2010-03-17

Activation of Non-Muscle Myosin IIB Helps Mediate TNF-Alpha Cell Death Signaling

Patrick G. Flynn
University of Miami, raldynn@hotmail.com

Follow this and additional works at: https://scholarlyrepository.miami.edu/oa_dissertations

Recommended Citation
https://scholarlyrepository.miami.edu/oa_dissertations/369

This Open access is brought to you for free and open access by the Electronic Theses and Dissertations at Scholarly Repository. It has been accepted for inclusion in Open Access Dissertations by an authorized administrator of Scholarly Repository. For more information, please contact repository.library@miami.edu.
UNIVERSITY OF MIAMI

ACTIVATION OF NON-MUSCLE MYOSIN IIB HELPS MEDIATE TNF-ALPHA CELL DEATH SIGNALING

By
Patrick G. Flynn

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida
May 2010
A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

ACTIVATION OF NON-MUSCLE MYOSIN IIB HELPS MEDIATE TNF-ALPHA
CELL DEATH SIGNALING

Patrick G. Flynn

Approved:

Kermit L. Carraway, Ph.D.
Professor of Cell Biology
and Anatomy

Terri A. Scandura, Ph.D.
Dean of the Graduate School

David M. Helfman, Ph.D.
Professor of Cell Biology
and Anatomy

Theodore J. Lampidis, Ph.D.
Professor of Cell Biology
and Anatomy

Sean P. Scully, M.D., Ph.D.
Professor of Orthopaedics
TNF-alpha can stimulate a variety of kinases with the ability to activate non-muscle myosin II. As a result, increases in actin filament formation and actomyosin contractility (AMC) have been reported in response to TNF-alpha. These events are thought to play an important role in mediating TNF-alpha induced apoptosis but how they do so is unclear. In this study we prevented non-muscle myosin II activation in response to TNF-alpha by treating cells with the myosin light chain kinase (MLCK) inhibitor ML-7 or through isoform specific siRNA knockdown of myosin IIA and IIB. We found that treatment with ML-7 or knockdown of myosin IIB, but not IIA, impaired the cleavage of caspase 3 and caspase 8 as well as nuclear condensation in response to TNF-alpha. During this cell death process myosin II seemed to function independent of AMC since treatment of cells with blebbistatin or cytochalasin D failed to inhibit TNF-alpha induced caspase cleavage. Immunoprecipitation studies revealed associations of myosin IIB with clathrin and FADD in response to TNF-alpha suggesting a role for myosin IIB in TNFR1 endocytosis and DISC formation. Taken together these findings suggest that myosin IIB activation promotes TNF-alpha cell death signaling in a manner independent of its force generating property.
Dedication

This work is dedicated to my parents, Michael and Teresa, their constant love and support has given me every opportunity in the world.

To my siblings and best friends; Tara, Kevin, Thomas and Terence.

To my aunt Mairead for being a source of inspiration.
Acknowledgements

I would first like to thank my mentor, David M. Helfman, for many years of patience and intellectual freedom. He brought a very positive attitude to the lab on a daily basis and created a wonderful environment to learn science. Thank you, David, for educating me as a scientist and instilling confidence in me to pursue my future goals.

I would like to thank my committee member, Dr. Sean P. Scully, for his support and encouragement. Despite an extremely busy schedule he was always able to make time for me. He played a crucial role in helping me to finish my Ph.D. and set an example for me in which I wish to follow.

I would like to thank my committee member, Dr. Kermit L. Carraway, for thoughtful insight and discussion. I would also like to thank him for his support throughout the years and assistance with helping me to further my career.

I would like to thank my committee member, Dr. Theodore J. Lampidis, for thoughtful insights and spirited discussions. I would like to thank Dr. Lampidis for his contagious passion for science as well as his encouragement and guidance throughout the years.

I would like to thank a former committee member, Dr. Lawerence Boise, for thoughtful insight and discussion that was extremely helpful to the development of my project. The students at Emory are lucky to have him.
I would like to thank the former members of the Helfman lab--Ali Saeed, Protiti Khan, and Fei Liu--for all their assistance, encouragement, support, and friendship which helped make these years at Miami great.

Last but not least I would like to especially thank Gregory Bigford, Gizelka David-West, Johnathan Maher, Sabrina Joseph, Indu Persaud, George Theodore, and all the other wonderful friends and colleagues I have met that have made these years at Miami some of the best years of my life.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures.............................................................................................viii</td>
</tr>
<tr>
<td>List of Tables..................................................................................................x</td>
</tr>
<tr>
<td><strong>Chapter I: Introduction</strong>...............................................................................1</td>
</tr>
<tr>
<td>TNF-alpha discovery...........................................................................................1</td>
</tr>
<tr>
<td>TNF-alpha signaling: physiological and pathological roles............................1</td>
</tr>
<tr>
<td>Involvement of the actin cytoskeleton in TNF-alpha signaling..........................4</td>
</tr>
<tr>
<td>TNF-alpha signaling.........................................................................................5</td>
</tr>
<tr>
<td>TNF-alpha involvement in the immune response, inflammation, and autoimmune</td>
</tr>
<tr>
<td>diseases...........................................................................................................12</td>
</tr>
<tr>
<td>Inflammation and Cancer..................................................................................16</td>
</tr>
<tr>
<td>Actin cytoskeleton involvement in inflammation.............................................19</td>
</tr>
<tr>
<td>TNF-alpha activated kinases involved in regulating myosin II and actin</td>
</tr>
<tr>
<td>cytoskeleton function....................................................................................20</td>
</tr>
<tr>
<td>Non-muscle myosin II: structure, regulation, and function..............................24</td>
</tr>
<tr>
<td>Isoform Specific Functions of Non-muscle myosin II......................................30</td>
</tr>
<tr>
<td>Non-muscle myosin II mutations and disease..................................................32</td>
</tr>
<tr>
<td>Summary.............................................................................................................35</td>
</tr>
<tr>
<td><strong>Chapter II: Materials and Methods</strong>..........................................................39</td>
</tr>
<tr>
<td>Cell Culture.......................................................................................................39</td>
</tr>
<tr>
<td>Antibodies and Reagents..................................................................................39</td>
</tr>
<tr>
<td>siRNA................................................................................................................40</td>
</tr>
<tr>
<td>Immunofluorescent Staining..............................................................................40</td>
</tr>
<tr>
<td>Apoptosis Assay...............................................................................................41</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Myosin II domain structure .................................................................25
Figure 2. Activation of non-muscle myosin II .....................................................27
Figure 3. Treatment with ML-7 impairs MLC phosphorylation in response to TNF-alpha .................................................................47
Figure 4. Treatment with ML-7 impairs cell death signaling in response to TNF-alpha .................................................................48
Figure 5. Inhibition of PKA or PKC does not effect TNF-alpha cell death signaling ....50
Figure 6. Treatment with ML-7 impairs TNF-alpha survival signaling ...............51
Figure 7. Knockdown of myosin IIB impairs cell death signaling in response to TNF-alpha .................................................................53
Figure 8. Knockdown of myosin IIB impairs nuclear condensation in response to TNF-alpha .................................................................54
Figure 9. Effects of blebbistatin and cytochalasin D treatment on actin cytoskeletal structure and myosin IIB localization .................................................................56
Figure 10. Treatment with blebbistatin does not impair TNF-alpha cell death signaling .................................................................57
Figure 11. Treatment with cytochalasin D does not impair TNF-alpha cell death signaling .................................................................58
Figure 12. Treatment with blebbistatin or cytochalasin D does not impair MLC phosphorylation in response to TNF-alpha .................................................................59
Figure 13. TNFR1 expression in Hela cells ..........................................................61
Figure 14. ML-7 treatment impairs TNFR1 internalization ..................................62
Figure 15. Knockdown of myosin IIB expression impairs TNFR1 internalization ....63
Figure 16. Treatment with blebbistatin does not impair TNFR1 internalization ....64
Figure 17. TNFR1 associates with clathrin in response to TNF-alpha ..................65
Figure 18. Myosin IIB associates with clathrin in response to TNF-alpha ............66
Figure 19. ML-7 treatment but not blebbistatin impairs myosin IIB association with clathrin in response to TNF-alpha………………………………………………………..67

Figure 20. Myosin IIB associates with pro-apoptotic signaling proteins FADD and caspsae 8 in response to TNF-alpha……………………………………………………...68

Figure 21. ML-7 impairs myosin IIB association with FADD………………………………69
LIST OF TABLES

Table 1. Isoform specific functions of non-muscle myosin II..........................31
Chapter I: Introduction

TNF-alpha discovery

In the late 19th century William B. Coley observed that tumors with bacterial infections underwent occasional regression. He concluded from these observations that there was a cytotoxic anti-tumor agent present in the immune response (Coley 1991). By 1968 lymphoid cell involvement in tumor immunity and allograft rejection had been well documented. Work based on this knowledge was done by Dr. Nancy H. Ruddle and Dr. Gale A. Granger which led to the identification of a toxic element released by lymphoid cells in response to target-cell-antigens that they named Lymphotoxin (LT) (Kolb and Granger 1968; Ruddle and Waksman 1968). Seven years later Dr. L.J. Old and Dr. B. Williamson of Memorial Sloan-Kettering Cancer Center transplanted mice with tumors and treated them with endotoxin to investigate the mechanism of “hemorrhagic necrosis”. Their studies revealed that treatment of these mice with endotoxin caused a release of a substance from macrophages that was selectively toxic for malignant cells and which they named tumor necrosis factor (TNF) (Carswell, Old et al. 1975). In 1984 the cDNA of TNF and LT were cloned and were determined to be very similar (Nedwin, Naylor et al. 1985). Their homology was confirmed by the ability of both to bind the TNF receptor and led to the renaming of TNF and LT to TNF-alpha and TNF-beta (Aggarwal 2003).

TNF-alpha signaling: physiological and pathological roles

Subsequent research has revealed a crucial role for TNF-alpha in the innate immune response. Macrophages that are activated by engulfing pathogens secrete large
amounts of TNF-alpha that are essential for the formation of granulomas to rid the body of infection (Lukacs, Chensue et al. 1994; Roach, Bean et al. 2002). Mice models deficient in TNF-alpha or its receptors cannot form granulomas in a timely or efficient manner in order to eliminate the pathogen and ultimately succumb to the infection (Bean, Roach et al. 1999; Allendoerfer and Deepe 2000; Roach, Bean et al. 2002). The interaction between TNF-alpha and its receptors activates the NFkappaB transcription factor which is crucial for inflammation and granuloma formation (Flynn, Goldstein et al. 1995; Hsu, Xiong et al. 1995; De Filippis, Russo et al. 2007). NFkappaB facilitates the immune response by promoting the expression of pro-inflammatory cytokines, adhesion molecules, and angiogenic promoters (Pahl 1999). However, these same signaling events and biological effects of TNF-alpha that help mount a beneficial immune response to pathogens can also support auto-immune diseases such as Chron’s disease and rheumatoid arthritis (RA). Development of TNF antagonists have proven to be very beneficial to treating the symptoms of RA and CD, however, the etiology of these diseases are still unknown (Tracey, Klareskog et al. 2008).

TNF-alpha induced chronic inflammation that gives rise to autoimmune diseases has also been linked to tumorigenesis. Chronic inflammation can be caused by foreign antigens that macrophages have difficulty breaking down such as asbestos particles that enter the lung (Yang, Bocchetta et al. 2006). The continual release of TNF-alpha from these activated macrophages can promote tumor growth by creating a microenvironment with increased levels of growth activating cytokines and vascular endothelial growth factor (VEGF). In addition to chronic inflammation, TNF-alpha autocrine/paracrine signaling has also been shown to promote tumorigenesis and metastasis of ovarian
surface epithelium (Kulbe, Thompson et al. 2007). Therefore, although TNF-alpha’s anti-tumor effects led to its discovery subsequent research has revealed that TNF-alpha also possesses strong tumor promoting properties.

As mentioned above TNF-alpha was originally discovered due to its detrimental effects on tumors, unfortunately, clinical trials determined that the systemic levels of TNF-alpha needed to treat human cancers were toxic to the patient (Spriggs, Sherman et al. 1988; Lejeune, Lienard et al. 1994). However, TNF-alpha has been shown to be effective in treating melanomas and sarcomas in the extremities through isolated limb perfusions (ILP) in combination with IFN-gamma and the chemotherapeutic drug melaphan (Lejeune, Lienard et al. 1994; Thom, Alexander et al. 1995). This combination of TNF-alpha with IFN-gamma and melaphan most likely suppress the pro-survival signaling of NFkappaB that increases anti-apoptotic proteins such as c-FLIP and inhibitors of apoptosis proteins (IAPs). In tumor cell environments in which Interfueron-gamma (IFN-gamma) is present crosstalk between IFN-gamma and TNF-alpha have been shown to induce apoptosis in different tumor cell types (Yamamoto, Zou et al. 1995; Wielockx, Lannoy et al. 2001; Zhong, Wang et al. 2009). For example, TNF-alpha/IFN-gamma treatment of human colon adenocarcinoma strongly activates NFkappaB to increase transcription of Fas leading to apoptosis (Kimura, Haisa et al. 2003). Alternatively, impairing NFkappa signaling can also lead to apoptosis as is the case in virally infected cells that compromise NFkappaB signaling (Schneider-Brachert, Tchikov et al. 2006).
Involvement of the actin cytoskeleton in TNF-alpha signaling

TNF-alpha is involved in diverse cellular processes such as permeability, inflammation, survival, metastasis, and apoptosis (Lukacs, Chensue et al. 1994; Lukacs, Strieter et al. 1995; Mark and Miller 1999; Kulbe, Thompson et al. 2007; Tracey, Klareskog et al. 2008). There are many variables that determine the biological effect of TNF-alpha such as cell type and the presence of other signaling molecules in the extracellular environment. Although many factors play a role in determining TNF-alpha’s biological effect the actin cytoskeleton seems to be one constant involved in helping to mediate many of the cell’s responses to TNF-alpha. TNF-alpha has been shown to activate a number of kinases such as Myosin light chain kinase (MLCK), Rho kinase, and Death associated protein kinase (DAPK) that share the ability to regulate actin dynamics through activation of non-muscle myosin II (Cohen, Inbal et al. 1999; Kuo, Lin et al. 2003; McKenzie and Ridley 2007). TNF-alpha activation of these kinases results in reorganization of actin filament dynamics and the production of actomyosin contractility (AMC) that is suggested to play an important role in mediating TNF-alpha signaling. For example, inhibition of MLCK through pharmacological or genetic means impairs TNF-alpha’s ability to activate myosin II, promote actin filament formation, NFkappaB signaling, and caspase activation (Petrache, Verin et al. 2001; Wadgaonkar, Linz-McGillem et al. 2005). Inhibition of Rho kinase and DAPK activity has also been shown to impair actin filament formation, permeability, and apoptosis in response to
TNF-alpha (Cohen, Inbal et al. 1999; Nwariaku, Rothenbach et al. 2003; Petrache, Crow et al. 2003). Since all three kinases share myosin II as a common substrate these data suggest a central role for myosin II activation in mediating TNF-alpha signaling especially since the only established substrate of MLCK is the MLC of myosin II.

**TNF-alpha signaling**

*Protein Expression*

Translation of TNF-alpha mRNA results in a homotrimer made up of 26kDa monomers. This protein does not contain a signal sequence and is therefore inserted into the plasma membrane as transmembrane TNF-alpha (tmTNF). From here tmTNF is cleaved and released from the membrane by TNF-alpha converting enzyme (TACE) to form a soluble form of TNF-alpha (sTNF) which is a homotrimer made up of 17kDa monomers. This homotrimer binds to its receptors causing oligomerization that results in recruitment of adaptor proteins, and activation of downstream signaling events (Tracey, Klareskog et al. 2008).

*TNF Receptors*

Both sTNF and tmTNF have the ability to bind to the TNFR1 (p55;CD120a) and TNFR2 (p75;CD120b) receptors. These receptors are part of the TNF receptor super family that include other receptors such as FAS, TRAILR1, HVE1, and a number of others (Hehlgans and Pfeffer 2005). Members of this family are distinguished by one to six cysteine-rich domains (CRDs) located in the extracellular region of the receptor. The monomeric chains of TNFR1 and TNFR2 posses four CRDs on their extracellular region. Prior to binding of TNF-alpha these receptors have the ability to form pre-ligand trimeric
structures that are believed to facilitate TNF-alpha binding. These trimeric structures are dependent upon the formation of the pre-ligand assembly domain (PLAD). The PLAD is formed when three monomeric receptors are bound to one another through their CRD furthest from the plasma membrane. It is the association of these three CRDs that forms the PLAD and allows for unbound TNFR1 and TNFR2 to form these trimeric structures. Since TNF-alpha can bind both TNFR1 and TNFR2 it is thought that the formation of the pre-ligand trimeric receptor helps prevent monomers from TNFR1 mixing with those of TNFR2 and vice-versa. This is important because the intracellular domains of TNFR1 and TNFR2 are different and mixing of the monomers could have detrimental effects on signaling (Idriss and Naismith 2000; Hehlgans and Pfeffer 2005; Deng 2007).

Although TNFR1 and TNFR2 share limited homology in their extracellular domain and some similarities in their ligand binding techniques they differ in cell type expression and intracellular structure. TNFR1 is ubiquitously expressed on nearly all cell types except erythrocytes whereas the expression of TNFR2 is more confined to hematopoietic cells but its expression can be induced in other cell types such as by Interfueron-gamma (IFN) in caco-2 cells (Wang, Schwarz et al. 2006; Tracey, Klareskog et al. 2008). The TNFR1 and TNFR2 receptors are distinct in terms of their intracellular domains where TNFR1 contains a death domain (DD) for recruitment of DD containing proteins whereas TNFR2 intracellular domain does not contain DDs and initiates most of its signaling through TRAF adaptor proteins (Micheau and Tschopp 2003; Till, Rosenstiel et al. 2005). Due to the absence of DDs from TNFR2 its activation mostly results in promoting cell survival, however, it has been shown to activate a RIP1 dependent cell death pathway and is able to augment TNFR1 induced apoptosis in certain
conditions. Although both receptors are versatile in their own right it is widely believed that TNFR1 mediates the biological effects of TNF-alpha due to its ubiquitous expression, intracellular domain structure, and higher affinity for soluble TNF-alpha (Till, Rosenstiel et al. 2005; Tracey, Klareskog et al. 2008).

**TNF-alpha Survival Signaling**

Since the cytoplasmic tails of the pre-assembled TNFR1 complex are brought into close proximity the DD of inactivated TNFR1 bind the silencer of death domain (SODD) to prevent against inadvertent signaling. However, once TNF-alpha has bound TNFR1 the PLAD interaction is replaced by the more stable ligand-receptor interaction and SODD is released from the intracellular domain to allow TNFR1 to initiate signaling (Hehlgans and Pfeffer 2005). TNFR1 is not known to have any intrinsic kinase activity, yet it has the ability to promote both survival and apoptosis depending on environmental context and molecular state of the cell. Upon the initial binding of TNF-alpha to TNFR1 survival signals are initiated via NFkappaB through the recruitment of DD containing adaptor proteins such as TNFR associated death domain (TRADD) and receptor interacting protein 1 (RIP1) (Micheau and Tschopp 2003). Recruitment of these adaptor proteins along with others, such as TNFR-associated factor-2 (TRAF-2), to the activated receptor forms a prosurvival signaling complex that promotes NFkappaB activation (Micheau and Tschopp 2003).

In quiescent cells the five members of the mammalian NFkappaB family; p65 (RelA), RelB, c-Rel, p50/p105 (NF-kB1), and p52/p100 (NF-kB2), are retained in the cytosol as hetero- and homodimers by their interactions with IkB proteins. NFkappaB family members are characterized by a conserved 300 amino acid Rel homology domain
(RHD) that is located in the N-terminal region and is involved in dimerization, interaction with IkB proteins, and DNA binding (Hayden and Ghosh 2004). In the cytosol the p65 nuclear localization sequence (NLS) is masked by binding of the IkBalpha protein which contains a nuclear export sequence (NES) keeping NFkappaB out of the nucleus (Huang, Kudo et al. 2000). However, the prosurvival signaling complex activates the IKK-alpha and IKK-beta kinases by promoting their association with the regulatory subunit IKK-gamma (NEMO- NFkappaB essential modifier) by recruiting these subunits through the K63-specific polyubiquitin chain on RIP1 (Hayden and Ghosh 2004). Activated IKK-alpha has the ability to phosphorylate the IkBalpha protein on serine 32 and 36 which then promotes polyubiquitination and targeting of IkBalpha for proteasomal degradation. This exposes the NLS of p65 and tilts the balance of NLSs of the p65 and p50 subunits in favor of nuclear translocation (Alkalay, Yaron et al. 1995).

NFkappa B is a transcription factor that is involved in the regulation of over 150 genes. Most of these target genes encode for proteins that are involved in the host immune response such as inflammatory cytokines, chemokines, immunoreceptors, and cell adhesion molecules (Pahl 1999). In addition to the immune response NFkappaB can also be activated by physiological and physical stress caused by ischemia/reperfusion, hemorrhagic shock, and irradiation (Li and Karin 1998; Hierholzer, Billiar et al. 2002; Zhang, Potrovita et al. 2005). These data indicate that NFkappaB is a versatile transcription factor of critical importance to many cellular processes needed for maintaining the survival and integrity of the cell.

In response to TNF-alpha, NFkappaB promotes both survival and anti-apoptotic signaling pathways to support viability of the cell. NFkappaB also plays an important
role in regulating TNF-alpha signaling by controlling both positive and negative feedback
loops through varying genes under its transcriptional control. For example, increasing
the transcription of IFN-gamma or TNF-alpha itself further increases the production of
TNF-alpha, therefore, acting as a positive feedback loop (Tracey, Klareskog et al. 2008).
However, NFkappa B also increases the transcription and production of negative
feedback regulators such as IL-10 that suppress TNF-alpha expression (Tracey,
Klareskog et al. 2008). This regulation of TNF-alpha signaling by NFkappaB is
important for regulating the immune response and to prevent inflammation from
becoming a chronic and pathological condition. Other transcriptional targets of
NFkappaB that are important in regulating TNF-alpha signaling promote the expression
of FLICE/inhibitor of caspase 8 protein (c-FLIP) and the inhibitor of apoptosis proteins
(IAPs) XIAP, xIAP1, and cIAP2 which help prevent TNF-alpha induced apoptosis
(Kreuz, Siegmund et al. 2001; Bai, Liu et al. 2004; Turner, Alaish et al. 2007).
Therefore, the ability of the cell to effectively transmit NFkappaB signaling often times
determines whether or not the cell will undergo apoptosis in response to TNF-alpha.
However, recent works have shown that TNF-alpha has the ability to initiate apoptosis
through two distinct caspase 8 activating pathways, one of which involves IAPs whose
expression is independent of NFkappaB transcriptional control (Wang, Du et al. 2008).

**TNF-alpha Apoptotic Signaling**

Both caspase 8 activating pathways seem to be dependent on the formation of the
prosurvival signaling complex (complex I) that is recruited to the activated TNFR1 at the
plasma membrane. Complex I is composed of TNFR1, TRADD, RIP1, TRAF-2, cIAP
and possibly other components that are yet to be identified (Micheau and Tschopp 2003). During the internalization process of TNFR1 complex I is released from the receptor and is modified in a manner that increases the molecular weight of its components. This modification of complex I allows it to recruit the Fas associated death domain (FADD) which in turn recruits procaspase 8 through interaction of their death effector domains (DEDs) to form the death inducing signaling complex (DISC) (Micheau and Tschopp 2003; Schneider-Brachert, Tchikov et al. 2004). Caspase 8 contains two DEDs and one protease domain that must be cleaved into the active p10 and p18 subunits (Alcivar, Hu et al. 2003). Activation of caspase 8 is thought to proceed through an oligomerization dependent manner meaning that when multiple caspase 8 proteins are recruited to the DISC complex they have the ability to promote the cleavage of one another’s protease domain. Interestingly, it has been shown in vitro that uncleavable caspase 8 mutants become active through dimerization, however, the physiological significance of this is yet to be determined (Donepudi, Mac Sweeney et al. 2003). Activation of caspase 8 at the DISC is a critical step in TNF-alpha induced apoptosis. However, if the cell is capable of promoting NFkappaB signaling the activation of caspase 8 by the FADD containing DISC complex will be inhibited by NFkappaB responsive genes that encode for antiapoptotic proteins such as cFLIP (Micheau and Tschopp 2003). Studies have shown that cFLIP is a protease dead homolog of caspase 8 with the ability to compete with caspase 8 for binding and association with FADD (Kreuz, Siegmund et al. 2001; Bai, Liu et al. 2004). If the cell is capable of transmitting the initial NFkappaB response to TNF-alpha in a timely and efficient manner caspase 8 activation at the DISC will be inhibited by the binding of cFLIP. However, if the cell is unable to activate NFkappaB and
promote the expression of antiapoptotic proteins such as cFLIP then caspase 8 will be activated through its association with DISC (Micheau and Tschopp 2003). In this manner the potential of the cell to maintain viability and promote survival in response to TNF-alpha is determined by its ability to activate NFkappaB signaling otherwise the apoptotic response to TNF-alpha will prevail.

An alternative pathway to activating caspase 8 in response to TNF-alpha involves the associations between RIP1, FADD, and caspase 8 (Vince, Wong et al. 2007). As mentioned above, RIP1 is recruited to the plasma membrane to help form complex I in response to TNF-alpha. Within complex I RIP1 is bound by IAPs but is still able to promote NFkappaB survival signaling by recruiting components of the IKK through its K63 polyubiquitin chain, thereby activating the kinase (Wang, Du et al. 2008). At this point the ability of RIP1 to promote apoptosis is inhibited by binding of IAPs. The level of IAP expression is much less sensitive to protein synthesis rate then cFLIP, instead, their expression is controlled by allosteric activation of their zinc ring finger domain ubiquitin ligase by the binding of Smac/Diablo to their BIR3 domain leading to their autodegradation (Varfolomeev, Blankenship et al. 2007; Wu, Tschopp et al. 2007; Wang, Du et al. 2008). Therefore, investigators developed a Smac mimetic which promoted the degradation of IAPs which then allows for the RIP1 K63 chain to be deubiquinated by CYLD. It is unclear if RIP1 gets deubiquinated before or after its release from the activated TNFR1. However, it is believed that RIP1 recruitment to complex I and the ubiquitination/de-ubiquitination process is essential for RIP1 pro-apoptotic activity since RIP1, FADD, and caspase 8 are all present in the cytosol but do not interact until RIP1’s recruitment and release from complex I (Wang, Du et al. 2008). Once RIP1 is released
from the receptor it enters the cytosol where it can now recruit FADD through its DD. From here FADD can now recruit caspase 8 to promote its activation and the execution of apoptosis (Wu, Tschopp et al. 2007; Wang, Du et al. 2008). Therefore, these works demonstrate another layer of complexity in TNF-alpha cell death signaling in which caspase 8 can be activated by two distinct pathways that are distinguished by their regulation by cFLIP and cIAPs.

**TNF-alpha involvement in the immune response, inflammation, and autoimmune diseases**

TNF-alpha and the TNFR1 receptor have been shown to be involved in development and function of the immune system. Knockout mice that do not express TNF-alpha or TNFR1 have defective formation of immune system components such as germinal centers, primary B-cell follicles, and Peyer’s Patches (Pfeffer, Matsuyama et al. 1993; Aggarwal and Natarajan 1996; Pasparakis, Alexopoulou et al. 1996). Although TNF-alpha and TNFR1 play a non-central role in development of the immune system they have an essential role in execution of an effective immune response. TNF-alpha is absolutely essential for fast and efficient innate immune response against many bacterial pathogens such as *Listeria monocytogenes* and *Mycobacterium tuberculosis* (Flynn, Goldstein et al. 1995; Pasparakis, Alexopoulou et al. 1996). During the innate immune response macrophages recognize the bacteria as foreign substances within the body through receptors on their surface. This recognition causes the macrophages to engulf the bacteria through a process known as phagocytosis (Aderem and Underhill 1999). Once the macrophages have engulfed the pathogen it begins to break it down through the use of
ROS (Swindle, Hunt et al. 2002). The cell wall of many bacteria possess a component known as lipopolysaccharide (LPS) that is a potent activator of TNF-alpha production in macrophages. Once a macrophage has engulfed a pathogen it begins to produce and secrete TNF-alpha which performs a number of functions to increase the immune response against the infectious agent.

TNF-alpha has the ability to recruit additional macrophages to the site of infection by forming a positive feedback loop in which TNF-alpha increases the production of chemokines as well as inflammatory cytokines such as Interfueron-gamma (IFN-gamma) and IL-6 from the activated macrophages (Wright, Pryhuber et al. 2004; Tracey, Klareskog et al. 2008). In order for macrophages to reach the site of inflammation they must cross the endothelium of blood vessels near the site of infection in a process known as leukocyte extravasation. TNF-alpha facilitates this process by binding TNFR1 and promoting the expression of adhesion molecules such as selectins on the surface of the endothelial cells to promote leukocyte binding (Eriksson, Xie et al. 2001). In addition, TNF-alpha can also increase the permeability of the endothelial cells near the site of infection by disrupting cell-cell junctions in a MLCK and Rho kinase dependent manner that will be discussed later.

Macrophages breakdown engulfed pathogens and present a section of the bacteria known as the antigen to neighboring helper T-cells. It does this by inserting the antigen into its cell membrane and displaying the antigen attached to the major histocompatability complex II (MHCII) so that the macrophage is not recognized as a pathogen even though it has antigens on its surface (Pai, Askew et al. 2002; Pai, Convery et al. 2003). Once the helper T-cell becomes activated by binding the antigen it begins
producing TNF-alpha to recruit more helper T-cells to the site of infection much like it does for macrophages. For example, T lymphocyte recruitment to inflamed colonic mucosa is suggested to be dependent on TNF-alpha induced expression of adhesion molecules mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and VCAM-1 (Watanabe, Miura et al. 2002). The recruitment of helper T-cells and macrophages is vital for the formation of granulomas, which is a conglomerate of immune cells that contain the pathogen preventing it from spreading and allowing for its removal from the body (Hernandez-Pando and Rook 1994; Roach, Bean et al. 2002). In TNF-alpha or TNFR1 deficient mice granuloma formation is significantly delayed and without TNF-alpha granuloma formation is aberrant and unable to contain the pathogen leading to eventual death (Lukacs, Chensue et al. 1994; Roach, Bean et al. 2002). Mouse studies have shown that TNF-alpha is also necessary to maintain granulomas once they have formed because treatment of these wild-type mice with TNF-alpha neutralizers breaks down the granuloma leading to spread of the infection (Wallis and Ehlers 2005).

Although the formation of these granulomas is essential to the innate immune response and clearing the body of infectious agents these same granulomas can cause autoimmune diseases such as Crohn’s disease (also known as granulomatous colitis) when their formation and degradation are not regulated properly (Matsumoto, Nakamura et al. 2001). Crohn’s disease (CD) is an inflammatory bowel disease and the formation of granulomas can occur anywhere on the gastrointestinal tract from the anus to the mouth. Significantly increased levels of TNF-alpha mRNA and protein are produced in the lamina propria of patients with CD (Stucchi, Reed et al. 2006). At the present time
there is no cure for the disease but TNF-alpha neutralizers such as the monoclonal anti-TNF antibody known as infliximab is effective in treating symptoms of CD (Tracey, Klareskog et al. 2008).

Rheumatoid arthritis (RA) is another inflammatory disease that TNF-alpha plays a crucial role in. RA usually effects women three times more often than men with the disease occurring mostly between the ages of 40 and 50, but it can develop in people of any age (Majithia and Geraci 2007). RA is caused by inflammation in synovial tissue at the joints that causes the breakdown of articular cartilage and bone destruction. Bone density is determined by the activities of osteoblasts, which promote bone formation, and osteoclasts, which promote bone degradation (Tracey, Klareskog et al. 2008). Osteoclasts are multinucleated cells derived from macrophage precursor and are sometimes referred to as ‘resident macrophages’ because they stay in a fixed location. TNF-alpha has the ability to promote osteoclast proliferation and function both directly and by increasing M-CSF and receptor activator of nuclear factor kappa-B ligand (RANKL) (Glantschnig, Fisher et al. 2003). It is these overactive osteoclasts at the site of joint inflammation that is believed to be a major cause of RA. Perhaps the most compelling evidence that TNF-alpha and osteoclasts play a central role in the development of RA come from clinical studies showing that TNF antagonists such as infliximab and etanercept slow or completely arrest bone destruction at sites of inflammation (Tracey, Klareskog et al. 2008). There has been a great deal of evidence demonstrating that TNF-alpha plays a central role in a number of autoimmune diseases
and TNF agonists have been effective in treating them, however, further work is needed to elucidate the etiology of these autoimmune diseases caused by TNF-alpha and the immune system.

**Inflammation and Cancer**

The relationship between tumors and cells of the immune system is complex and there is still many questions left to be answered, however, it is generally accepted that inflammation and cancer are linked. Epidemiological studies have shown that chronic inflammation predisposes individuals to certain types of cancer and that chronic inflammation is linked to 15-20% of all cancer deaths worldwide (Balkwill and Mantovani 2001). Two examples where links between inflammation and cancer have been seen are inflammatory bowel disease (IBD) which increases the risk of developing colon cancer and chronic inflammation in the lung in response to asbestos poisoning leading to mesothelioma (Coussens and Werb 2002).

From the earliest stages of development inflammatory cells, cytokines, and chemokines are present in the microenvironment of most, if not all, tumors found in animal models and humans (Mantovani, Allavena et al. 2008). The presence of these cells and the involvement of inflammation in tumor development can be induced by an intrinsic pathway dependent on oncogenes or by an extrinsic pathway dependent on inflammation caused by infection or autoimmune response. Intrinsic mutations have the ability to initiate a transcription pattern that is similar to what is seen in the inflammatory response. In human papillary thyroid carcinogenesis a mutation of the protein tyrosine kinase RET promotes transcription of colony-stimulating factors (CSFs), IL-1B, and
COX2 which results in leukocyte recruitment, inflammation, and chemokine production which is believed to facilitate tumorigenesis (Mantovani, Allavena et al. 2008). Extrinsic factors can also initiate inflammation that leads to tumor formation. On certain occasions macrophages engulf foreign material that they have great difficulty degrading and clearing from the body such is the case after exposure to asbestos that enter the lung. These asbestos activated macrophages therefore promote a state of chronic inflammation in response to these foreign particles and there is a strong link between asbestos poisoning and the development of mesothelioma (Choe, Tanaka et al. 1997; Jaurand, Renier et al. 2009).

In addition to activated macrophages, tumor cells have the ability to produce TNF-alpha to initiate autocrine signaling that facilitates tumor development. Studies done in ovarian cancer cell lines illustrated that TNF-alpha autocrine signaling resulted in increased production of the proinflammatory cytokine IL-6, the angiogenesis promoting factor VEGF, and chemokine receptor CXCR4 and its ligand CXCL12 as compared to ovarian cell lines that had little to no TNF-alpha autocrine signaling (Kulbe, Hagemann et al. 2005; Kulbe, Thompson et al. 2007). The ability of TNF-alpha to promote the expression of VEGF and facilitate angiogenesis plays a crucial role in its involvement in tumor progression by providing the growing tumor with additional nutrients and oxygen while promoting the recruitment of inflammatory cells to produce even more cytokines such as IL-6 to further promote tumor cell growth and proliferation (Tracey, Klareskog et al. 2008). One of the more detrimental effects of TNF-alpha production during tumor development is increasing the expression of the CXCR4 receptor. Many cells begin to express CXCR4 during transformation because it is important for metastasis and in
primary human tumors the amount of CXCR4 expressed correlates with the extent in which metastasis occurs to the lymph nodes in colorectal, liver, oesophageal, and breast cancer (Mantovani, Allavena et al. 2008). TNF-alpha’s ability to promote metastasis has also been demonstrated in breast cancer and colon carcinoma cells where it is a potent simulator of epidethelial-mesenchymal transition (EMT). In LIM 1863 colon cancer organoids it has been shown that TNF-alpha produced by activated macrophages drastically increases the EMT that is promoted by TGF-beta in a p38MAPK dependent pathway (Bates and Mercurio 2003). Interestingly, in the colon adenocarcinoma cell line RPMI4788 TNF-alpha is pro-survival by itself but in combination with IFN-gamma it increases caspase 3 activity, PARP cleavage, and diminishes tumor cell survival. Signaling studies illustrated that TNF-alpha/IFN-gamma treatment was able to significantly enhance NFkappaB mediated transcription of Fas to promote apoptosis (Kimura, Haisa et al. 2003). The ability of NFkappaB to initiate the transcription of both survival and apoptotic genes has made its activity critically important in regulating inflammation and tumor development. Indeed, many works seem to suggest that the effect TNF-alpha exerts on tumor cells and the tumor microenvironment is determined by its interactions with other cytokines and the manner in which they activate the NFkappaB pathway.

The interplay between tumor cells and the microenvironment containing inflammatory cells, chemokines, and cytokines such as TNF-alpha is complex. Overall, the majority of cytokines and chemokines produced at tumor sites seem to support tumor growth, however, there are cytokines such as IL-10, which is a negative regulator of TNF-alpha, that helps suppress tumor progression. Ironically, in spite of its name, TNF-
alpha seems to support the growth of many tumor cell types. TNF-alpha’s role in tumor cell regulation is much more complex than first thought and although it can have influential effects on its own what seems to determine TNF-alpha’s overall biological effects is what other cytokines and chemokines it is interacting with in the tumor cell microenvironment.

**Actin cytoskeleton involvement in TNF-alpha mediated inflammation**

TNF-alpha serves many functions during inflammation one of which is increasing endothelial cell permeability. TNF-alpha has been shown to destabilize microtubules and initiate signaling pathways that increase actomyosin contractility to destabilize endothelium cell-cell junctions (Verin, Birukova et al. 2001; Vandenbroucke, Mehta et al. 2008). The endothelium forms adhesive cell-cell junctions composed of the transmembrane protein vascular endothelial cadherin (VE-cadherin) that is connected to the actin cytoskeleton through adaptor proteins alpha and beta catenin. Associations between these proteins and the actin cytoskeleton are crucial to regulating endothelium permeability (Lampugnani, Corada et al. 1995; Dejana, Lampugnani et al. 2000). TNF-alpha has the ability to re-organize actin filaments into stress fibers through the Rho kinase mediated activation of myosin II (Wojciak-Stothard, Entwistle et al. 1998). Formation of actin stress fibers and activation of myosin II generates actomyosin contractility (AMC) that is believed to pull VE-cadherin inward thereby disrupting endothelial cell-cell junctions allowing for increased permeability and leukocyte recruitment to the site of infection (Vandenbroucke, Mehta et al. 2008).
TNF-alpha activation of actin cytoskeleton associated kinases has also been shown to play a vital role in TNF-alpha survival signaling involving NFkappaB as well as the cell death response involving caspase activation (Petrache, Birukov et al. 2003; Petrache, Crow et al. 2003; Wadgaonkar, Linz-McGillem et al. 2005). The central role of the actin cytoskeleton in endothelial permeability, survival, and apoptosis indicates its importance in mediating TNF-alpha signaling. A better understanding of how the actin cytoskeleton mediates the response to TNF-alpha may identify drug targets that inhibit the detrimental signaling events that lead to autoimmune diseases and cancer.

**TNF-alpha activated kinases involved in regulating myosin II and actin cytoskeleton function**

The actin cytoskeleton and its associated proteins have been shown to play an important role in mediating TNF-alpha signaling. Myosin light chain kinase (MLCK), Rho kinase, and Death associated protein kinase (DAPK) are some of the major regulators of actin cytoskeletal dynamics that are activated in response to TNF-alpha (Cohen, Inbal et al. 1999; Kuo, Lin et al. 2003; McKenzie and Ridley 2007). MLCK and DAPK kinase activity are regulated in similar fashions by the binding of calcium/calmodulin to autoinhibitory domains that promotes the unfolding of the kinase to expose their catalytic domain (Bialik and Kimchi 2006). Despite the fact that the only established substrate of MLCK is the MLC of myosin II it has still been shown to be involved in a variety of cellular processes. MLCK activation of MLC is important for cell motility, focal adhesion formation, cell division, phagocytosis, and more (Mansfield, Shayman et al. 2000; Matsumura, Totsukawa et al. 2001; Totsukawa, Wu et al. 2004;
Choi, Ryu et al. 2006; Connell and Helfman 2006). There are various works highlighting a role for MLCK in mediating survival, apoptosis, and endothelial permeability in response to TNF-alpha. Mice injected intraperitoneally with recombinant TNF-alpha had a 4.2 fold increase in jejuna epithelial cell MLCK (Graham, Wang et al. 2006). This increased expression of MLCK in response to TNF-alpha is consistent with MLCK involvement in mediating TNF-alpha signaling. Wadgaonkar et al. also demonstrated a role for MLCK in TNF-alpha signaling by showing that impairing the kinase activity of MLCK through pharmacological and genetic means impairs NFkappaB translocation to the nucleus and its ability to promote gene expression as measured by a luciferase reporter assay (Wadgaonkar, Linz-McGillem et al. 2005). After the initial NFkappaB response there are works that suggest MLCK, Rho kinase, and DAPK help to promote TNF-alpha induced apoptosis. Inhibition of MLCK through treatment with the kinase inhibitor ML-7 and expression of antisense or mutant constructs have illustrated a role for MLCK in TNF-alpha induced apoptosis (Petrache, Verin et al. 2001; Petrache, Birukov et al. 2003). Jin et al. demonstrated the importance of MLCK in caspase activation through expression of a dominant negative kinase-dead MLCK in MDCK cells that impaired the TNF-alpha induced recruitment of caspase 8 to FADD (Jin, Atkinson et al. 2001).

DAPK is another calcium/calmodulin serine/threonine kinase that shares 44% identity with the corresponding catalytic domain of MLCK and has the ability to activate myosin II through the phosphorylation of MLC (Bialik and Kimchi 2006). Although MLCK and DAPK are similar kinases there have been more downstream substrates identified for DAPK such as Ser283 on tropomyosin-1 which promotes stress fiber formation in endothelial cells in response to oxidative stress (Houle, Poirier et al. 2007).
DAPK expression is also evident in the hippocampus and cerebral cortex and its ability to activate syntaxin-1A regulates synaptic transmission by mediating vesicle docking and fusing at the neuronal membrane (Tian, Das et al. 2003). Another important function of DAPK is its role as a tumor suppressor due to its ability to sensitize cells to apoptotic signals that are encountered during tumorigenesis. DAPK is involved in mediating cell death in response to a variety of stimuli such as c-myc oncogene expression, ceramide, TGF-beta, and activation of death receptors such as Fas and TNFR1 (Bialik and Kimchi 2006). TNF-alpha present in culture cell medium used to grow activated macrophages was added to HCT116 cells and was shown to activate DAPK induced apoptosis in a p-38 MAPK dependent manner (Bajbouj, Poehlmann et al. 2009). Other works have shown that expression of DAPK antisense RNA impaired TNF-alpha induced apoptosis and deletion mutants demonstrated that the death domain of DAPK plays a crucial role in mediating TNF-alpha cell death in cell lines such as Hela (Cohen, Inbal et al. 1999). The involvement of DAPK in mediating a variety of apoptotic signals and its ability to activate myosin II makes it a relevant kinase to this study.

Rho kinase also forms an autoinhibitory loop structure in the inactive state, however, Rho kinase is activated by binding of Rho GTPases such as RhoA, RhoB, and RhoC (Narumiya, Tanji et al. 2009). There are two isoforms of Rho kinase that have 65% amino acid identity overall and a 92% amino acid identity in the kinase domain and so far there is no evidence that they phosphorylate different substrates (Nakagawa, Fujisawa et al. 1996). Although Rho kinase has a variety of downstream substrates many of them seem to be related to regulation of cell structure through modulation of the cytoskeleton. Adducin and vimentin are examples of Rho kinase substrates that influence
both actin and intermediate filaments respectively (Kimura, Fukata et al. 1998; Inada, Togashi et al. 1999). Rho kinase can also promote myosin II activation through direct phosphorylation of the MLC on ser19/thr18 and through inhibition of the myosin II phosphatase MYPT which help to stabilize actin filament bundles (Yazaki, Tamaru et al. 2005). Rho kinase activation of LIM kinase also helps stabilize actin filament bundles by inhibiting the function of the actin severing protein cofilin (Riento and Ridley 2003).

The ability of Rho kinase to stabilize actin filaments and activate myosin II produces AMC which is important to many cellular processes such as motility, adhesion, and apoptosis (Sebbagh, Renvoize et al. 2001; Kolega 2003; Totsukawa, Wu et al. 2004).

Work done in bovine endothelial cells showed that treatment with TNF-alpha resulted in stress fiber formation and increased MLC phosphorylation that coincided with caspase 8 activation (Petrache, Crow et al. 2003). Treatment with the Rho kinase inhibitor Y27632 or expression of a dominant negative Rho significantly impaired actin filament formation, MLC phosphorylation, and caspase activation in response to TNF-alpha (Petrache, Crow et al. 2003). This study concluded that Rho dependent AMC was involved in early apoptotic signaling events, possibly in the formation of the DISC complex to activate caspase 8. However, inhibition of Rho kinase can have a wide range of effects on the cell and this study along with others, failed to directly analyze the involvement of stress fiber formation and AMC by failing to directly target actin filaments and myosin II.

Although MLCK, Rho kinase, and DAPK have varying substrates one common downstream effector in response to TNF-alpha is that of myosin II. Inhibition of these kinases through pharmacological and genetic means impairs myosin II activation and
signaling in response to TNF-alpha. Myosin II is involved in a variety of cellular processes and preventing its activation in response to TNF-alpha impairs both survival and apoptotic signaling. These were interesting findings suggesting that myosin II may be involved in helping to mediate the life and death decision many cells make in response to TNF-alpha. Therefore, I wanted to take a closer look at the role myosin II plays in mediating TNF-alpha cell death signaling in the hopes of elucidating new functions for myosin II in this process and uncover therapeutic targets that could manipulate the TNF-alpha cell death pathway.

Non-muscle myosin II: structure, regulation, and function.

Three different genes in mammalian cells (MYH9, MYH10, and MYH14) encode for the isoforms of myosin II known as myosin IIA (IIA), myosin IIB (IIB), and myosin IIC (IIC). All three isoforms are hexamers made up of two heavy chains, two essential light chains (ELC), and two regulatory light chains (MLC) (Bresnick 1999; Vicente-Manzanares, Ma et al. 2009). Association of the two essential light chains and two regulatory light chains at the neck of the protein are important for structural stability and regulation of myosin II activation. Myosin II can be pictured as having three main domains (Figure 1), first, there is the N-terminal S1 domain that contains the globular head which possesses ATPase function and an actin binding site. In addition, the S1 domain also contains the neck of the myosin II protein where the pairs of essential and regulatory light chains are located. The neck connects the globular head to the second domain of myosin II, the long alpha-helical coiled coil tail which is involved in
dimerization with other myosin II molecules. The third domain is a carboxy terminal non-helical tailpiece that differs substantially between the myosin II isoforms and antibodies to this region have been developed to distinguish the myosin II isoforms from one another (Figure 1) (Vicente-Manzanares, Ma et al. 2009).

![Myosin II domain structure](image)

**Figure 1.** Myosin II domain structure. The above illustration demonstrates the three major domains of the myosin II protein: S-1, coiled-coil rod, and non-helical tail domain.

One of myosin II’s most well characterized functions is its ability to bind to actin filaments and pull them in opposing directions to generate a force known as actomyosin contractility (AMC). This contractile activity is most evident in skeletal and cardiac muscle, however, it also occurs in non-muscle cells to promote cell-cell adhesions, motility, cell division and other processes that require force generation (Yamada and Nelson 2007; Reichl, Ren et al. 2008; Okeyo, Adachi et al. 2009). Although the ‘powerstroke’ involving ATP processing by myosin II is the same in muscle and non-
muscle cells there is an important difference in the initial regulation of AMC. The primary regulation of skeletal and cardiac myosin II is dependent upon the actin associated proteins tropomyosin and troponin (el-Saleh, Warber et al. 1986). Tropomyosin is an actin binding protein that normally sits within the groove of the actin helix. However, in resting skeletal and cardiac muscle, troponins T, I, and C (named for tropomyosin binding, inhibitory property, and calcium binding respectively) pull tropomyosin out of the actin helix and place in a position where myosin II heads cannot interact with actin. When intracellular calcium levels rise in response to nerve impulses troponin C binds calcium causing troponin I to release its hold on actin. This removal of troponin results in tropomyosin returning to the groove of the actin helix thereby allowing the myosin II head to bind actin filaments and generate force (Gordon, Regnier et al. 2001; Szczesna and Potter 2002).

In non-muscle cells the phosphorylation of the MLC by activated kinases such as MLCK play an important role in initiating the production of AMC. This is an important difference from skeletal and cardiac muscle because it allows non-muscle cells to produce varying quantities of AMC in specific locations within the cell depending on the intracellular or extracellular signal. Inactive non-muscle myosin II has an alpha-helical tail that forms an autoinhibitory loop which distorts globular head structure thereby interfering with the actin binding site and the ATPase function of the S1 head domain (Conti and Adelstein 2008; Vicente-Manzanares, Ma et al. 2009) (Figure 2A). Upon phosphorylation of the MLC by kinases such as MLCK, Rho kinase, or DAPK myosin II is converted to its active state through the unfolding of the heavy chains which allows the S1 domain to now bind ATP in a large cleft located in the ‘back’ of the head region. The
cleft closes around the ATP molecule and hydrolyzes the ATP binding tightly to the ADP and inorganic phosphate (Pi) that is produced. Myosin II with bound ADP and Pi are now in the cocked position and once myosin II comes into contact with an actin filament Pi is released allowing myosin II to bind tightly to the actin filament. From here ADP is released and myosin II pulls on the filament to induce a powerstroke resulting in AMC. Myosin II must now bind a new ATP molecule so that it can be released from actin and repeat the process (Craig, Smith et al. 1983; Tyska and Warshaw 2002; Kovacs, Toth et al. 2004; Sellers and Knight 2007). Therefore, although the events leading up to ATP binding in muscle and non-muscle cells are different the powerstroke itself is the same.

(Vicente-Manzanares, Ma et al. 2009)

**Figure 2.** Activation of non-muscle myosin II. (A) The regulatory light chain (RLC) is unphosphorylated on inactive non-muscle myosin II (NM-myosin II). This allows the coiled-coil rod domain to fold over on itself to distort the globular head region and prevent binding to actin filaments. (B) Phosphorylation of the RLC by kinases such as
MLCK convert NM-myosin II into an active conformation that allows myosin II to form bipolar filaments with other myosin II proteins. (C) These activated myosin II bundles can now bind and pull on multiple actin filaments by processing ATP to generate a force known as actomyosin contractility (AMC).

As previously mentioned the production of AMC in non-muscle cells is crucial to many cellular processes such as apoptosis, motility, and cytokinesis. The localization of myosin II and its temporal activation by upstream kinases play an important role in determining the biological effects of AMC produced by myosin II. For example, integrin binding to components of the extracellular matrix during migration initiate signaling pathways involving Rho kinase and MLCK that activate myosin II. AMC produced by myosin II promotes integrin clustering through actin adaptor molecules such as talin and vinculin (Vicente-Manzanares, Ma et al. 2009). Therefore, a positive feedback is created between bound integrins and activated myosin II for the maturation of adhesions and the promotion of survival signaling. Work done in migrating fibroblasts demonstrate that inhibition of MLCK blocks MLC phosphorylation and focal adhesion formation at the cell periphery while maintaining MLC phosphorylation and adhesions at the cell center. Whereas inhibition of Rho kinase had the opposite effect preventing MLC phosphorylation and focal adhesion formation at the cell center while MLC phosphorylation and focal adhesion formation was maintained at the cell periphery (Totsukawa, Yamakita et al. 2000). Furthermore, Rho kinase has been shown to preferentially phosphorylate the MLC of myosin IIA during migration (Sandquist, Swenson et al. 2006). These works demonstrate the possibility that the regulation of myosin II activation is quite complex involving different initiating signals and upstream kinases that can utilize the myosin II isoforms for different biological processes.
In addition to migration there is also evidence that activation of myosin II by upstream kinases and production of AMC play a central role in the execution phase of apoptosis. Disrupting AMC through the use of cytochalasin D or impairing myosin II ATPase activity by blebbistatin treatment prevents membrane blebbing, cellular fragmentation, and nuclear disintegration which are all morphological hallmarks of the execution phase of apoptosis (Croft, Coleman et al. 2005; Orlando, Stone et al. 2006). In addition, disrupting AMC through inhibition of upstream kinases MLCK and Rho kinase decreases MLC phosphorylation and also impairs membrane blebbing, cellular contraction, and nuclear condensation (Mills, Stone et al. 1998; Lai, Hsieh et al. 2003; Petrache, Birukov et al. 2003). Work done in NIH3T3 cells showed that Rho kinase was cleaved into a constitutively active fragment in a caspase dependent manner which increased MLC phosphorylation and AMC that was required for nuclear disintegration in response to TNF-alpha (Croft, Coleman et al. 2005). Another study using NIH3T3 demonstrated that treatment with TNF-alpha resulted in the caspase dependent degradation of myosin IIA while IIB expression remained constant (Solinet and Vitale 2008). These works did not examine a Rho kinase specific activation of myosin IIB or myosin IIC and it did not examine the contribution of MLCK. However, these works do suggest a possible isoform specific function of myosin II during apoptosis that may be determined in response to upstream signaling events by activation of differing upstream kinase.
Isoform Specific Functions of Non-muscle myosin II

Since AMC is involved in a variety of cellular processes it is not surprising that the ubiquitously expressed non-muscle myosin II isoforms can possess separate and distinct functions within the same cell. Evidence suggests that spatial regulation and temporal activation of the myosin II isoforms allows them to perform distinct functions in biological processes such as neuronal development, receptor internalization, and migration (Rey, Vicente-Manzanares et al. 2002; Chantler and Wylie 2003; Kolega 2003). Work done in CHO-K1 and Rat2 cells using siRNA knockdown of the myosin IIA and IIB isoforms demonstrated a role for myosin IIA in promoting cell adhesion formation at the central region of the cell and disassembly of adhesions at the rear. Myosin IIB was shown to establish front to back polarity involving centromosome, golgi, and nuclei positioning (Vicente-Manzanares, Zareno et al. 2007; Vicente-Manzanares, Koach et al. 2008). Interestingly, expression of a myosin IIB mutant with a point mutation in its motor domain that impaired its ATPase activity by 75% was able to rescue front to back polarity in myosin IIB deficient cells (Vicente-Manzanares, Zareno et al. 2007). This mutant was still able to cross-link actin filaments indicating myosin IIB may be playing a structural role in determining front-back polarity in migrating cells. Published works have also used antisense techniques to knockdown the expression of myosin IIA and IIB in Swiss 3T3 fibroblasts to reveal distinct roles for these isoforms in vesicle trafficking during cell membrane repair (Togo and Steinhardt 2004). In addition, myosin II plays an important role in the structural development of neurons with myosin IIB being responsible for nerve process outgrowth while myosin IIA is responsible for adhesion to the substrate as well as retraction of the nerve cell process (Chantler and
Ma et al. demonstrated that myosin IIB was essential for migration for a distinct set of neurons in the developing mouse brain. Mice generated with a point mutation impairing the motor domain of myosin IIB had distorted migration of cerebellar granule cells and pontine neurons resulting in ataxia and the inability of pups to maintain proper balance (Ma, Kawamoto et al. 2004).

<table>
<thead>
<tr>
<th>Biological Process Involving Myosin II</th>
<th>Isoform Specific Function</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myosin IIA</td>
</tr>
<tr>
<td>Motility</td>
<td>Dynamic adhesion formation</td>
</tr>
<tr>
<td>Neurite Outgrowth</td>
<td>Maintain adhesion and retraction of nerve cell process</td>
</tr>
<tr>
<td>Presynaptic Nerve Function</td>
<td>—</td>
</tr>
<tr>
<td>Receptor Internalization</td>
<td>SDF-1α induced CXCR4 internalization</td>
</tr>
<tr>
<td>Apoptosis</td>
<td></td>
</tr>
<tr>
<td>• Execution</td>
<td>N.D.</td>
</tr>
<tr>
<td>• Initiation</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Table 1. Isoform specific functions of non-muscle myosin II. The above table gives examples of different biological processes involving the isoforms of myosin II such as myosin IIB involvement in presynaptic nerve function where myosin IIA has been shown not to play a role (-). Myosin IIB is suggested to play a role in TNF-alpha induced cell retraction but its involvement in receptor internalization or the initiation of apoptosis has not yet been demonstrated (N.D.).
Non-muscle myosin II mutations and disease

The myosin II isoforms have been shown to play important physiological roles, therefore, it is not surprising that mutations in myosin II can give rise to pathological conditions. Evidence for this comes from work done in mice demonstrating that ablation of non-muscle myosin IIA results in death by embryonic day 6.5 (Conti, Even-Ram et al. 2004). In addition, mutations in approximately 40 different sites along the head and rod domains of myosin IIA have been shown to give rise to pathological conditions. Human platelets express only myosin IIA and mutations in conserved locations effect platelet morphology that is characterized by thrombocytopenia with giant platelets and large clusters of IIA in granulocytes that are visible under microscopy and termed Dohle-like bodies. A major defect in these platelets rising from the myosin IIA mutation is that their actin cytoskeleton is unable to reorganize and respond in a proper manner to stimulants such as thrombin receptor activating peptide. The inability of myosin IIA and the actin cytoskeleton to respond to stimulants is likely to effect the platelets migration and ability to form cell-cell adhesions which is detrimental to blood clot formation (Canobbio, Noris et al. 2005; Calaminus, Auger et al. 2007; Johnson, Leis et al. 2007).

Currently there are no known mutations in myosin IIB that have been reported to cause human disease, however, mouse models with mutated or ablated myosin IIB provide evidence that impairing myosin IIB function can indeed give rise to pathological conditions. Ablation of myosin IIB results in cardiac mutations such as membranous ventricular septal defect (VSD) and dextroposition of the aorta as well as defects in the nervous system that are lethal during development or on the day of birth (Tullio, Accili et
al. 1997; Tullio, Bridgman et al. 2001). Therefore, studies in mice use methods to
decrease the expression of myosin IIB or create myosin IIB mutants with point mutations
that are not lethal. One study showed a correlation between decreasing myosin IIB
expression and the onset of myocyte hypertrophy. When expression of myosin IIB was
diminished by 88% in the heart cardiac myocyte hypertrophy developed between 7 and
11 months of age whereas hypertrophy developed within one month in mice with
approximately 96% decrease in myosin IIB. This study demonstrated a relationship
between myosin IIB expression and the onset and severity of defects in the heart (Uren,
Hwang et al. 2000).

In addition to effects on cardiac function, decreases in myosin IIB expression also
impair neuronal cell migration and the formation of cell-cell adhesions in the cells lining
the spinal canal. These defects can lead to a condition known as hydrocephalus in which
there is an abnormal build up of cerebral spinal fluid (CSF) in the ventricles of the brain.
In healthy individuals CSF circulates through the brain and is continuously drained away
into the circulatory system through the spinal canal. In 2-3 out of every 1,000 live births
this flow is obstructed making hydrocephalus one of the most common congenital
conditions that can lead to severe brain damage if not treated (Schulman, Landau et al.
2000). Compared with myosin IIA and IIC, myosin IIB is enriched in the brain,
especially in neural cells. The cells lining the spinal chord in mice contain only the non-
muscle myosin IIB isoform that is shown to colocalize with the cell-cell junction proteins
Beta-catenin and N-cadherin. Immunoflorescent images revealed that myosin IIB forms
a polarized mesh-like structure at the apical region of the cells lining the spinal chord
(Ma, Bao et al. 2007). This mesh-like structure was collapsed and became discontinuous
in mice knockin models that decreased myosin IIB expression by 75% and the IIB that was being expressed contained a point mutation in the motor (Arg709Cys) impairing its motor activity by 70%. The collapse of the mesh-like barrier allowed the underlying neuroepithelial cells to invade and obstruct the spinal chord. It was this blockage of the spinal chord that seemed to be the primary cause of hydrocephalus in this model. Interestingly, the development of hydrocephalus can be prevented by increased expression of the Arg709Cys myosin IIB mutant (Ma, Bao et al. 2007). The ability of this myosin IIB with impaired ATPase function to prevent neuroepithelial invasion of the spinal canal indicates more of a structural role for myosin IIB as opposed to a force generating role in restoring the apical mesh-like barrier. These findings are consistent with the enzymatic properties of myosin IIB that make it the ideal isoform for structure related functions. Kinetic properties between the isoforms vary with myosin IIA having the highest rate of ATP hydrolysis and the ability to propel actin filaments more rapidly then myosin IIB and IIC. However, the kinetics of myosin IIB have a significantly higher duty ratio which is the time myosin II stays bound to actin in a force generating state. This high duty ratio stems from myosin IIB’s very high affinity for ADP which slows its release and keeps myosin IIB bound to actin (Rosenfeld, Xing et al. 2003; Vicente-Manzanares, Ma et al. 2009). This property of myosin IIB makes it ideal for stabilizing structural components of the cell because of its ability to exert tension on actin filaments for longer periods of time with less expenditure of energy. This property of myosin IIB seems to be important for the proper formation of the spinal canal and perhaps other biological processes within the cell.
As described above, the importance of myosin II isoform specific functions is evident for a variety of cellular processes, however, little work has been focused on distinct functions during cell death. While it is important to determine if there is a predominant myosin II functioning during the execution phase of apoptosis it is also important to determine if there are isoform specific roles during the initial apoptotic signaling events that lead to caspase activation. Elucidation of isoform specific roles of myosin II in cell death signaling will provide a better understanding of how the actin cytoskeleton may mediate survival and apoptotic signals from the cells extracellular environment. Since the actin cytoskeleton is central to a variety of cellular processes it can play an important roll in the cell’s response to extracellular signals such as TNF-alpha. Therefore, studying isoform specific functions of myosin II in cell death signaling may give rise to therapeutic targets that can modulate the cells response to stimulants such as TNF-alpha. Understanding how to modulate the cells response to extracellular ligands through manipulation of the actin cytoskeleton may prove useful in treating pathological conditions caused by extracellular stimulants such as TNF-alpha. Our work investigated the roles of actin filament stabilizing and AMC producing myosin IIA and IIB in mediating TNF-alpha cell death signaling. Our data demonstrate that myosin IIB plays an important role in mediating TNF-alpha cell death signaling and its function in doing so may be based on structural properties of the protein.

Summary

Myosin II activation is important for a variety of biological processes in response to TNF-alpha such as survival, permeability, and apoptosis (Wojciak-Stothard, Entwistle
et al. 1998; Petrache, Birukov et al. 2003; Petrache, Crow et al. 2003; Croft, Coleman et al. 2005; Wadgaonkar, Linz-McGillem et al. 2005; Mong, Petrulio et al. 2008). Although the production of AMC is important in many of these processes there has been little work focused on possible AMC independent roles for myosin II. Evidence for AMC independent functions of myosin II come from works showing alternative splicing of the MYH10 gene giving rise to a myosin IIB-2 isoform that does not generate AMC after phosphorylation of its MLC. However, this myosin IIB-2 isoform can bind actin in the absence of ATP and is released in its presence (Kim, Kawamoto et al. 2008). Ablation of myosin IIB-2 gives rise to abnormalities in the cerebellar Purkinje cells that effect mice motor coordination. Surprisingly, these abnormalities cannot be rescued by expression of the non-spliced isoform myosin IIB-0 that contains ATPase motor activity (Kim, Kawamoto et al. 2008). These observations suggest myosin IIB-2 may rely more on its structural properties as opposed to force generating properties to effect certain biological processes. These observations coincide with previously mentioned works illustrating a structural role for myosin II in which expression of myosin IIB with impaired ATPase activity was able to rescue the formation of a mesh like structure in nueroepithelial cells to prevent hydrocephalus (Ma, Bao et al. 2007). Therefore, it is possible that there is a structural role for myosin II in other cellular processes such as apoptosis. Although it has been well established that AMC generated by myosin II is required to carry out the morphological hallmarks of apoptosis such as membrane blebbing, cellular contraction, and nuclear disintegration only a few studies have looked at possible roles of myosin II in early apoptotic signaling events that promote cell death. Our study was designed to gain a better understanding of how myosin II and its isoforms contribute to the apoptotic cell
death process. This study looks at the involvement of myosin II activation in early cell death signaling events in TNF-alpha induced apoptosis such as the internalization of TNFR1.

The MLC is an important substrate for a number of the kinases activated by TNF-alpha such as DAPK, Rho kinase, and MLCK (Cohen, Inbal et al. 1999; Kim, Kim et al. 2002; Kuo, Lin et al. 2003; McKenzie and Ridley 2007). Interestingly, signal transduction pathways leading to myosin II activation have been shown to be involved in both apoptotic and survival signaling in response to TNF-alpha. Studies have revealed that inhibiting myosin II activation by impairing MLCK activity through pharmacological and genetic means hinders both NFkappaB signaling and caspase activation (Petrache, Verin et al. 2001; Wadgaonkar, Linz-McGillem et al. 2005). Since the only established substrate for MLCK at this time is the MLC of myosin II I have hypothesized that MLCK along with other kinases activate the myosin II isoforms in separate and distinct manners to mediate the life and death response to TNF-alpha. Works supporting this hypothesis come from studies demonstrating that myosin II isoforms can have separate and distinct functions within the same cell depending on their localization and temporal activation by varying upstream kinases such as MLCK and Rho kinase (Totsukawa, Yamakita et al. 2000; Sandquist, Swenson et al. 2006). Since the activation of myosin II seems to be important for mediating a variety of TNF-alpha’s biological effects we examined the possibility that the non-muscle myosin II isoforms myosin IIA and myosin IIB were being utilized in distinct manners to mediate TNF-alpha cell death signaling.
We present evidence that myosin IIB has an isoform specific role in mediating TNF-alpha induced cell death signaling and that its function in doing so is independent of force generation (AMC).
Chapter II: Materials and Methods

Cell Culture

Hela cells were cultured in Dulbecco’s modification of Eagles medium Cat # 10-017-CV (Mediatech Inc, Herndon Va) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% of penicillin/streptomycin and grown in 5% CO2 at 37°C.

Antibodies and Reagents

Rabbit anti-cleaved caspase 3 (Asp175)(5A1), anti-phospho-MLC (ser18/thr19), anti-TNFR1 (C25C1), anti-phospho-I-kappa-B-alpha (Ser32), anti-I-kappa-B-alpha, anti-clathrin heavy chain, and mouse anti-cleaved caspase 8 (1C12) antibodies were all purchased from Cell Signaling (Danvers, MA). Rabbit anti-MLC2, anti-TNFR1 (H-271), anti-Clathrin HC (H-300), anti-FADD (H-181), and mouse anti-caspase 8 p20 (D-8) antibodies was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-nonmuscle myosin IIA heavy chain and anti-nonmuscle myosin IIB heavy chain antibodies were purchased from Covance (Emeryville, California). Recombinant human TNF-alpha was purchased from R&D systems. ML-7 and Blebbistatin were purchased from BioMol International, LP. Cycloheximide was purchased from Sigma. Cytochalasin D was purchased from Calbiochem. 4',6-diamidino-2-phenylindole (DAPI) was purchased from Invitrogen.
siRNA

The siRNA oligonucleotides used to knockdown myosin IIA and IIB expression were purchased from Dharmacon; the antisense sequence for Myosin IIA (MYH9) is 5’- AAAUCGGUCACAUUGAUACUU-3’. The antisense sequence for Myosin IIB (MYH10) is 5’-UAUUCUCAGAGUAAAUUGGUU-3’. The protocol recommended by the supplier Dharmacon was followed. Briefly, cells were seeded overnight so that they were approximately 50% at the time of siRNA transfection. siRNA was suspended in the supplied RNase free buffer then incubated with the dharmafect transfection reagent (dharmafect 1) at a 11:1 ratio of siRNA to transfection reagent in a total of 400 ul of serum free medium (SFM) for 20min. This mixture was then added to 1.6 ml of complete medium that did not contain any antibiotics. This mixture totaling 2 ml was then added to one well of a 6-well plate in a drop wise manner. The cells were incubated with the medium containing the siRNA transfection complex for 24 hrs. After this time the siRNA containing medium was replaced with new fresh complete medium not containing any antibiotics. Seventy-two hours post transfection was the optimal time for myosin IIA and IIB knockdown as determined through western blot analysis. At this time TNF-alpha and CHX was added to the conditioned medium for approximately 2-3 hours to analyze the effects knockdown of IIA and IIB had on TNF-alpha + CHX induced apoptosis.

Immunofluorescent Staining

Cells were plated on glass coverslips for 24hrs. After treatments the cells were fixed in 3% PFA for 15 min, washed 3x in PBS, then permeabilized with 0.1% TritonX-100 in
PBS for 15 minutes, washed 3x with PBS, and blocked with 5% BSA at room temperature for 30 min. The cells were then incubated with myosin IIB Ab from Covance at a 1:500 dilution in 5% BSA for 30 minutes. Cells were then washed 3 times with PBS and then incubated with Alexa Fluor 594 goat anti-rabbit IgG secondary (Invitorgen, Molecular Probes, Eugene, Oregon) at a 1:500 dilution in 5% BSA for 30 minutes. The 5% BSA also included Oregon green 488 Phalloidin (Molecular Probes). Cells were then washed 3 times with PBS and incubated in a solution containing DAPI nuclear staining for 5 minutes then washed 2 more times. The coverslips containing the cells were placed on glass slides containing ProLong Gold anti-fade reagent (Invitorgen, Molecular Probes, Eugene, Oregon) overnight at room temperature. Cells were viewed and pictures were taken on a Zeiss Axiovert 200M microscope.

**Apoptosis Assay**

Apoptosis was assessed by viewing DAPI stained nuclei through an immunofluorescent scope for the presence of nuclear condensation. The percentage of apoptotic cells present was determined by performing a blind count in which the number of cells with nuclear condensation was divided by the total number of cells counted. Each treatment was done in triplicate and a minimum of 200 cells were counted for each treatment.

**Isolation of Cell Surface TNFR1**

Isolation of cell surface TNFR1 was accomplished by using the cell surface labeling accessory pack from Pierce (Rockford, IL) and following the manufacture’s protocol. Briefly, after TNF-alpha treatment (5ng/ml) cells were washed with cold PBS and
incubated in a biotin/PBS solution for 30 minutes after which time a quenching reagent was added to the dish and cells were harvested by gentle scraping. Cell pellets were spun down and washed once in TBS and then lysed for 30 minutes on ice with vortexing every 5 minutes along with pippeting of samples through a 25 1/8 needle. The samples were spun down and the supernatant was added to a column containing streptavidin beads that were previously incubated in 5% BSA for 30 minutes to help reduce non-specific binding. The supernatant containing the biotinylated protein and the streptavidin beads were rotated in a column for 2 hrs at 4 degrees Celsius. After the incubation the supernatant was spun through the column’s filter and the beads/streptavidin/protein complex was washed 3 times. Laemmli sample buffer + DTT was then added in order to allow proteins to be spun through the column’s filtered bottom and gathered in an eppendorf tube. Samples were then ran on SDS-PAGE gel to analyze the presence of TNFR1.

**Co-Immunoprecipitation**

Cells were seeded to obtain 90% confluency at the time of treatment. The dosage and time of treatments with TNF-alpha, CHX, and drugs are indicated in the figure legends. After treatment cells were kept on ice and washed with ice cold PBS two times then IP lysis buffer containing 50mM Tris HCl (pH 7.4), 150 mM NaCl, 1% NP40, and 0.5% SodiumDeoxycholate was added to cells at a 1.2x concentration. The cells were rocked back and forth for 15 minutes at 4 degrees Celsius and were then gathered into eppendorf tubes through gentle scraping. The cell lysis was then inverted several times and then gently pipetted through a 25 1/8 needle 3 times. Next, the lysis sample was spun down
at an RCF of 16,000g for 12 minutes to form a solid pellet. The supernatant was carefully separated from the pellet and placed in an eppendorf tube containing washed protein A/G agarose beads and rotated for 1 hour at 4 degrees Celsius in order to pre-clear the supernatant. The pre-cleared supernatant was then removed from the beads and placed in a new eppendorf tube. Then, either myosin IIB from Covance, TNFR1(H-271) from santa cruz, FADD (H-181) from Santa Cruz, or caspase 8 p20 (D-8) from santa cruz was added to the supernatant and allowed to rotate overnight. The next day TruBlot anti-Rabbit or anti-Mouse IgG IP beads were added to the supernatant and the Beads/Ab/Supernatant mixture were rotated for 2-3 hours at 4 degrees Celsius. After 2-3 hours the mixture was spun down and the supernatant was removed so that the beads could be washed three times with IP lysis buffer. After the washing SDS-Lamelli sample buffer was added and the samples were vortexed, spun down, and then boiled for 10 minutes.

**Western Blot Analysis**

Cells were harvested in 1x SDS Laemmli sample buffer from Biorad, Hercules CA (cat#161-0737) containing beta-mercaptoethanol and Halt phosphatase (Thermo Scientific) and protease inhibitor cocktail (Sigma). Samples were placed in boiling water for 5 min and loaded into the appropriate percentage of SDS–PAGE gel. After running the lysate samples for approximately 2 hours to get adequate separation of proteins, the samples were transferred to nitrocellulose membrane at 40 volts for 90 min. The nitrocellulose membrane (Hybred-ECL 0.2uM, Amersham Bioscience) was incubated in Ponceau (Sigma) stain for 3 min in order to view the levels of protein present on the
membrane, this helps determine if even loading has been achieved. The membrane was then blocked in 5% milk for one hour. Membranes were then washed and incubated with appropriate primary antibody at a 1:1,000 ratio in 5% BSA (Calbiochem) overnight. Membranes were then washed 3 times for 5 minutes in TTBS at which time the appropriate HRP-conjugated secondary antibody (Cell Signal, Danvers MA) was added to the membrane at a 1:1,000 ratio in 2.5% milk for 1 hour. The membrane was then washed 3 times for 5 minutes then exposed to Super signal west pico chemilumenescent reagent (Pierce). The membrane was then exposed to Blue lite autorad film (ISC Bioexpress). Densitometry analysis of western blots was performed using Image J.

RT-PCR

A reverse transcriptase PCR was performed in order to compare the amounts of TNFR1 and TNFR2 present in Hela cells. RNA isolation was performed using the RNeasy mini kit from Qiagen. Hela cells were harvested using an RNAse free buffer and cells were homogenized by passing them through a 20-gauge needle at least 5 times. The samples had 1 volume 70% ETOH added to them and they were then spun down in an RNeasy column in order to get rid of the ETOH. The samples were washed one time then DNase I was added to degrade any DNA that was present. The samples were then washed multiple times and the RNA was eluted into a collection tube using RNAase free water. A reverse transcriptase (RT) reaction was then performed using reagents and protocol from Applied Biosystems. A master mix (MM) composed of RT-buffer, dNTP mix, Random Primer, MultiScribe RT, and RNAase free water. 50ul MM was added to 50ul
of RNA sample and then mixed by pipetting. Samples were then put at 25°C for 30min and 37°C for 2 hours in order to complete the RT reaction.

PCR was then performed on Hela cDNA samples using forward and reverse primers targeting human TNFR1 and TNFR2. The TNFR1 forward primer sequence used was: 5’-TGGTGGAATATACCCCTCAG; and the reverse TNFR1 primer sequence used was: 5’-GCACTTGGTACAGCAAATCGAAT. The TNFR2 forward primer sequence used was: 5’-CGCTCTTCCAGTTGGACTGAT; and the reverse TNFR2 primer sequence used was: 5’-CACAAGGGCTTCTTTTCACCT. These primers were placed into a master mix then added to Hela and SkBr3 cDNA to perform a PCR reaction overnight in a Veriti 96-well Thermal Cycler. SkBr3 was used as a positive control for TNFR2. In addition, a PCR reaction was performed on the Hela and SkBr3 cDNA samples using GAPDH for loading control.
Chapter III: Results

ML-7 treatment impairs TNF-alpha signaling

TNF-alpha promotes the translocation of NFκB to the nucleus in order to promote the expression of a variety of anti-apoptotic proteins such as Bcl-xl, FLIP, and IAPs (Pahl 1999). Therefore, the use of a protein synthesis inhibitor such as cycloheximide (CHX) is often times employed to prevent the expression of these anti-apoptotic proteins to allow TNF-alpha cell death signaling to proceed. It is important to note that the use of CHX does not alter TNF-alpha cell death signaling that occurs through formation of the DISC complex (Micheau and Tschopp 2003). Therefore, Hela cells were treated with 5ng/ml TNF-alpha in combination with 10ug/ml CHX in order to analyze the relationship between TNF-alpha cell death signaling and myosin II activation.

Myosin light chain (MLC) phosphorylation results in the activation of non-muscle myosin II and this event has been shown to play an important role in TNF-alpha cell death signaling. MLCK, DAPK, and Rho kinase all play important roles in promoting TNF-alpha induced apoptosis through their ability to phosphorylate MLC and activate myosin II (Cohen, Inbal et al. 1999; Jin, Atkinson et al. 2001; Petrache, Verin et al. 2001; Kuo, Lin et al. 2003). In order to examine the role of myosin II activation in TNF-alpha cell death signaling we impaired MLC phosphorylation by treating cells with the MLCK inhibitor ML-7. Figure 3A shows that concentrations as low as 10uM of ML-7 was able to impair MLC phosphorylation by approximately 20% indicating a role for MLCK in mediating increases in MLC phosphorylation in response to TNF-alpha. As the concentration of ML-7 increases it has the ability to impair other kinases that are
activated by TNF-alpha that can phosphorylate the MLC such as PKC and DAPK (Schumacher, Velentza et al. 2002; Fazal, Gu et al. 2005). Therefore, figure 3A illustrates that as the concentration of ML-7 increases to 75uM MLC phosphorylation is drastically reduced by approximately 80%. Figure 3B provide western blots demonstrating the decrease seen in MLC phosphorylation at ML-7 concentrations of 10uM and 75uM.

**Figure 3.** Treatment with ML-7 impairs MLC phosphorylation in response to TNF-alpha. (A) Hela cells were pretreated with increasing amounts of ML-7 from 10uM to 75uM for 30 minutes then 10ng/ml of TNF-alpha (TNF) + 10ug/ml of cycloheximide (CHX) was added to the medium for 3.5 hours. Myosin II activation was then assessed by western blotting for di-phosphorylation of the MLC (Thr18/Ser19). The graph represents the percentage of pp-MLC in cells treated with ML-7 as compared to the amount of pp-MLC seen in the TNF-alpha + CHX control (n=3). (B) Representative western blots for pretreatment of cells with 10uM and 75uM ML-7 illustrate the decrease in pp-MLC as the concentration of ML-7 increases.
Previous works have suggested that MLC phosphorylation by upstream kinases such as MLCK, Rho kinase, and DAPK are important for mediating TNF-alpha cell death signaling. In order to determine if MLC phosphorylation played a role in mediating TNF-alpha induced cell death signaling Hela cells were pretreated with increasing amounts of ML-7. Figure 4A shows that a concentration of 10uM ML-7 was able to impair cleavage of caspase 3 and 8 by approximately 20% indicating a role for MLCK in mediating TNF-alpha cell death signaling. ML-7 has also been shown to impair DAPK activity at concentrations above 50uM and figure 4A illustrates an 80% decrease in caspase cleavage at 75uM suggesting a possible role for DAPK in this system.

**Figure 4.** Treatment with ML-7 impairs cell death signaling in response to TNF-alpha. (A) Hela cells received the same ML-7 and TNF-alpha + CHX treatments as Figure 1 and cell death signaling was assessed by western blotting for cleavage of caspases 3 and
8. Percentage of caspase cleavage was determined by dividing the values obtained for TNF-alpha + CHX cells that were pretreated with ML-7 by the values obtained for TNF-alpha + CHX control cells. These values were arbitrary units obtained through densometric analysis of western blots (n=3). (B) Representative western blots for pretreatment of cells with increasing amounts of ML-7 illustrate decreased amount of caspase cleavage as the concentration of ML-7 increases.

As mentioned above, increasing concentrations of ML-7 can impair other kinases activated by TNF-alpha such as protein kinase A (PKA) and protein kinase C (PKC). Therefore, Hela cells were treated with the PKA inhibitor H-89 and the PKC inhibitor BisI to determine if these kinases were playing a role in TNF-alpha induced cell death signaling. Results show that increasing amounts of H-89 and BisI of up to 40 uM were unable to prevent caspase cleavage in response to TNF-alpha (Figures 5A and 5B respectively). This is consistent with reports illustrating that PKA and PKC help regulate endothelial cell permeability in response to TNF-alpha and that inhibition of PKC can actually sensitize B16 melanoma B6 cells to TNF-alpha induced apoptosis (Ferro, Neumann et al. 2000; Nishida, Yoshioka et al. 2003; Qiao, Huang et al. 2003). These results demonstrate that ML-7 can impair MLC phosphorylation in response to TNF-alpha making it possible to study the involvement of myosin II activation in TNF-alpha cell death signaling.
Figure 5. Inhibition of PKA or PKC does not effect TNF-alpha cell death signaling. (A) Hela cells were treated with increasing amounts of the PKA inhibitor H-89 (B) and the PKC inhibitor BisI for 30 minutes prior to treatment with TNF + CHX to the medium. These inhibitors were unable to impair TNF-alpha induced cell death signaling as illustrated by western blot of cleaved caspase 3 and 8.

In addition to the cleavage of caspases TNF-alpha can also promote the translocation of NFkappaB to the nucleus. In quiescent cells NFkappaB is retained in the cytosol through its interaction with the IkBalpha protein. Upon TNF-alpha stimulation IKK kinase is activated resulting in the phosphorylation of IkBalpha which targets it for proteasomal degradation thereby freeing NFkappaB and allowing it to translocate to the nucleus (Klement, Rice et al. 1996; Hayden and Ghosh 2004). To determine if a TNF-alpha induced activation of myosin II might play a role in mediating NFkappaB signaling in Hela cells we examined the effects of ML-7 treatment on IkBalpha phosphorylation. The graph in figure 6A demonstrates that pretreatment of cells with 75uM of ML-7
impairs IkBalpha phosphorylation by approximately 56% at the 15 minute time point. This inhibition of phosphorylation impairs the cells ability to degrade IkBalpha by approximately 71% at the 60 minute time point. The western blot in figure 6B illustrates that ML-7 treatment significantly reduced the amount of IkBalpha phosphorylation seen at the 15 minute time point as compared to control. This inhibition of IkBalpha phosphorylation in ML-7 treated cells also prevented the degradation of total IkBalpha at the 30 and 60 minute time point as compared to control. The inability of cells treated with ML-7 to phosphorylate and degrade IkBalpha suggests a role for myosin II activation in promoting NFkappaB signaling in response to TNF-alpha. Collectively, our data suggests a central role for myosin II activation in mediating both the survival and apoptotic response of Hela cells to TNF-alpha.

Figure 6. Treatment with ML-7 impairs TNF-alpha survival signaling. (A) Hela cells were pretreated with 75uM of ML-7 for 30 minutes then treated with TNF-alpha + CHX for the indicated times. The graph represents the inverse relationship between an increase
in IkBalpha phosphorylation and the decrease in protein expression in control cells (n=3). This relationship is impaired by ML-7 treatment which prevents IkBalpha phosphorylation and thereby impairs proteasomal degradation of IkBalpha. (B) This is a representative western blot illustrating that IkBalpha phosphorylation at 15 minutes and degradation by 60 minutes is impaired by treatment with 75uM ML-7.

Myosin IIB helps mediate TNF-alpha cell death signaling

Myosin II activation plays an important role in mediating TNF-alpha signaling, therefore, we wanted to determine if myosin IIA or myosin IIB were being utilized in an isoform specific manner to mediate the TNF-alpha cell death response. In order to address this question the use of siRNA against myosin IIA (siIIA), myosin IIB (siIIB), and a scrambled siRNA control (siCon) was employed. Although the myosin II isoforms share a great deal of amino acid identity with 85% similarity in the myosin motor domain and 72% similarity in the alpha-helical tail domain the siRNA knockdown of myosin IIA and IIB is isoform specific, figure 7A is representative of the knockdown obtained in our siRNA experiments (Bresnick 1999). Once the specificity of the isoform knockdown was verified Hela cells were treated with siCon, siIIA, or siIIB and then exposed to TNF + CHX to determine the ability of these cells to carry out TNF-alpha cell death signaling. The western blot in Figure 7B illustrates that cells treated with siIIB had a significant decrease in the amount of caspase 3 cleavage in response to TNF-alpha as compared to the siCon and siIIA treated cells. The graph in Figure 7C illustrates that cells treated with siIIB had a 38% decrease in the amount of caspase 3 cleavage in response to TNF-alpha as compared to the siCon treated cells and a 45% decrease as compared to the siIIA treated cells.
Figure 7. Knockdown of myosin IIB impairs cell death signaling in response to TNF-alpha. (A) Hela cells were treated with siRNA against myosin IIA (silIA), myosin IIB (silIB), and siRNA control sequence (siCon) as described in experimental procedures. Isoform specific knockdown was assessed by western blotting for the expression of the myosin IIA and myosin IIB protein. (B) Hela cells were treated with siRNA in order to knockdown the expression of myosin IIA or IIB. These cells were then treated with 10ng/ml TNF-alpha + 10ug/ml of CHX for 3hrs and the ability of these cells to transmit TNF-alpha cell death signaling was determined through western blot analysis of effector caspase 3 cleavage. (C) Two-tailed student’s t-test determined that the decrease of caspase 3 cleavage seen in silIB treated cells as compared to siCon (*) or silIA (#) is statistically significant with p-values < .05 (n=3).

Caspase 3 plays an important role in nuclear condensation during the execution phase of apoptosis by breaking down components of the nuclear matrix (Kivinen, Kallajoki et al. 2005). Since knockdown of myosin IIB impairs caspase 3 cleavage we
wanted to determine if siIIB treatment was also able to impair nuclear condensation. Immunofluorescence images in Figure 8A illustrate that cells treated with siIIB had much less nuclear condensation as compared to the siCon and siIIA treated cells in response to TNF-alpha (white arrowheads). The graph in figure 8B illustrates that the siCon and siIIA treated cells had a 19% and 24% increase in nuclear condensation in response to TNF-alpha as compared to their respective CHX controls whereas siIIB treated cells only had a 7.4% increase as compared to its CHX control. Taken together these data suggest an important role for myosin IIB, but not IIA, in mediating TNF-alpha induced cell death.

**Figure 8.** Knockdown of myosin IIB impairs nuclear condensation in response to TNF-alpha. (A) Hela cells were treated with the indicated siRNA, then TNF-alpha + CHX was added to the medium for 3.5 hours. The cells were then stained with DAPI and the nuclei were visualized under an immunofluorescent microscope. The white arrowheads indicate nuclei undergoing nuclear condensation. The percentage of apoptosis was assessed through visualization of nuclear condensation as described in experimental procedures. (B) Two-tailed student’s t-test determined that the decrease of nuclear condensation in siIIB treated cells as compared to siCon (*) or siIIA (*) is statistically significant with p-values < .05 (n=3).
TNF-alpha cell death signaling does not require AMC

Myosin light chain (MLC) phosphorylation resulting in activation of non-muscle myosin II has been shown to effect cell signaling events independent of AMC. For example, MLC phosphorylation has been shown to promote RAP1 signaling at the plasma membrane to facilitate collagen phagocytosis by fibroblasts independent of AMC (Arora, Conti et al. 2008). Therefore, in order to gain a better understanding of the role activated myosin II plays in TNF-alpha induced apoptosis we wanted to determine if AMC was required for TNF-alpha cell death signaling. In order to address this question we treated Hela cells with the pharmacological agent blebbistatin which specifically inhibits non-muscle myosin II motor activity without impairing MLC phosphorylation (Kovacs, Toth et al. 2004; Limouze, Straight et al. 2004; Allingham, Smith et al. 2005; Ponsaerts, D'Hondt et al. 2008). Blebbistatin treatment impairs the ability of myosin II to process ATP resulting in disruption of myosin II association with actin filaments leading to actin cytoskeletal destabilization (Figure 9).
Figure 9. Effects of blebbistatin and cytochalasin D treatment on actin cytoskeletal structure and myosin IIB localization. (A) Immunofluorescent images of the effects that a 30 minute treatment with 100uM blebbistatin and 10uM cytochalasin D have on the structure of actin filaments (green) and distribution of myosin IIB (red) in Hela cells. Blebbistatin disrupts actin filament formation causing a widely dispersed localization of myosin IIB. Cytochalasin D treatment completely inhibits actin filament formation causing smaller collapsed cells to form and promoting small aggregates of myosin IIB (Scale bar 20uM).

The IC50 for inhibiting non-muscle myosin II ATPase activity with blebbistatin is approximately 4uM. Therefore, we performed a dose response experiment using 5uM to 100uM of blebbistatin to determine if AMC is necessary for TNF-alpha cell death signaling. The graph in figure 10A demonstrates that treatments with blebbistatin were unable to significantly impair caspase 3 and 8 cleavage in a dose dependent manner in response to TNF-alpha. The representative western blots in figure 10B also illustrate that increasing concentrations of blebbistatin were unable to impair cell death signaling in response to TNF-alpha.
Figure 10. Treatment with blebbistatin does not impair TNF-alpha cell death signaling. (A) Hela cells were pretreated with increasing amounts of blebbistatin from 5uM to 100uM for 30 minutes then 10ng/ml of TNF-alpha + 10ug/ml of cycloheximide (CHX) was added to the medium for 3.5 hours. The graph represents the percentage of caspase 3 and 8 cleavage in cells pretreated with blebbistatin as compared to the amount of caspase 3 and 8 cleavage seen in the TNF-alpha + CHX control (n=3). (B) Representative western blots illustrate that increases in blebbistatin concentration were unable to inhibit the cleavage of caspases 3 and 8.

Although blebbistatin treatment is established as an effective method to impair AMC, an alternative means of impairing AMC that does not directly target the myosin II protein is through treatment with cytochalasin D. This pharmacological agent disrupts the formation of actin filaments (Figure 9) providing another approach in which to examine the involvement of AMC and actin filament re-organization in TNF-alpha cell death signaling. Although cytochalasin D disrupts actin filaments thereby impairing force generation by myosin II, it was unable to inhibit the cleavage of caspases 3 and 8 in
response to TNF-alpha (Figure 11A). The western blots in figure 11B illustrate that increasing amounts of cytochalasin D from 10uM to 30uM did were unable to prevent cleavage of caspase 3 and 8.

**Figure 11.** Treatment with cytochalasin D does not impair TNF-alpha cell death signaling. (A) Hela cells were pretreated with increasing amounts of cytochalasin D (Cyt D) from 10uM to 30uM for 30 minutes. Next, 10 ug/ml of TNF-alpha + 10 ug/ml CHX was added to the medium for 3.5 hours. The graph represents arbitrary units of caspase cleavage from experiments using 10uM cytochalasin D treatments (similar results were found for 20uM and 30uM cytochalasin D). Arbitrary units were determined using Image J densitometry analysis of western blots (n=3). (B) A representative western blot illustrates that increasing amounts of Cyt D from 10uM to 30uM was unable to inhibit the cleavage of caspases 3 and 8.
It is important to note that MLC phosphorylation in response to TNF-alpha was not inhibited by blebbistatin or cytochalasin D treatments (Figure 12A). Western blots illustrate that 100um blebbistatin and 30uM cytochalasin D were unable to impair MLC di-phosphorylation (Figure 12B); similar results were found for lower concentrations of blebbistatin and cytochalasin D. Therefore, these data suggest a role for activated myosin II in TNF-alpha cell death independent of AMC.

**Figure 12.** Treatment with blebbistatin or cytochalasin D does not impair MLC phosphorylation in response to TNF-alpha. (A) Hela cells were pretreated with 100uM blebbistatin and 30uM Cyt D for 30 minutes then MLC di-phosphorylation in response to TNF-alpha + CHX was measured at the 15 minute time point. The graph represents arbitrary units from 3 independent experiments measuring MLC di-phosphorylation (pp-MLC) through densometric Image J analysis of western blots. (B) A representative
western blot illustrates that 100μM blebbistatin and 30μM Cyt D was unable to inhibit pp-MLC in response to TNF-alpha (similar results were found for lower concentrations of blebbistatin and Cyt D; data not shown).

**ML-7 treatment and knockdown of myosin IIB expression impair TNFR1 internalization**

Impairing the phosphorylation of the MLC disrupts both survival and apoptotic signals in response to TNF-alpha suggesting that myosin II activation is an event that is upstream of these two signaling pathways. One crucial upstream event that is required for TNF-alpha induced apoptosis is the internalization of the TNFR1 receptor which has been shown to occur within minutes after TNF-alpha binding (Schutze, Machleidt et al. 1999; Schneider-Brachert, Tchikov et al. 2004; Schneider-Brachert, Tchikov et al. 2006). Therefore, we wanted to determine if myosin II activation was mediating the TNF-alpha cell death response through an involvement in TNFR1 internalization. However, since TNFR2 has been shown to bind TNF-alpha and has been shown to induce apoptosis in certain cell systems we first wanted to ensure that TNF-alpha induced apoptosis was more then likely proceeding through signaling initiated by TNFR1 (Depuydt, van Loo et al. 2005). Therefore, we performed a qualitative PCR against TNFR1 and TNFR2 mRNA present in Hela cells and found that TNFR1 mRNA was present in far greater amounts then that of TNFR2 which is consistent with other works reporting that TNFR1 is the predominantly expressed TNF-alpha receptor on Hela (Figure 13) (Grell, Wajant et al. 1998; Jupp, McFarlane et al. 2001). Therefore, since TNFR1 contains a death domain on its cytoplasmic tail while TNFR2 does not and TNFR1 is expressed in a far greater amount suggests it is the main receptor mediating TNF-alpha induced apoptosis in Hela cells.
Figure 13. TNFR1 expression in Hela cells. RNA isolation was performed on whole cell lysate samples of Hela and SkBr3 cells followed by a reverse transcriptase reaction to acquire cDNA. A qualitative PCR reaction was performed using primers against TNFR1 and TNFR2. PCR reactions against GAPDH in Hela and SkBr3 were performed to ensure equal loading between the samples.

Figure 14A is a representative western blot from a cell surface biotinylation procedure to measure the amount of cell surface TNFR1 that is present before and after treatment with TNF-alpha. The blot shows that treatment with TNF-alpha causes a significant decrease in the amount of cell surface TNFR1 by the 30 minute time point. The presence of TNFR1 in whole cell lysate samples indicates that the decrease in cell surface TNFR1 in response to TNF-alpha was due to internalization and not to release of the receptor from the cell membrane. The graph in figure 14B illustrates that ML-7 treatment prevented the 77% decrease seen in cell surface TNFR1 at 30 minutes indicating a role for myosin II activation during the internalization process.
Figure 14. ML-7 treatment impairs TNFR1 internalization. (A) Cells were pretreated with 75uM of ML-7 for 30 min then 10ug/ml of TNF-alpha + 10ug/ml CHX was added to the medium for an additional 30 min at which time cell surface TNFR1 was isolate as described in experimental procedures. Samples were then ran out on western blot to measure decreases in cell surface TNFR1. Whole cell lysates were ran to ensure that the decrease in cell surface TNFR1 was due to internalization and not the cleavage and release of the receptor from the plasma membrane. (B) Arbitrary units representing cell surface TNFR1 were acquired through Image J densometric analysis. (C) Cells were grown on glass coverslips overnight then pretreated with 75uM ML-7 for 30 minutes at which time TNF-alpha + CHX was added to the medium for an additional 30 minutes. The first set of Hela cells were fixed with paraformaldehyde (PFA) then stained for cell surface TNFR1. The second set was fixed with PFA then permeabilized with Triton X-100 to stain for the total amount of TNFR1 present in the cell.
To determine if myosin IIA and IIB have isoform specific roles in mediating TNFR1 internalization we silenced their expression through siRNA treatment before addition of TNF-alpha to the medium. The graph in figure 15A illustrates a 2 fold increase in the amount of cell surface TNFR1 seen in cells treated with siIIB as compared to the siIIA or siCon treatments. The representative western blot in figure 15B shows that knockdown of myosin IIB impairs TNFR1 internalization as compared to the internalization seen for siIIA and siCon treated cells. These results suggest a role for activation of myosin IIB, but not IIA, in promoting the internalization of the TNFR1 receptor.

**Figure 15.** Knockdown of myosin IIB expression impairs TNFR1 internalization. (A) Cells were treated with siCon, siIIA, and siIIB as described in experimental procedures then 10ug/ml of TNF-alpha + 10ug/ml CHX was added to the medium for 30 min. The
graph illustrates an approximate 2-fold increase in the amount of cell surface TNFR1 present in the siIIB treated cells. Two-tailed student’s t-test determined that the decrease of TNFR1 internalization seen in siIIB treated cells as compared to siCon (*) or siIIA (#) is statistically significant with p-values < .05 (n=3). (B) Representative western blot illustrating the increased presence of cell surface TNFR1 in siIIB treated cell sample.

The contractile forces generated by myosin II as well as myosin VI and myosin 1E have been suggested to play varying roles during receptor endocytosis (Samaniego, Sanchez-Martin et al. 2007; Ungewickell and Hinrichsen 2007). In order to determine if AMC produced by myosin II was involved in TNFR1 internalization we treated cells with blebbistatin to specifically inhibit the ATPase function of myosin II (Kovacs, Toth et al. 2004; Limouze, Straight et al. 2004; Allingham, Smith et al. 2005). Figure 16 illustrates that blebbistatin treatment was unable to prevent the decrease seen in cell surface TNFR1 in response to TNF-alpha. This result indicates that AMC produced by TNF-alpha activation of myosin II is not required for TNFR1 internalization and is consistent with blebbistatin treatment being unable to prevent caspase cleavage in response to TNF-alpha.

<table>
<thead>
<tr>
<th></th>
<th>TNF</th>
<th>CHX</th>
<th>Blebbistatin</th>
<th>Surface TNFR1</th>
<th>Total Biotinylated Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>_</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surface TNFR1</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Biotinylated Protein</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 16.** Treatment with blebbistatin does not impair TNFR1 internalization. Cells were pretreated with 100uM of blebbistatin for 30 min then 10ug/ml of TNF-alpha + 10ug/ml CHX was added to the medium for an additional 30 min at which time cell surface TNFR1 was isolated and ran out on western blot to measure the amount of TNFR1 internalization. Western blot illustrates triplicate samples of cells pretreated with blebbistatin that were unable to prevent TNFR1 internalization in response to TNF-alpha.
Activated myosin IIB associates with proteins involved in TNF-alpha signaling

Since internalization of TNFR1 has been shown to be clathrin mediated (Mosselmans, Hepburn et al. 1988) we wanted to determine if there was an association between clathrin and TNFR1 in Hela cells treated with TNF-alpha. Internalization of TNFR1 occurs as early as 7.5 minutes with a concomitant increase in myosin II activation (Figure 17A). Therefore, we performed an immunoprecipitation (IP) against TNFR1 at 15 minutes and western blotted for its interaction with clathrin. The representative blot in figure 17B illustrates an increased association between TNFR1 and clathrin at the 15 minute time point. This association was impaired by pretreatment of cells with ML-7 but not blebbistatin indicating a role for MLC phosphorylation and myosin II activation in their interaction independent of AMC.

**Figure 17.** TNFR1 associates with clathrin in response to TNF-alpha. (A) Hela cells were treated with 10ng/ml TNF-alpha + 10ug/ml CHX for 7.5 minutes at which time cell surface TNFR1 was isolated and the amount present was analyzed through western blot.
A western blot illustrating a concomitant increase in di-phosphorylated MLC (pp-MLC) is also shown for the 7.5 min time period. (B) Hela cells were treated with ML-7 (75uM) or blebbistatin (100uM) for 30 minutes at which time the above concentrations of TNF-alpha + CHX was added to the cells for 15 minutes. Cells were harvested for IP of TNFR1 and its association with clathrin in response to TNF-alpha was analyzed by western blot (n=3).

Based on the previous data myosin IIB was the isoform implicated in TNFR1 internalization, therefore, we wanted to determine if there was any interaction between myosin IIB with clathrin or TNFR1. We performed an IP against myosin IIB and found that there was an increased association with clathrin, but not actin, in response to TNF-alpha indicating that clathrin pulldown is not a result of its association with actin filaments (Figure 18).

<table>
<thead>
<tr>
<th></th>
<th>WCL</th>
<th>Ig</th>
<th>IP: IIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-alpha</td>
<td>–</td>
<td>+</td>
<td>– +</td>
</tr>
<tr>
<td>CHX</td>
<td>+</td>
<td>+</td>
<td>+ +</td>
</tr>
<tr>
<td>Clathrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IIB</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Figure 18.** Myosin IIB associates with clathrin in response to TNF-alpha. Hela cells were treated with TNF-alpha + CHX for 15 minutes and cells were harvested for IP of IIB. Whole cell lysates (WCL) and Ig negative controls were ran out on western blot with IPs of IIB to analyze the presence of actin and clathrin within the samples (n=3).
Next, we performed IPs in the presence of ML-7 and blebbistatin and found association between clathrin and myosin IIB was impaired by pretreatment of cells with ML-7 but not blebbistatin suggesting a role for MLC phosphorylation and myosin II activation in these protein interactions independent of force generation (Figure 19). Western blot analysis of myosin IIB IP samples revealed that there was no detectable association between myosin IIB and TNFR1 (data not shown).

<table>
<thead>
<tr>
<th></th>
<th>TNF-alpha</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>CHX</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ML-7</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Blebbistatin</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

**Figure 19.** ML-7 treatment but not blebbistatin impairs myosin IIB association with clathrin in response to TNF-alpha. Cells were pretreated with 75uM ML-7 and 100uM of blebbistatin then TNF-alpha + CHX was added to the medium for 15 minutes. Cells were then harvested for IP of myosin IIB and then ran out on western blot to analyze the presence of clathrin and myosin IIB in the samples (n=3).
In addition to TNFR1 internalization the formation of the DISC complex is another event that is crucial for initiating TNF-alpha induced apoptosis (Micheau and Tschopp 2003; Schneider-Brachert, Tchikov et al. 2004). For this reason we wanted to examine the possibility of myosin IIB interacting with key members of the DISC complex such as FADD and caspase 8. Figure 20 illustrates that at the 3.5 hour time point in which cleavage of caspases 3 and 8 are detectable there is also an increased association of FADD and caspase 8 with myosin IIB.

**Figure 20.** Myosin IIB associates with pro-apoptotic signaling proteins FADD and caspase 8 in response to TNF-alpha. Immunoprecipitations for FADD and caspase 8 were performed at 3.5 hours after treatment with TNF-alpha + CHX. Association of FADD and caspase 8 with myosin IIA and IIB was analyzed through western blot (n=3).
Increased associations of FADD with myosin IIA were inconsistent and when interactions were detected between FADD and IIA they were significantly less than what was seen with myosin IIB (Figure 21). FADD interaction with both isoforms was inhibited by ML-7 treatment indicating a role for myosin II activation in these associations. It is worth noting that attempts to co-IP FADD and caspase 8 in their associated state to detect the formation of the DISC complex were unsuccessful. Therefore, it is still unclear whether or not myosin IIB is an actual member of the DISC complex, however, these observations do suggest a possible role for myosin IIB in DISC formation.

Figure 21. ML-7 impairs myosin IIB association with FADD. (A) Cells were pretreated with 75uM ML-7 for 30 minutes then treated with TNF-alpha + CHX for 3.5 hours at which time IP against FADD was performed and its association with myosin IIA and IIB was analyzed through western blot. (B) The graph represents fold increase in association of FADD with either myosin IIA or IIB with CHX control value set at 1. This representative IP experiment illustrates myosin IIA association with FADD increasing by 14 fold while myosin IIB association with FADD increased by 98 fold.
Chapter IV

Discussion

This investigation has provided new insights into the roles for myosin IIA versus IIB in mediating TNF-alpha cell death signaling. Previous evidence that myosin II was involved in mediating the biological effects of TNF-alpha comes from works demonstrating that inhibition of MLCK and Rho kinase through pharmacological and genetic means impaired MLC phosphorylation and cell signaling events associated with cell death and survival in response to TNF-alpha (Petrache, Birukov et al. 2003; Petrache, Crow et al. 2003). Work done in MDCK cells showed that treatment with ML-7 and MLCK kinase dead mutants impaired TNFR1 translocation to the plasma membrane and delayed the formation of FADD and caspase 8 aggregates (Jin, Atkinson et al. 2001). Work done in bovine endothelial cells also suggested a role for MLCK in mediating TNF-alpha induced apoptosis by demonstrating that ML-7 treatment impaired MLC phosphorylation, Annexin-V staining, and caspase 8 activity (Petrache, Verin et al. 2001).

Use of the pharmacological agent ML-7 is commonly used to inhibit MLCK activity. Our data show that at 10uM ML-7 there is approximately a 20% decrease in MLC-phosphorylation and caspase 3 and 8 cleavage. This data is consistent with previous works that suggest MLCK plays an important role in mediating TNF-alpha cell death, however, there is still 80% of the cells that are able to phosphorylate MLC and cleave caspase 3 and 8. One study reported that since the Ki of ML-7 is 0.3uM that 20uM of ML-7 inhibits 90% of the MLCK present in the cell, therefore, using this work
as a reference that means approximately 45% of the MLCK present in the cell is inhibited by 10uM (Fazal, Gu et al. 2005). Therefore, at 10uM ML-7 there is still a significant amount of cell death signaling occurring which is most likely due to the uninhibited MLCK, Rho kinase, DAPK and possibly other kinases that are activated by TNF-alpha that promote apoptosis. However, as ML-7 concentration increases TNF-alpha cell death signaling begins to decrease. At 20uM of ML-7 more MLCK is being inhibited resulting in caspase 3 and 8 cleavage decreasing by approximately 40% to 45%. This increased inhibition of MLCK and caspase cleavage may also be having an indirect effect on Rho kinase pro-apoptotic activity since it has been shown that active caspase 3 can cleave Rho kinase giving rise to a constitutively active fragment that supports apoptosis in response to TNF-alpha (Croft, Coleman et al. 2005). Therefore, as increased amounts of ML-7 inhibit cell death signaling Rho kinase apoptotic function may also be impaired by the decreased presence of active caspase 3.

DAPK is another kinase that promotes TNF-alpha induced apoptosis with catalytic activity that can be impaired by ML-7. DAPK’s catalytic domain shares 44% identity with the corresponding catalytic domain of MLCK and both are under calcium/calmodulin regulation (Bialik and Kimchi 2006). At concentrations above 50uM ML-7 has been shown to impair DAPK’s serine/threonine catalytic activity (Schumacher, Velentza et al. 2002). Our results show that at 75uM MLC phosphorylation and caspase cleavage were drastically reduced by approximately 80% as compared to control. At 75uM the MLC phosphorylating kinases involved in TNF-alpha cell death are severely impaired, however, at 75uM there is also likely to be non-specific effects of the drug. Protein kinase A (PKA) and protein kinase C (PKC) are involved in the regulation of
endothelial cell permeability and their kinase activity has been shown to be impaired by 21uM and 42uM ML-7 respectively (Ferro, Neumann et al. 2000; Qiao, Huang et al. 2003; Fazal, Gu et al. 2005). However, our data showed that inhibition of these kinases did not effect TNF-alpha cell death signaling. These data suggest that ML-7’s ability to inhibit TNF-alpha cell death signaling is not due to impairment of PKA or PKC activity. Therefore, although the exact mechanism of how 75uM ML-7 inhibits TNF-alpha cell death is not clear, these results demonstrate a dose sensitive response of MLC phosphorylation and caspase cleavage to ML-7 indicating the involvement of a cell death signaling pathway(s) that involves myosin II activation in response to TNF-alpha.

The function of myosin II and its force generating property in membrane blebbing, nuclear condensation, and cellular contraction during the execution phase of apoptosis have been reported (Cotter, Lennon et al. 1992; Coleman, Sahai et al. 2001; Croft, Coleman et al. 2005). Caspase 3 mediated cleavage of Rho kinase in NIH3T3 cells treated with TNF-alpha forms a constitutively active fragment that promotes the formation of actin stress fibers through LIMK and myosin II activation (Croft, Coleman et al. 2005). Increases in MLC phosphorylation allow myosin II to generate AMC that is essential for membrane blebbing and nuclear disintegration. The importance for an intact actin cytoskeleton and generation of AMC for the execution of apoptosis is demonstrated through the inhibition of nuclear disintegration by treatment with cytochalasin D and blebbistatin (Croft, Coleman et al. 2005; Moss and Lane 2006). Interestingly, work done in NIH3T3 cells demonstrated that myosin IIA protein was degraded in a caspase 3 dependent manner leaving myosin IIB as the essential isoform needed for AMC during the execution phase of TNF-alpha induced apoptosis, however, this degradation of
myosin IIA in response to TNF-alpha was not seen in our Hela cell system (Solinet and Vitale 2008). Although the role of myosin II in the execution phase of apoptosis has been established its involvement in apoptotic signaling prior to the execution phase has not been as thoroughly studied.

Previous works demonstrated a role for myosin II in both survival signaling through NFkappaB and apoptosis signaling through caspase activation in response to TNF-alpha (Petrache, Birukov et al. 2003; Wadgaonkar, Linz-McGillem et al. 2005). Data suggesting that myosin II activation plays a role in both of these signaling pathways indicated a possibility that the myosin II isoforms may be utilized in a separate and distinct manner to mediate the life and death response to TNF-alpha. This investigation examined the role that myosin II isoforms play in mediating the TNF-alpha cell death response by inhibiting the expression of myosin IIA and IIB through siRNA treatment. The results show knockdown of myosin IIB expression, but not myosin IIA, impairs caspase 3 cleavage in response to TNF-alpha. These data suggest an isoform specific function for myosin IIB in mediating TNF-alpha cell death signaling prior to the execution phase. Preventing early cell death signaling events can impair the ability of the cell to carry out the execution phase of apoptosis. For example, interfering with the initial activation of caspase 3 impairs a cell’s ability to breakdown components of the nuclear matrix such as lamins A, B1, or C to allow AMC forces to promote nuclear condensation (Croft, Coleman et al. 2005; Kivinen, Kallajoki et al. 2005). My results show that knockdown of myosin IIB impairs caspase 3 cleavage which is consistent with my finding showing the inability of these same cells to undergo nuclear condensation providing further evidence of a central role for myosin IIB in TNF-alpha cell death. It
was also observed that knockdown of myosin IIA or myosin IIB does not appear to effect NFκB signaling as measured by IκBα phosphorylation (data not shown).

Whether myosin IIC is involved in survival signaling or myosin IIA and IIB have redundant functions in NFκB signaling remains to be investigated and is beyond the scope of the present study. Although the roles for myosin IIA and IIB in TNF-alpha survival signaling are yet to be determined, to our knowledge these data provide the first report of a myosin IIB isoform specific function in mediating cell death signaling prior to the execution phase.

Previous studies have proposed AMC produced by myosin II facilitates TNF-alpha cell death signaling prior to the execution phase. These works have suggested an involvement for AMC in TNFR1 translocation to the plasma membrane as well as a possible role in DISC formation (Jin, Atkinson et al. 2001; Petrache, Crow et al. 2003). Work done in bovine endothelial cells demonstrates a concomitant increase in MLC phosphorylation and stress fiber formation with TNF-alpha induced caspase activation. These events are thought to be MLCK and Rho kinase dependent since pharmacological and genetic inhibition of these kinases prevents these cytoskeletal events and caspase activation from occurring in response to TNF-alpha (Petrache, Verin et al. 2001; Petrache, Crow et al. 2003). Therefore, investigators have suggested that increases in AMC and remodeling of the actin cytoskeleton facilitate translocation of the TNFR1 receptor and formation of the DISC complex (Jin, Atkinson et al. 2001; Petrache, Crow et al. 2003). The involvement of MLCK and Rho kinase in promoting TNF-alpha cell death can be accurately concluded by these studies since they directly inhibit the activity of
these kinases. However, the involvement of AMC was not directly analyzed and, therefore, to conclude that AMC is essential for TNF-alpha cell death would take further investigation.

The involvement of AMC in apoptotic signaling events leading to caspase activation is based on increases seen in MLC phosphorylation and actin filament formation in response to TNF-alpha, which indeed can lead to increases in AMC. Although MLC phosphorylation is required to initiate AMC it is not sufficient to do so on its own. Activation of the myosin II motor domain through ATP hydrolysis and binding to actin filaments must also be achieved to initiate AMC (Tyska and Warshaw 2002; Sellers and Knight 2007). Therefore, MLC phosphorylation, hydrolysis of ATP, and binding to actin filaments can be considered three distinct events all leading to the production of AMC. Further evidence that MLC phosphorylation can be considered a distinct event from force generation come from works showing myosin II functions that are activated by MLC phosphorylation and are independent of AMC (Ben-Ya'acov and Ravid 2003; Arora, Conti et al. 2008; Vicente-Manzanares, Koach et al. 2008). For example, studies show that inhibition of MLCK with ML-7 prevents the localization of myosin II to the contractile ring during cytokinesis while inhibition of myosin motor activity by deletion of the myosin motor domain or through blebbistatin treatment does not (Zang and Spudich 1998; Murthy and Wadsworth 2005). These studies demonstrate that MLC phosphorylation can effect cellular processes independent of AMC.

Therefore, we wanted to determine if the function of myosin II in TNF-alpha cell death signaling involved AMC. In order to answer this question we directly inhibited
AMC through use of blebbistatin, which is a selective inhibitor for non-muscle myosin II. As previously described, myosin II must bind ATP, hydrolyze ATP into ADP and Pi, then release Pi in order to bind actin and generate force (Tyska and Warshaw 2002; Sellers and Knight 2007). Blebbistatin functions by preferentially binding to the ADP + Pi intermediate at the ATPase active site thereby preventing release of Pi and impairing the ability of myosin II to bind actin filaments and produce AMC (Kovacs, Toth et al. 2004; Limouze, Straight et al. 2004; Allingham, Smith et al. 2005). Therefore, the use of blebbistatin is a useful tool to determine the involvement of AMC in TNF-alpha cell death signaling by directly inhibiting myosin II’s force generating property as opposed to indirectly inhibiting AMC by impairing the kinase activity of MLCK and Rho kinase. In the present study we show that inhibition of AMC with blebbistatin did not prevent the cleavage of caspases in response to TNF-alpha. Therefore, given that multiple studies including our own illustrate a decrease in MLC phosphorylation impairing caspase cleavage while inhibition of AMC does not suggests that phosphorylation of the MLC is the crucial signaling event in promoting cell death, independent of AMC.

Activated myosin II can participate in the formation and stabilization of actin microfilament bundles (Bresnick 1999; Conti and Adelstein 2008). This property of myosin II has been suggested to play an important role in TNF-alpha induced apoptosis since pharmacologic and genetic inhibition of MLCK and Rho kinase impairs actin filament organization while preventing cell death (Petrache, Verin et al. 2001). In this investigation actin filament dynamics were disrupted by directly targeting the actin cytoskeleton through treatment with cytochalasin D. My data along with work done in NIH3T3 cells suggests that increases in actin microfilament formation and rearrangement
are not required for cell death signaling since treatment with cytochalasin D does not inhibit caspase cleavage in response to TNF-alpha (Croft, Coleman et al. 2005). It should be noted that although cytochalasin D treatment does destabilize the proper formation and reorganization of actin microfilament bundles in response to TNF-alpha the presence of filamentous aggregates or actin foci have been reported and could be playing a role in apoptosis (Schliwa 1982). Nevertheless, cytochalasin D disrupts actin dynamics, but not MLC phosphorylation, in response to TNF-alpha suggesting that the function of activated myosin II during cell death is independent of actin filament formation and AMC.

Since the data suggested that the function of myosin II during TNF-alpha cell death was independent of AMC, the role of myosin II in this process remained to be determined. Therefore, I examined alternative functions of myosin II that MLC phosphorylation may be promoting. Phosphorylation of the MLC converts myosin II into an active conformation that can allow multiple myosin II proteins to associate with one another to form myosin II filaments (Bridgman 2002). Myosin IIA filaments have been shown to form docking sites for the signaling protein RAP1 which promotes integrin activation. Impairing myosin II activation through treatment of cells with ML-7 prevented myosin IIA association with RAP1 thereby disrupting RAP1’s localization and signaling at the plasma membrane. Interestingly, inhibition of myosin II motor activity through treatment of cells with blebbistatin did not affect RAP1 localization or signaling (Arora, Conti et al. 2008). This work demonstrates how activated myosin II can affect a cellular process independent of AMC and gives rise to a possible role for myosin II function in mediating TNF-alpha induced apoptosis independent of AMC.
One crucial upstream event of TNF-alpha induced apoptosis is the internalization of the TNFR1 receptor which has been shown to occur within minutes of TNF-alpha binding (Schutze, Machleidt et al. 1999; Schneider-Brachert, Tchikov et al. 2004; Schneider-Brachert, Tchikov et al. 2006). This internalization process is important for the formation of the DISC complex and the activation of caspase 8 (Micheau and Tschopp 2003; Schneider-Brachert, Tchikov et al. 2004). Published work has demonstrated that preventing endocytosis of the activated TNFR1 receptor through treatment with monodansylcadaverine (MDC) inhibits the induction of apoptosis. MDC is an inhibitor of transglutaminase which is a membrane bound enzyme that is involved in the internalization of a number of receptor systems such as clathrin mediated endocytosis (CME) (Schutze, Machleidt et al. 1999). CME is involved in the internalization of a variety of ligand-receptor complexes such as the CXCR4 receptor in response to SDF-1alpha (Rey, Valenzuela-Fernandez et al. 2007). This internalization process has been suggested to rely upon myosin IIA to couple the activated receptor to vital endocytic proteins such as B-arrestin. Myosin II has also been shown to be important for the positioning of clathrin coated endocytic structures to the uropod of moving T-lymphocytes for the uptake of transferrin (Samaniego, Sanchez-Martin et al. 2007). Since published works have demonstrated a possible role for myosin II involvement in CME, which is also important for the uptake of TNFR1 into the cell (Mosselmans, Hepburn et al. 1988), I examined the role of myosin II in mediating TNFR1 internalization.

This investigation presents data that suggests activated myosin IIB forms a docking site for clathrin during TNFR1 internalization independent of its force generating
property. First, data showed that treatment with ML-7 and siRNA knockdown of myosin IIB, but not IIA, impairs the internalization of TNFR1 while blebbistatin did not. These data suggest that activation of myosin IIB through MLC phosphorylation plays a role in TNFR1 internalization independent of AMC. Next, data showed that the associations of clathrin with TNFR1 and myosin IIB were also impaired by ML-7 but not blebbistatin indicating these associations are also dependent upon phosphorylation of the MLC yet independent of force generation. Taken together these data suggest that the activation of myosin IIB promotes the internalization of TNFR1 by promoting the association of TNFR1 with clathrin. Similar to the role of myosin IIA in RAP1 signaling it is possible that MLC phosphorylation of myosin IIB can promote filament formation that could be responsible for proper positioning of clathrin with TNFR1 independent of AMC. This hypothesis is supported by evidence in the literature suggesting that myosin IIB can play structural roles that influence cellular processes independent of its force generating property (Ma, Bao et al. 2007; Vicente-Manzanares, Zareno et al. 2007; Kim, Kawamoto et al. 2008).

Although the data demonstrates that myosin IIB plays a role in the internalization of TNFR1, we wanted to examine if myosin IIB might also be involved in caspase activation. Our data illustrates that internalization of TNFR1 occurs as early as 7.5 minutes but caspase cleavage is not detected until the 2 hour time period. Therefore, we wanted to examine the protein interactions of myosin IIB that were occurring after a time in which cell death signaling was detectable. IPs of the pro-apoptotic proteins FADD and caspase 8 demonstrated an isoform specific association with myosin IIB at a time in which caspase cleavage was occurring. Although there were times in which myosin IIA
association was detected with FADD its association was significantly less than that of myosin IIB. The interactions between myosin IIB with FADD were inhibited by ML-7 treatment suggesting that activating myosin II through MLC phosphorylation plays a vital role in their association. Attempts to pulldown TRADD, FADD, and caspase 8 in their associated state to determine the involvement of myosin IIB in the formation of the DISC complex was unsuccessful. Since Hela cells have been shown to express viral proteins of the human papillomavirus (HPV) it is possible that these viral proteins interfere with IP of the DISC complex (Rodier, Bertrand et al. 2000). Although activation of myosin IIB in response to TNF-alpha seems to be essential for its association with FADD and caspase 8 further work will be needed to determine the significance of these interactions.

In conclusion, our studies provide evidence that TNF-alpha promotes cell death by utilizing the myosin IIB protein in an isoform specific manner. Myosin II is ubiquitously expressed and its force generating property is crucial to many cellular processes, however, this investigation presents an example of myosin II playing a critical role in cell death signaling independent of AMC. Furthermore, our study demonstrates that myosin IIB may be involved in multiple facets of TNF-alpha cell death signaling from receptor internalization to cleavage of caspases through its associations with clathrin, FADD, and caspase 8. Further work will be required to establish the mechanism by which myosin IIB participates in apoptotic signaling.

Future directions will involve testing the findings of this study in both transformed and non-transformed cell lines to determine the significance of myosin IIB’s role in mediating TNF-alpha cell death signaling. Future works will also focus on elucidating possible redundant and non-redundant roles of myosin IIA, IIB, and IIC in
helping to mediate TNF-alpha induced apoptosis and survival signaling. Experiments will be performed to examine a possible role for the myosin II isoforms as docking sites/scaffolding proteins that help promote signaling in response to TNF-alpha. Identifying interaction sites among important protein-protein associations in response to TNF-alpha could lead to the development of small molecule inhibitors that may be used as an anti-inflammatorys, immune modulators, or anti-tumor agents in the treatment of certain cancers.
Chapter V: References


