London health research day
Platform presentation 2014

Physiology and Pharmacology research day
Poster presentation 2013 and 2014