A Thesis

entitled

The Role of Mixed Lineage Kinase 3 in MAVS- Dependent Signaling

by

Samantha Jean Stefl

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in

Cellular and Molecular Biology

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An Abstract of

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The innate immune system is the first line of defense against infection. Specific pathways are activated by detection of pathogen-specific molecules that are distinct from host antigens. The RIG- like helicase (RLH) pathway includes the cytoplasmic pattern recognition receptors RIG-I and MDA-5, two related RNA helicases that recognize and bind cytoplasmic dsRNA and ssRNA of viral origin. Once activated, RIG-I and MDA5 associate with the mitochondrial antiviral signaling adaptor protein (MAVS), leading to activation of downstream transcription factors NF-κB and IFN regulatory factors (IRFs) such as IRF3. These transcription factors translocate to the nucleus where they induce expression of type I IFNs, including IFNβ, and other pro-inflammatory genes.

The MAPK pathway has also been implicated in virus detection and response. Environmental stimuli such as inflammatory cytokines trigger the MAPK pathway. MAP3Ks including Mixed lineage kinase 3 (MLK3) phosphorylate and activate MAP2Ks, which then phosphorylate and activate MAPKs such as p38, JNK and ERK.

The MAPKs then activate transcription factors that regulate expression of genes involved in cell proliferation, differentiation and apoptosis.

The current understanding of MLK3 is limited. Within the MAPK system, MLK3 has been implicated as a regulator of signaling pathways JNK, ERK and p38. MLK3 is activated by various cellular stressors or through TNF–α stimulated recruitment of TRAF2. MAPK pathways assist in virus detection as shown by the MAVS recruitment of MKK4/7 and the presence of TRAF2 and other adaptor proteins in the RLH activated pathways.

Our data suggest that MLK3 overexpression negatively regulates MDA5 and/or MAVS-dependent signaling and suppresses IFNβ promoter activation. Suppression of endogenous MLK3 using siRNA upregulated IFNβ gene induction by dsRNA. The negative regulatory role of MLK3 on IFNβ production is dependent on its kinase activity. TRAF2-MLK3 association is observed in unstimulated SKOV3 cells. We have also observed an inhibitory effect of MLK3 on IRF3 activation in cells with transient MAVS or TRAF2 overexpression. Collectively our results suggest MLK3 is a regulator of IFNβ signaling downstream of RLH signaling.

This work is dedicated to my supportive family and loving partner

“Not all those who wander are lost”- J. R. R. Tolkien

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List of Abbreviations

APS ..... Ammonium persulfate
ATCC ..... American Type Culture Collection

β-gal ..... β- galactosidase
BSA ..... Bovine serum albumin

CARD ..... Caspase Activation and Recruitment Domain
CRIB ..... Cdc42/Rac interactive binding protein

DAPI ..... 4',6-diamidino-2-phenylindole
DLK ..... Dual leucine zipper-bearing kinase
DMEM ..... Dulbecco's Modified Eagle Medium
DNA ..... Deoxyribonucleic acid
dNTP ..... deoxyribonucleotide triphosphate
dsRNA ..... double stranded ribonucleic acid
DTT..... Dithiothreitol

EDTA ..... ethylenediaminetetraacetic acid
ERK ..... Extracellular signal-regulated kinase
FADD ..... Fas-associated death domain protein
FBS ..... Fetal bovine serum

GFP ..... Green fluorescent protein
GST ..... Glutathione S-transferase

HEK ..... Human embryonic kidney

IB ..... Immunoblot
IFN ..... interferon
IKK ..... IκB kinase
IP..... Immunoprecipitation
IRF .....interferon regulatory factor
ISG ..... interferon stimulated gene
IκBα ..... inhibitor of kappa B alpha

JAK ..... Janus Kinase 1
JNK ..... c-Jun N-terminal kinase

LPS ..... Lipopolysaccharide
LZ ..... Leucine zipper

MAP2K ..... MAPK kinase
MAP3K ..... MAPK kinase kinase
MAPK ..... Mitogen activated protein kinase
MAVS ..... mitochondrial antiviral signaling
MDA5 ..... Melanoma differentiation-associated gene-5
MEK ..... MAPK/ERK kinase
MEKK ..... MEK kinase
MLK ..... Mixed lineage kinase

NF-κB ..... Nuclear factor kappa-light-chain enhancer of activated B cells

p ..... Phosphoprotein gene
PAMP..... pathogen associated molecular pattern
PBS ..... Phosphate buffered saline
PCR ..... polymerase chain reaction
PMSF ..... Phenylmethylsulphonyl fluoride
PRR ..... pattern recognition receptor
PVDF ..... Immobilon-P Polyvinylidene Flouride

RIG-I ..... retinoic acid inducible gene 1
RLH ..... RIG-I-like helicase

SDS-PAGE ..... sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser ..... Serine
SH ..... Src homology
siRNA ..... small interfering RNA
SKOV..... Human ovary: ascites cells
ssRNA ..... single-stranded ribonucleic acid
STAT..... Signal transducer and activator of transcription

TBST..... tris-buffered saline Tween-20
TEMED ..... tetramethylethylenediamine
Thr .....Threonin
Tyr .....Tyrosine

List of Symbols

α …….... Alpha
β ……..…….. Beta
γ …….…….. Gamma
ω …….…….. Omega
τ ……..….…….. Tau
δ …….…….. Delta
ε ……..…….. Epsilon
Fig ……...…….…….. Figure
h …….…….. Hour
κ ……...…….. Kappa
Min ……...…… Minute(s)
x g …….…..……. Gravity

Chapter 1

1.1 Type I interferon production

The human innate immune system is the first line of defense against pathogens. Innate immune signaling pathway respond to a variety of pathogens and rely on several stages of recognition and communication within and between signaling cascades [1]. Initiating many pathogen-activated cellular signaling cascades are pattern recognition receptors (PRRs) that include a variety of receptor classes, such as Toll-like receptors (TLRs) and the cytoplasmic viral detection receptors retinoic acid inducible gene I (RIG-I) and melanoma differentiation-associated gene-5 (MDA5), commonly referred to together as "RIG-like helicases (RLHs)" [1]. Both RIG-I and MDA5 contain two N terminal caspase activation and recruitment domains (CARDs) and a helicase domain capable of recognizing viral pathogen-associated molecular patterns (PAMPs), such as double stranded RNA (dsRNA) and uncapped single stranded RNA (ssRNA) [2]. RIG-I detects the 5' triphosphate groups and blunt ends of short dsRNAs or ssRNA hairpins often present in a wide range of negative strand viruses, such as influenza A, and some positive-and double-stranded RNA viruses [3, 4]. RIG-I resides in the cytoplasm and binds to dsRNA as a monomer [5]. MDA5 detects long-duplex RNAs such as dsRNA virus genomes or dsRNA replication intermediates of positive-strand viruses [6]. MDA5 exists in the cytoplasm as monomer units. However, MDA5 assembles into

filamentous oligomers along dsRNA, which is important for high-affinity interaction with long dsRNA [5]. The initial binding to dsRNA leads to the formation of filament oligomers around dsRNA. As MDA5 monomers begin to recognize and stack along the dsRNA, the CARDs oligomerize [5]. MDA5 oligomers interact with mitochondrial antiviral-signaling (MAVS) protein (also known as VISA/IPS-1/Cardiff) which is required for activation of downstream signaling events in response to both RIG-I and MDA5 [7].

The localization of MAVS to the mitochondrial membrane is mediated by a C-terminal transmembrane domain and is required for downstream antiviral signaling events [8-11]. MDA5 and RIG-I CARDs initiate MAVS activity by promoting formation of prion-like filaments consisting of the N-terminal CARD of MAVS, however, the mechanism behind this remains elusive [12]. In addition to the CARD interaction between RLHs and MAVS, MAVS-mediated antiviral signaling is propagated through assembly of a MAVS "signalosome" containing tumor necrosis factor (TNF) receptor associated factor (TRAF) 3, TRAF2, TRAF6, TRAF family member-associated nuclear factor κB (NF-κB) activator (TANK), and TNFR1-associated death domain protein (TRADD). The formation of MAVS signaling complexes results in the phosphorylation and nuclear translocation of interferon regulatory factor 3 (IRF3) by TANK binding kinase 1 (TBK1) and/or IKKε, as well as activation of NF-κB to induce type I interferons (IFNs) and pro-inflammatory cytokines [13-16] (Fig 1). The recruitment of TRAFs to MAVS is essential for MAVS-mediated antiviral signaling. However, there is some debate as to the role of each TRAF member within the pathway. MAVS contains binding sites for several TRAF proteins [8, 11] but their individual roles in MAVS signaling have

been enigmatic because cells lacking individual TRAF proteins, including TRAF2, TRAF3, TRAF5, and TRAF6, still induce IFNβ normally in response to virus infection [11, 17, 18]. Importantly, MAVS binds to TRAFs in a manner that depends on virus infection and MAVS polymerization. Mutations in the CARD domain of MAVS that disrupt its polymerization also abolish the recruitment of the TRAF proteins [19].

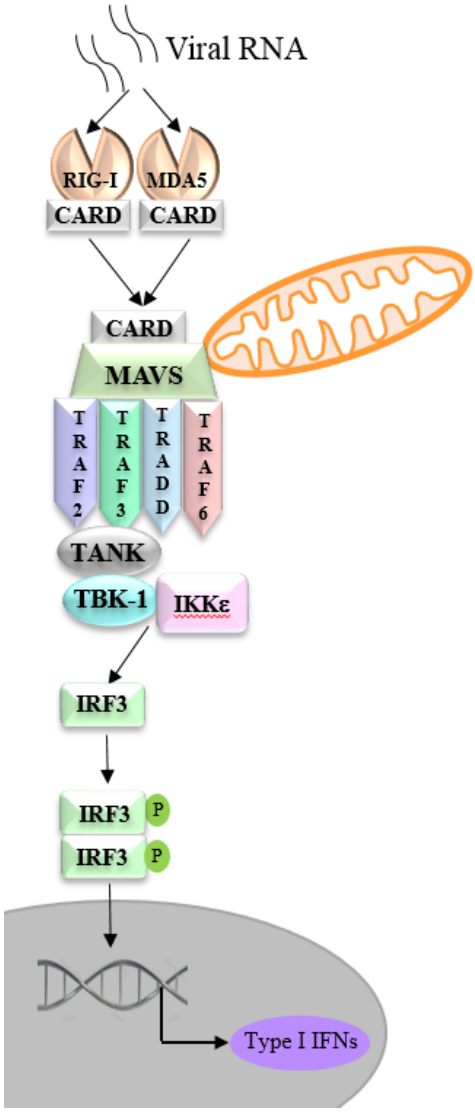


Figure 1: Innate Immune System Signaling through MAVS recruitment of adaptor proteins. Once a signal is transduced from RLHs, MAVS begins to recruit adaptor proteins for the next juncture of the RLH signaling pathway. Formation of the signalosome involves recruitment of TRAF3, 6, TANK and TRADD. Also recruited are TRAF2, 5 and TBK1. Together these proteins assist in the propagation of the signal to the nucleus where gene expression is modified.

Following MAVS signaling complex formation, TRAF2 and TRAF6 participate in downstream activation of IRF3. In unstimulated cells, IRF3 is present in the cytoplasm in a monomeric, unphosphorylated state. Viral infection results in the phosphorylation of latent cytoplasmic IRF3 by TBK1 and IKKε on serine and threonine residues in the C-terminal region, leading to dimerization, nuclear translocation, association with the p300/CREB-binding protein (CBP) coactivator, and stimulation of DNA binding and transcriptional activities [20, 21]. This activation of IRFs results in transcriptional activation of responsive genes, including type I IFNs. Type I IFNs are protective in acute viral infections but can have either protective or deleterious roles in bacterial infections and autoimmune diseases, making their balanced regulation critical to host protection [22]. Up-regulation of cytokines, IFNs in particular, is a critical aspect of cellular innate immunity. In mammals, three distinct families of IFN have been identified, designated type I (IFN-α, -β, -ω, -τ, -δ, -ε etc), type II (IFNγ) and type III (IFNλ) [23]. Type I IFNs secreted by infected cells have three major functions. First, they induce cell-intrinsic antimicrobial states in infected and neighboring cells that limit the spread of infectious invaders, particularly viral pathogens. Second, they promote antigen presentation and natural killer cell functions while restraining pro-inflammatory pathways and cytokine production. Third, they activate the adaptive immune system, thus promoting the development of high-affinity antigen-specific T and B cell responses and immunological memory [24].

1.1.2 JAK-STAT Signaling Pathway

Once transcribed, translated and secreted, type I IFNs bind to transmembrane IFNα receptors (IFNAR) composed of heterodimeric subunits. IFNAR engagement activates the receptor-associated protein tyrosine kinases Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which phosphorylate cytoplasmic transcription factors signal transducer and activator of transcription 1 (STAT) 1 and STAT2 [25, 26]. Tyrosine-phosphorylated STAT1 and STAT2 dimerize and translocate to the nucleus, where they assemble with IFN-regulatory factor 9 (IRF9) to form a trimolecular complex called IFN-stimulated gene factor 3 (ISGF3). ISGF3 binds to its cognate DNA sequences, which are known as IFN-stimulated response elements (ISREs), activating the transcription of IFN stimulated genes (ISGs) [24]. ISGs are then translated into proteins that assist in establishing a cellular antiviral state, protecting the cell from subsequent viral infection. Collectively the dsRNA response and interferon stimulated pathways are one mechanism the innate immune system uses to protect the host (Fig 2).

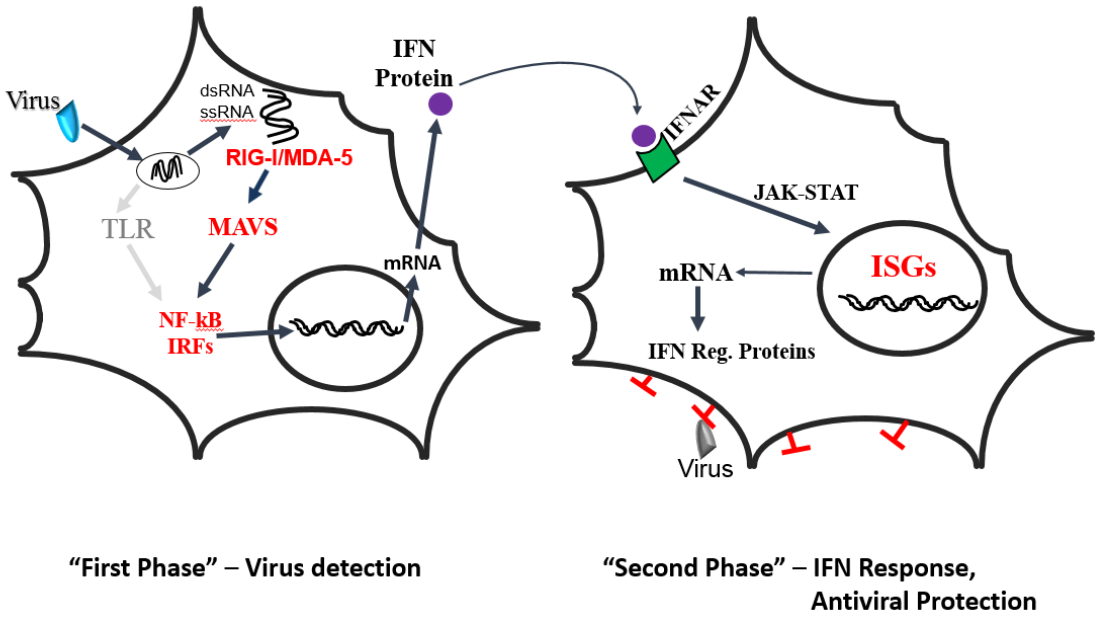


Figure 2: Viral Detection and Interferon Response Pathway. RIG-I and MDA5 recognize and bind to cytoplasmic viral nucleic acids, this leads to a CARD to CARD interaction between RLHs and MAVS. MAVS then transmits a signal for adaptor proteins to be recruited; these adaptor proteins assist in the activation of transcription factors, including NF-κB and IRF3, responsible for type I IFN production. Once produced, Type I IFNs are released locally and distally and bind to IFNARs on the surface of target cells, triggering JAK-STAT signaling. Successful JAK-STAT signaling induces ISGs, the proteins products of which establish an antiviral state.

1.2 Mitogen-activated protein kinase pathways in innate immunity

The mitogen-activated protein kinase (MAPK) signaling pathways are evolutionarily conserved, functioning to transmit a wide range of extracellular signals from the cell surface to the nucleus, which results in the regulation of gene expression [27]. Activated by environmental factors such as pro-inflammatory cytokines, tumor necrosis factor (TNF), osmotic stress, heat shock and ionization radiation, the three-tiered MAPK system is regulated by phosphorylation events [27]. Once activated, the top tier MAP kinase kinase kinases (MAP3Ks) phosphorylate MAP2Ks on serine (Ser) and threonine (Thr) residues, which leads to MAP2K activation [27]. Activated MAP2Ks are dual specificity kinases that, phosphorylate tyrosine (Tyr) and Thr residues of MAPKs, which results in MAPK activation [27]. MAPKs have diverse cellular substrates, and phosphorylate cytoplasmic substrates as well as transcription factors within the nucleus [28].

The three major MAPK signaling pathways are the extracellular signal-regulated kinase (ERK1/2 or p44/p42), the stress activated protein kinases (SAPK) or c-Jun N-terminal kinases (JNK1/2/3), and the p38 kinases ($\alpha,\beta,\gamma,\delta$) [29]. ERK1/2 responds predominantly to mitogenic stimuli [30], while JNK/SAPKs are predominantly stimulated by inflammatory cytokines and environmental stresses such as heat shock, oxidative stress and UV radiation [31]. The p38 $\alpha,\beta,\gamma,\delta$ MAP kinases are stimulated by various stressors including UV radiation, pro-inflammatory cytokines, osmotic stress, hypoxia, and hematopoietic growth factors [32-35]. These MAPK pathways execute a diverse range of cellular activities including proliferation, differentiation and apoptosis.

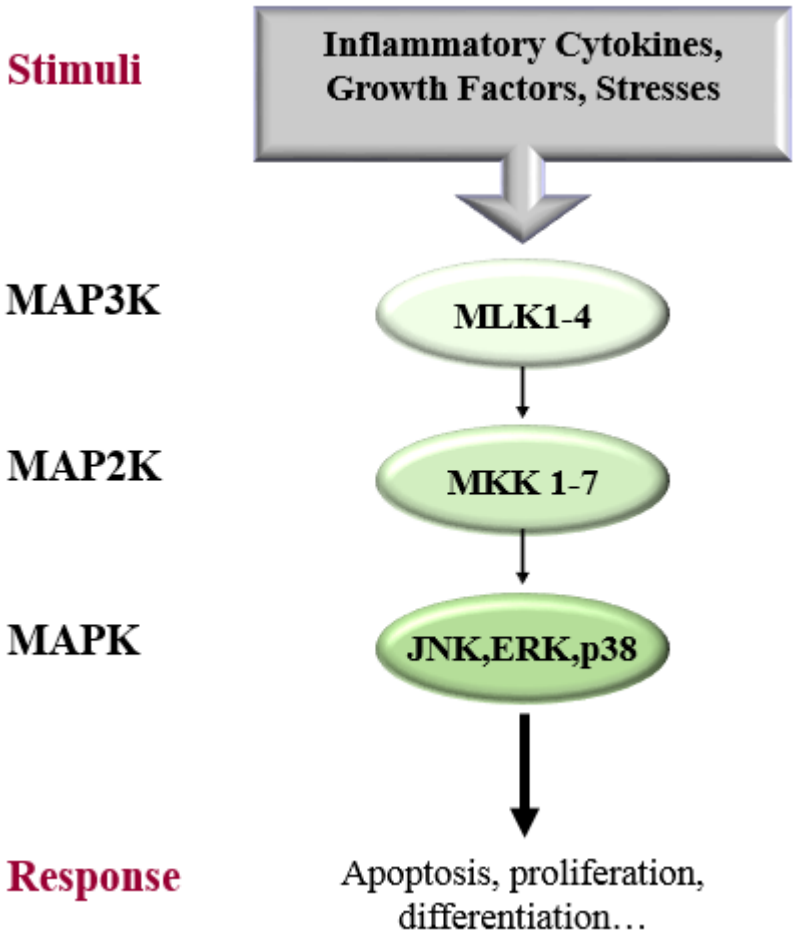


Figure 3: MLK Signaling Pathways. MAPK signaling pathways are stimulated by mitogens and environmental stressors leading to the phosphorylation and activation of MAP3Ks. MAP3Ks, including MLK3, phosphorylate and activate MAP2Ks, which phosphorylate and activate MAPKs including ERK, JNK and p38. MAPKs are responsible for carrying out an assortment of cellular responses based on the signal received.

1.3 Mixed-lineage kinase 3 structure and function

Mixed-lineage kinase 3 (MLK3) belongs to the MAP3K family of serine/threonine protein kinases responsible for phosphorylating and activating MAP2Ks [49]. MLKs 1–3, 4a, and 4β are categorized based on sequence similarity and domain organization. MLKs 1-4 contain an amino-terminal Src-homology 3 (SH3) domain, a kinase domain, a Cdc42–Rac interactive binding (CRIB) motif, and a central leucine zipper region that mediates homodimerization [36, 37]. Variations in the carboxyl termini of MLK proteins suggests that these regions might serve different regulatory functions [37]. MLK3 protein contains additional variation including a Gly–Pro-rich amino terminus that is absent from MLK1, MLK2 or MLK4 [37]. (Fig 4)

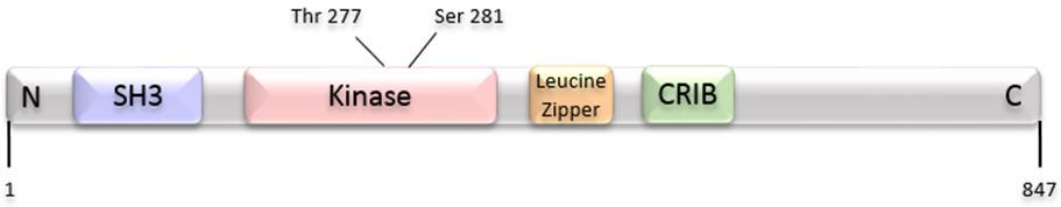


Figure 4: MLK3 Domains. MLK3 domains include a SH3 domain, kinase domain, leucine zipper and CRIB motif which assist MLK3 in autophosphorylation, dimerization, and other regulatory events.

MLK3 auto-phosphorylates on the sequence TTXXS (residues 277–281; where X represents any amino acid, S represents serine and T represents Threonine) within the

activation loop upon stimulation with factors such as TNFα [38, 39]. Once activated, MLK3 activity can be regulated through leucine zipper-mediated protein dimerization or oligomerization by forming coiled coils that are stabilized primarily by leucine or other non-aromatic hydrophobic residues that interact at the interfaces of opposing helices [40-42]. MLK3 auto-inhibition is mediated through the N-terminal SH3 domain, causing a 'closed' structure, which prevents MLK3 oligomerization and auto-phosphorylation. The MLK3 SH3 domain may bind intramolecularly to a region between the leucine zipper and the CRIB motif, and mutation of a single proline here prevents SH3 binding and increases kinase activity. These results suggest that MLK3 is auto-inhibited by binding of its SH3 domain to an auto-regulatory sequence [43]

1.3.1 MLK3 Biological Function

MLK3 is important for the downstream activation of JNK, ERK and p38 signaling cascades in different cell types and in response to certain stimuli. MLK3 is critical for promoting neuronal cell death [44]. Additionally the scaffold functions of MLK3 assists in the regulation of ERK signaling and in limiting Rho GTPase activity [45, 46]. MLK3 can promote microtubule instability and is required for cell proliferation in epithelial and fibroblast cells [47, 48]. MLK3 activity and protein levels vary in different types of cancers. Overexpression of wild type MLK3 can transform NIH3T3 cells [49] . High expression of MLK3 has been noted in breast cancer cell lines as compared with nontumorigenic mammary epithelial cell lines [50, 51]. MLK3 is necessary for breast and ovarian cancer (SKOV3) cell invasion [50-53]. SKOV3 ovarian cancer cells also have high MLK3 activity in comparison to normal ovarian epithelial cells [52]. TNF and

Interleukin-1β (IL-1β) stimulation rapidly activate MLK3 kinase activity in SKOV3 cells [54]. TNF stimulates an interaction between MLK3 and TRAF2, and IL-1β stimulates an interaction between MLK3 and TRAF6 [54, 55] Binding of MLK3 to TRAF2 occurs through the TRAF-N domain of TRAF2 and the C-terminal domain of MLK3 [54, 55]. (Fig 5)

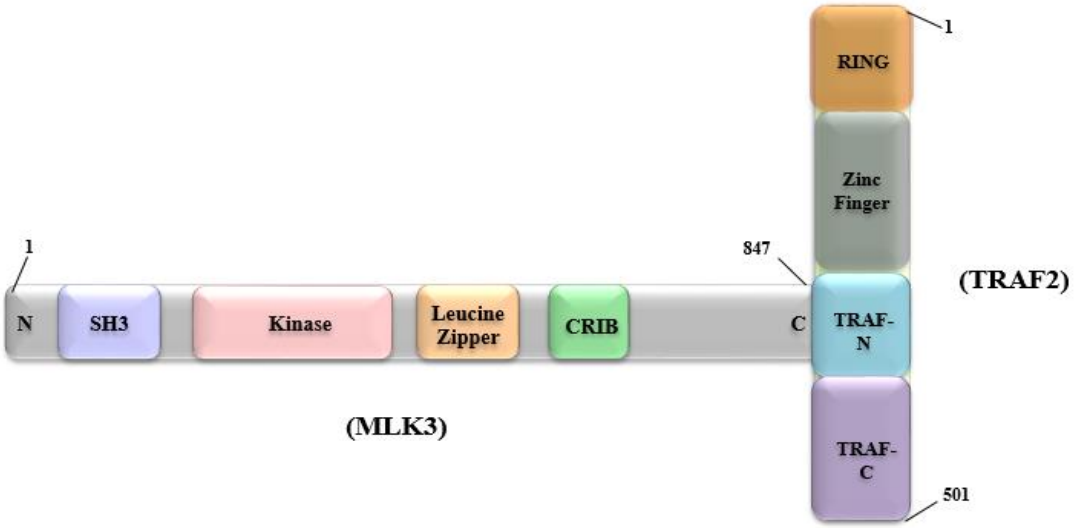


Figure 5: MLK3-TRAF2 association. The full length domain structure of MLK3 and TRAF2 are shown. The MLK3-TRAF2 association occurs at the C- terminus domain of MLK3 and the TRAF-N domain of TRAF2.

TRAF proteins, with the exception of TRAF1, contain a RING domain and multiple zinc-finger structures, which are essential for their effector functions [56, 57]. TRAF2, TRAF5 and TRAF6 can all bind to MLK3, however only TRAF2 can activate the MLK3 kinase activity when stimulated with TNFα, in turn causing significantly higher kinase activity in TRAF2- associated MLK3 complexes compared to total the MLK3 pool [55].

Additionally MLK3 expression suppresses NF-κB activation by inhibiting IKK activity in SKOV3 human ovarian carcinoma cells and HEK293 human embryonic kidney cells [58]. Silencing of MLK3 leads to a reduction in the level of IκBα protein, elevated basal IKK activity, and elevated expression of NF-kB regulated genes [58].

1.4 Significance

MLK3 negatively regulates NF-kB activity [58]. MLK3 has also been implicated in the TNF signaling pathway, where it binds with TRAF2. TRAF2 participates in activation of JNK and NF-κB pathways, both of which contribute to inflammatory responses [58, 59]. The impact of MLK3 in RLH and IFN signaling has yet to be investigated. In this study we analyzed how the presence and absence of MLK3 impacts the RLH and IFN signaling pathways. We have observed that MLK3 overexpression inhibits MDA5- and MAVS-dependent activation of IFNβ, while suppression of endogenous MLK3 augments dsRNA-induced IFNβ mRNA expression. Overexpression of MLK3 also leads to inhibition of IRF3 translocation. We have also identified a constitutive MLK3-TRAF2 association that can be sustained through dsRNA treatment. Collectively these data propose a role for MLK3 in the RLH signaling pathway.

Chapter 2

Materials and Methods

2.1 Cell lines

Human ovarian cancer (SKOV3) cells were obtained from Dr. Deborah Chadee (The University of Toledo). Human Embryonic kidney (HEK293) cells were obtained from American Type Culture Collection (ATCC) (Rockville, MD,). SKOV3 and HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, South Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, South logan, UT, USA). All culture media was supplemented with 1% penicillin and streptomycin (Cellgro, Manassas, VA, USA). Cells were cultured at 37°C with 5.0% CO_2. Cells were passaged regularly using trypsin-EDTA to disrupt the monolayer**.**

2.2 Plasmids

GST-MLK3 and Kinase-Dead MLK3 expression plasmids were provided by Dr. Deborah Chadee (The University of Toledo). UAS- luciferase and IRF3-GAL4 expression plasmids were provided by Dr. Malathi Krishnamurthy (The University of Toledo). pIRF3-GFP expression plasmid was provided by Dr. Travis Taylor (The

University of Toledo). IFN-β- luciferase was provided by Dr. Jon Hiscott (McGill University). 9-27- luciferase was previously cloned by Dr. George Stark (Cleveland Clinic). pcDNA(-) was purchased from Invitrogen. SV40 β-Gal, FLAG-MAVS, FLAG-MDA5, FLAG-TRAF2, expression vectors were previously cloned in our lab.

2.3 Transfections

Transient transfections of HEK293 were carried out using Polyjet (Signagen, Rockville, MD, USA) while transient transfection of SKOV3 were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Transfections were performed in serum free DMEM media at 37°C for 3h and then DMEM media containing serum and antibiotics was added. Plasmid and treatment amounts are specified in figures legends.

2.4 Luciferase and β-galactosidase Assay

HEK293 cells were transfected at 70% confluency in a 12 well plate. 24 h after transfection the cells were resuspended in the media and centrifuged at 1500 x g for 5 min, washed 2X with cold PBS and lysed with 150 µl of 1X Cell culture lysis buffer (Promega, Madison, WI, USA). Cells were placed at 4°C for 20 min. In addition an ATP mixture (735 µl ATP assay buffer (20 ml of 250 mM Glycyl-glycine, 0.304 g EGTA, 0.362 g $MgSO_4$, 20 ml K_2HPO_4, total volume 200 ml with H_2O), 7.5 µl DTT, 15 µl 100 mM ATP, luciferin mixture (75 µl Glycyl-glycine, 7.5 µl DTT and 475 µl water), β-galactosidase buffer (1.6 µl β-mercaptoethanol, 100 µl ortho-Nitrophenyl β-galactoside (ONPG)), and 500 µl of β- galactosidase buffer [8.5 g Na_2HPO_4, 4.75 g NaH_2PO_4, 10 ml

1M KCl, and .246 g $MgSO_4$; total volume 1L with H_2O) were made and placed in 4°C for 20 min. Cells were centrifuged at 12,000 x g for 10 min, 50 μl of lysate was added in a clear 96 well plate along with 50 μl of the β-galactosidase buffer. The plate was incubated at 37°C for 2 h. The β-galactosidase absorbance was then read at 414 nm on a SpectraMax plate reader. 70 μl of sample lysate was placed in an opaque 96 well plate. Rapidly, 200 μl luciferin was added to the luciferine mixture which was then added to the ATP mixture. Using a multichannel pipette, 100 μl of the solution was added to each well in the opaque 96 well plate. Using a SpectraMax plate reader, the luminescence of each sample was read rapidly (100 ms integration period). Luminescence values were normalized to the corresponding β-galactosidase absorbance values.

2.5 siRNA and gene knockdown

Human MLK3 and non-target small interfering RNAs (siRNAs) were purchased from Dharmacon (Lafayette, CO, USA) and Santa Cruz Technologies, (Santa Cruz, CA, USA), MLK3 siRNAs oligo sequences were as previously described [47]. SiRNA Gene knockdown in SKOV3 cells was carried out in 60 mm dishes when the cells were approximately 70% confluent. Dishes containing complete DMEM and FBS were washed with PBS and serum free DMEM, 1.5 ml of fresh serum free DMEM was added and the dishes were returned to 37°C. In 1.5 ml Eppendorf tubes, 7 μl of MLK3 or non-target siRNA was added to 500 μl of serum free DMEM. 7 μl of Lipofectamine 2000 was added to 500 μl serum free DMEM and incubated for 5 min. The Lipofectamine 2000 solution was mixed with the siRNA solution and gently inverted 5 times, incubated at room temperature for 20 min and then added drop wise to the dish. The final siRNA

concentration in the transfection was 100nM. After 5 h at 37°C, 2.5 ml DMEM containing 20% FBS and antibiotics was added. After 24 h, the cells were either harvested as described below or treated with 3 μg/ml double stranded RNA (dsRNA) and 7μl Lipofectamine 2000.

2.6 Preparation of whole cell extracts

24 h after transfection or siRNA knockdown the media was aspirated and the cells were washed with cold PBS. 4X SDS sample buffer (4 ml glycerol, 1 ml β-mercaptoethanol, 0.4 g SDS, 2.4 mg Bromophenol blue) was added to each well: 200 μl for a 60 mm dish, 150 μl for a 6 well plate and 100 μl for a 12 well plate. The cells were scraped off the plate and transferred to a 1.5 ml Eppendorf tube. DNA was then sheared with a 23 gauge needle 5 times per sample, using a new needle for each sample. The samples were then boiled for 5 min, centrifuged for 10 seconds and then subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE)followed by immunoblot analysis.

2.7 Immunoblotting

In a 10% SDS-PAGE 40 μl of sample was loaded into each well, and the gel was run (PROTEAN Tetra at 80V and PROTEAN II XL at 60V). After SDS-PAGE electrophoresis proteins were transferred to Immobilon-P Polyvinylidene Fluoride (PVDF) membrane using a Bio-Rad Trans-Blot SD Semi-Dry Electrophoretic Transfer. The PVDF membrane was soaked in methanol and then transfer buffer (12 g Tris, 57.6 g glycine and 800 ml methanol, 40 ml 10% SDS, final volume 4 L) sandwiched between sheets of chromatography paper (Fisher, Pittsburgh, PA, USA), also soaked in transfer

buffer. The transfer ran for 60 min at a constant 20 volts. PVDF membrane was blocked in 5% non-fat milk in Tris-Buffered Saline and Tween 20 (TBST) (400 ml Tris/1M HCl pH 7.4, 600 ml 5M NaCl, 80 ml .5M EDTA pH 8.0, 40 ml Tween 20 total volume 4L with H_20) or 2% -5% Bovine Serum Albumin (BSA) (Boston BioProducts, Boston, MA, USA) in TBST, for 1h at room temperature. The membrane was then washed 3X (5 min each) in TBST and placed in primary antibody (diluted in either 5% milk or 2-5% BSA according to the manufacturer's instructions) overnight on a 4°C rotator. The gel was placed in destaining buffer (100 ml glacial acetic acid, 300 ml methanol, final volume 1 L with H_2O) overnight at room temperature in a rotator. The membrane was then washed 3 times with TBST, secondary antibody was added 1:5000 in 5% milk or 2-5% BSA and incubated for 1 h at room temperature on a rotator. The membrane was washed 3X in TBST and chemiluminescent substrate was added. The membrane was then moved to a ChemiDoc-It^2 Imager where the signal was observed and imaged.

2.8 Antibodies

Antibodies used to conduct these experiments included TRAF2 (C-20) (Santa Cruz), Rabbit IgG (sc-2027) (Santa Cruz), Mouse IgG (sc-2025) (Santa Cruz), GST (B-14) (Santa Cruz), light chain specific mouse anti-rabbit secondary antibody, β-Actin (N-21) and Alexa Fluor 594 Goat anti mouse secondary antibody (Life Technologies), MLK3 (C-20) (Santa Cruz) and Anti-Flag M2 (Sigma-Aldrich Corp., St. Louis, MO, USA) and goat anti-mouse secondary (Thermo Scientific) and Goat anti-rabbit secondary (Thermo Scientific).

2.9 Immunofluorescence

24 h before transfection, poly-L-Lysine treated coverslips were added to each well and growth media containing HEK293 cells was added. HEK293 cells at 30% confluency were transiently transfected with p-IRF3-GFP plasmid and/or FLAG-MAVS, FLAG-TRAF2, GST-MLK3 for 24 h as described above. After 24 h, growth media was removed and cells were fixed by immersion in 3.7% paraformaldehyde for 30 min at 37 °C, then washed in cold PBS for. The coverslips were removed from the wells and placed in a chamber lined with aluminum foil and each coverslip covered with blocking buffer (1% BSA in PBS) and incubated for 30 min at room temperature. The blocking buffer was removed and the coverslips were incubated with Anti-Flag M2 primary antibody (diluted 1:100 in blocking buffer) for 1 h at room temperature. The primary antibody was removed and the coverslips were washed 3X with PBS (5 min each). The coverslips were then covered with Alexa-Fluor 594 secondary antibody diluted 1:200 in blocking buffer for 1 h at room temperature. The secondary antibody was removed and the coverslips were washed 3X with PBS for 5 min each. The coverslips were incubated with DAPI (1:1000 dilution in PBS) for 15 min at room temperature. The DAPI dilution was removed and the cells washed 2X with PBS. A drop of ProLong Gold antifade reagent was then placed on a slide and a coverslip was slowly placed on top, cell side down, avoiding air bubbles. The samples cured for 24 h at room temperature in the dark and were then sealed with clear nail polish. Prepared slides were imaged using an epi-fluorescent microscope (Olympus IX81).

2.10 Immunoprecipitation

Prior to cell harvest, 25µl/ sample of protein-A agarose beads (Pierce Thermo Scientific, Rockford, IL, USA) were placed in an Eppendorf tube, centrifuged for 10 seconds, then washed in lysis buffer (20 mM Tris pH 7.4, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% β-mercaptoethanol, 1.0% Triton X-100) at an equal ratio and placed in 4°C until needed. Confluent 10 cm dishes of SKOV3 cells were washed with cold PBS, lysed in 1 ml lysis buffer, and 1 µl of PMSF protease inhibitor were added to each plate. The cells were scraped and placed into 1.5 ml Eppendorf tubes and spun at 10,000 rpm for 10 min at 4°C and the supernatant was then collected. A Bradford assay was performed to verify the protein concentration of each sample. 4 µl of appropriate antibody was added to each tube along with 25 µl of the agarose bead mixture. The samples were placed on a rotator at 4°C for 2 h. The tubes were centrifuged for 10 seconds and the supernatant was removed, 1 ml of lysis buffer was added and the samples were rotated at 4°C for 5 min. The samples were centrifuged for 10 seconds and the supernatant was removed, 1 ml of high salt wash buffer (20 mM Tris pH 7.4, 2 mM EGTA, 10 mM $MgCl_2$, 0.1% β-mercaptoethanol, 0.1% Triton X-100, 1M LiCl) was added to each sample and rotated in 4°C for 5 min. The samples were centrifuged for 10 seconds and the supernatant was removed, 1 ml of wash buffer (20 mM Tris pH 7.4, 20 mM EGTA, 10 mM $MgCl_2$, 0.1% β-mercaptoethanol, 1.0% Triton X-100) was added to each sample and rotated in 4°C for 5 min. The samples were centrifuged for 10 seconds and the supernatant was removed

and 40 µl of 4X SDS sample buffer was added. The samples were boiled for 5 min and centrifuged for 10 seconds, and the immunoblotting protocol was then followed.

2.11 Real-time PCR

Isolation of RNA was carried out using Trizol (Life Technologies) according to the manufacturer's instructions. A Reverse Transciptase (RT) protocol was then followed to obtain cDNA. In an Eppendorf tube, 1 µg of RNA, 1 µl of random hexamer primer with a concentration of 1 µg/ul and H_2O to a total of 7 µl were combined and placed in a 70°C water bath for 10 min. The samples were then placed on ice for 10 seconds and 13 µl of 'master mix' (6 µl H_2O, 4 µl M-MLV RT 5X buffer, 2 µl dNTP, 0.5 µl optimiyze Ribonuclease inhibitor, 0.5 µl M-MLV RT) was added and the samples were places in a 42°C water bath for 1 h. Quantitative real-time PCR was performed with the SsoFast Evagreen Supermix master mix (Biorad). Each reaction consisted of 10 µl SsoFast Supermix, 8 µl H_2O, 0.5 µg sense and 0.5 µg anti sense primer and 1µl cDNA. PCR was carried out on a Bio-Rad C1000 Thermal Cycler CRX96 Real-Time system. A 3 minute denaturation step at 95°C was followed by 40 cycles of 30 seconds at 95°C and 60 seconds at 60°C. Fluorescence was read at the end of each elongation phase. Samples were normalized using the human β-Actin housekeeping gene. Gene expression levels were calculated using a ΔΔCT method. The primers used in this study are listed in Table 1.

2.12 primers

Table 1

Primer	Sequence
Human IFNβ	Sense : 5'AGCACTGGCTGGAATGAGA3' Antisense : 5'TCCTTGGCCTTCAGGTAATG3'
Human β-Actin	Sense: 5'GTGCCCATTTATGAGGGCTA3' Antisense: 5'CTGGCAGCAGCTCGTAGCTCTTT3'

2.13 Statistical analysis

Data were analyzed by unpaired student t-test (http://graphpad.com/quickcalcs/PValue1.cfm). Values represent mean ± standard deviation and p-values below 0.05 were considered as significant. Experiments were repeated a minimum of three times.

Chapter 3

Results

3.1 MLK3 inhibits the RLH signaling pathway

3.1.1 MLK3 inhibits MDA5- and MAVS-dependent activation of IFNβ

Viral or dsRNA induced cytoplasmic signaling pathways rely on effective downstream signaling from RIG-I or MDA5 to MAVS to produce type I IFNs, such as IFNβ. Disrupting this pathway could result in decreased production of IFNβ and a delayed or reduced immune response. To gain insight into a possible role for MLK3 in the RLH signaling cascade, we transiently overexpressed FLAG-MDA5 or FLAG-MAVS with or without GST-MLK3 and the effect on IFNβ expression was determined using a co-transfected luciferase construct driven by the IFNβ promoter. Transient overexpression of MDA5 or MAVS potently activates the IFN-β promoter [11, 60]. MLK3 inhibited MDA5-and MAVS- dependent activation of IFNβ in a dose dependent manner (Fig 6). To determine if the kinase activity of MLK3 was important for its suppressive effect on this signaling pathway, a kinase-dead MLK3 plasmid was co-transfected along with FLAG-MAVS or GST-MLK3 in HEK293 cells and IFNβ luciferase activity was assessed (Fig 7). The kinase-dead MLK3 did not significantly inhibit IFNβ luciferase activity, suggesting that the kinase activity of MLK3 is required

for its inhibitory effects. Collectively, these data suggest that MLK3 might negatively regulate MDA5 and MAVS signaling, and that the kinase activity of MLK3 is required for this effect.

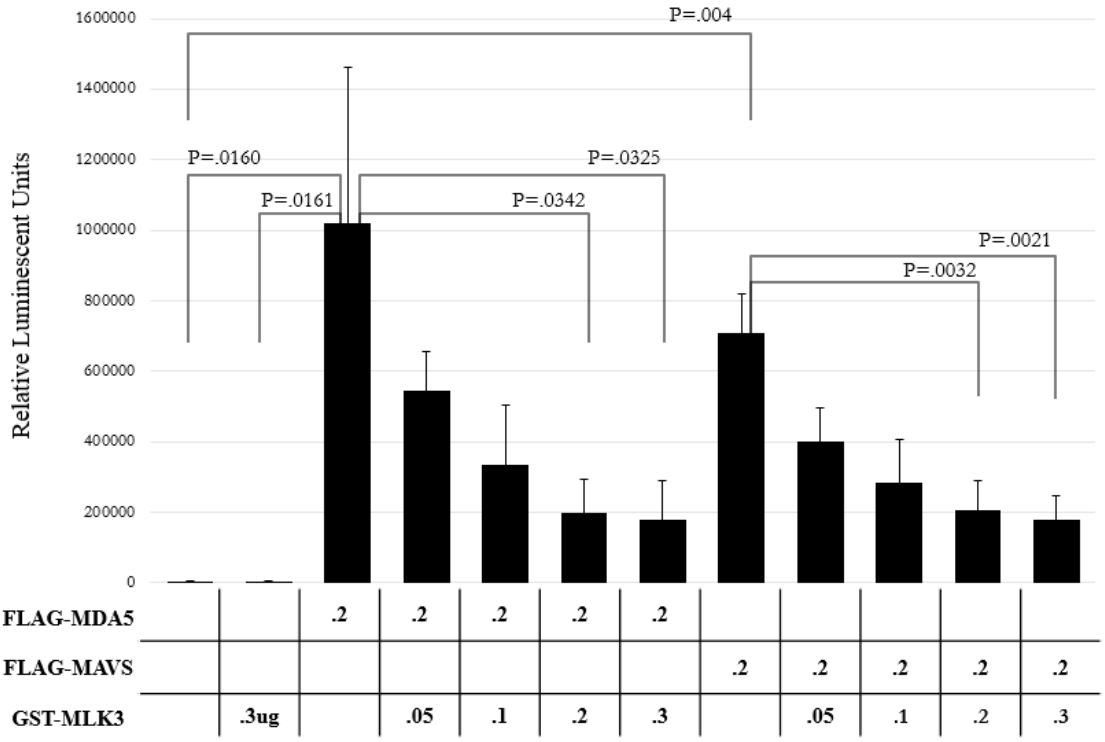


Figure 6: MLK3 inhibits MDA5- and MAVS-dependent activation of IFNβ. HEK 293 cells were transiently co-transfected with IFNβ-luciferase (0.2 µg) and β-galactosidase (0.2 µg). The transfection also contained the indicated concentrations of FLAG-MDA5, FLAG-MAVS or GST-MLK3 plasmids. Empty pcDNA vector was used to balance each sample to 1 µg DNA. The cells were harvested 24 h after transfection and luciferase and β-galactosidase assays performed. The figure represents luciferase activity measured and normalized to the co-transfected β-galactosidase expression construct

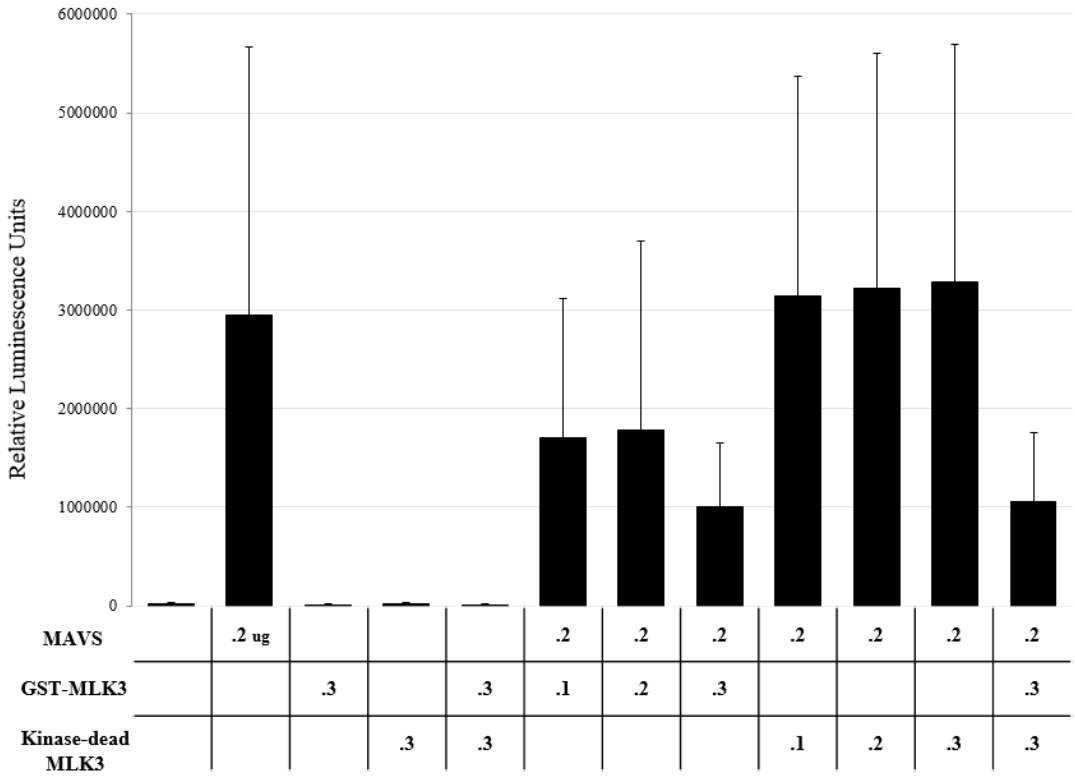
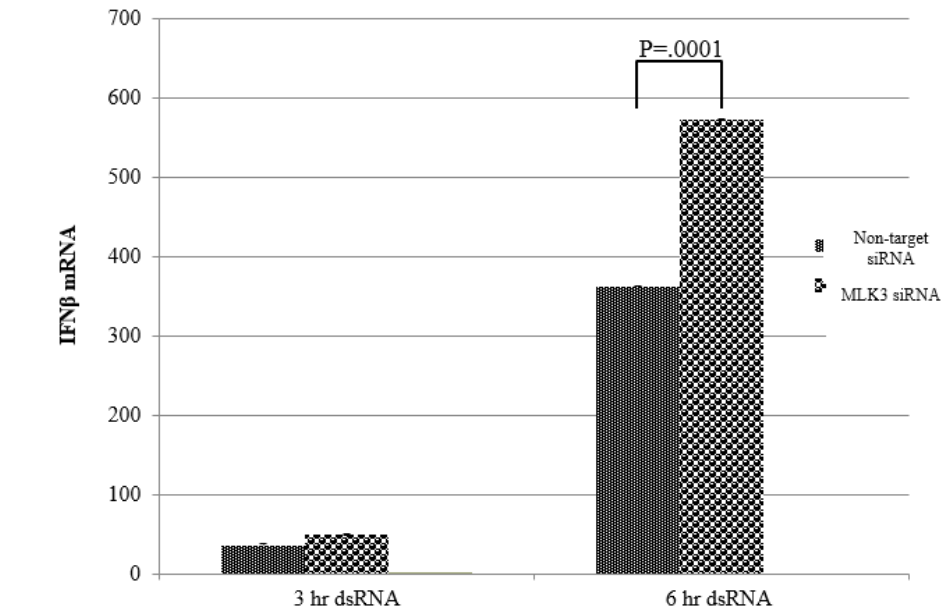


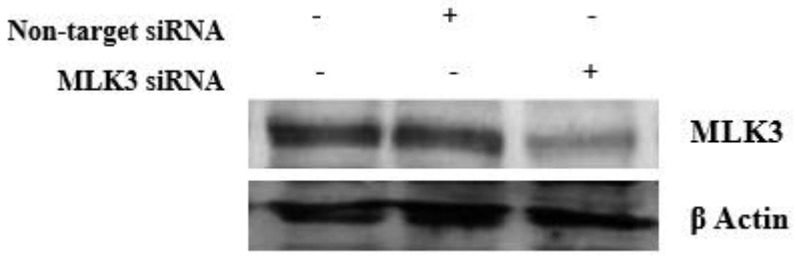
Figure 7: MLK3 inhibition of IFNβ is kinase dependent. HEK 293 cells were transiently co-transfected with IFNβ-luciferase (0.2 μg) and SV40 β-gal (0.2 μg), along with the indicated amounts of FLAG-MAVS, GST-MLK3 and/or kinase-dead MLK3 plasmids. Empty pcDNA vector was used to balance each sample to 1 μg DNA. The cells were harvested 24 h after transfection and luciferase and β-galactosidase assays performed. Luciferase activity measured and normalized to the co-transfected β-galactosidase expression construct.

3.1.2 Suppression of Endogenous MLK3 Augments dsRNA-induced IFNβ mRNA Expression

Our IFNβ luciferase data suggested that overexpression of MLK3 negatively regulated MDA5 and MAVS induced IFNβ luciferase. To assess the role of endogenous MLK3 in IFNβ gene expression, MLK3 mRNA knockdown in SKOV3 cells was achieved with MLK3 siRNA. A non-target siRNA was used as a control. 24h after siRNA transfection, cells were transfected with dsRNA for 3 or 6 h. DsRNA-induced IFNβ mRNA levels were determined by quantitative real-time PCR. Suppression of MLK3 expression significantly upregulated IFNβ mRNA after 6 h of dsRNA treatment, as compared to control siRNA treated cells (Fig 8). In absence of MLK3 from the RLH signaling pathway IFNβ mRNA expression levels are significantly increased suggesting when present, MLK3 has an inhibitory role in IFNβ mRNA expression in the RLH signaling pathway.



A



B

Figure 8: Suppression of Endogenous MLK3 Augments dsRNA-induced IFNβ mRNA. A. SKOV3 cells were subjected to gene knockdown with non-target or MLK3 specific siRNA. After 24 h, cells were treated with dsRNA (3 μg/ml) for 3 or 6 h. RNA was isolated, followed by RT-PCR. IFNβ mRNA expression was assessed through quantitative real-time PCR (qRT-PCR). Samples were normalized to human β Actin mRNA levels, and relative IFNβ mRNA quantified using the ΔΔCT method. B. SKOV3 cells were subjected to gene knockdown with non-target or MLK3 specific siRNA. After 24 h, cells were harvested and analyzed by SDS-PAGE.

3.1.3 MLK3 andTRAF2 association during dsRNA treatment

TNFα induces association between TRAF2 and MLK3 that is mediated by the TRAF N domain on TRAF2, TNFα treatment also leads to ubiquitination and activation of MLK3 [54]. To determine whether MLK3 and TRAF2 associate in response to dsRNA treatment, SKOV3 cells were treated with dsRNA for 0-6 h and TRAF2 was immunoprecipitated from whole cell lysates at each time point (Fig 9) and then quantified (Table 3.1/Fig 10). We observed a constitutive TRAF2-MLK3 association.

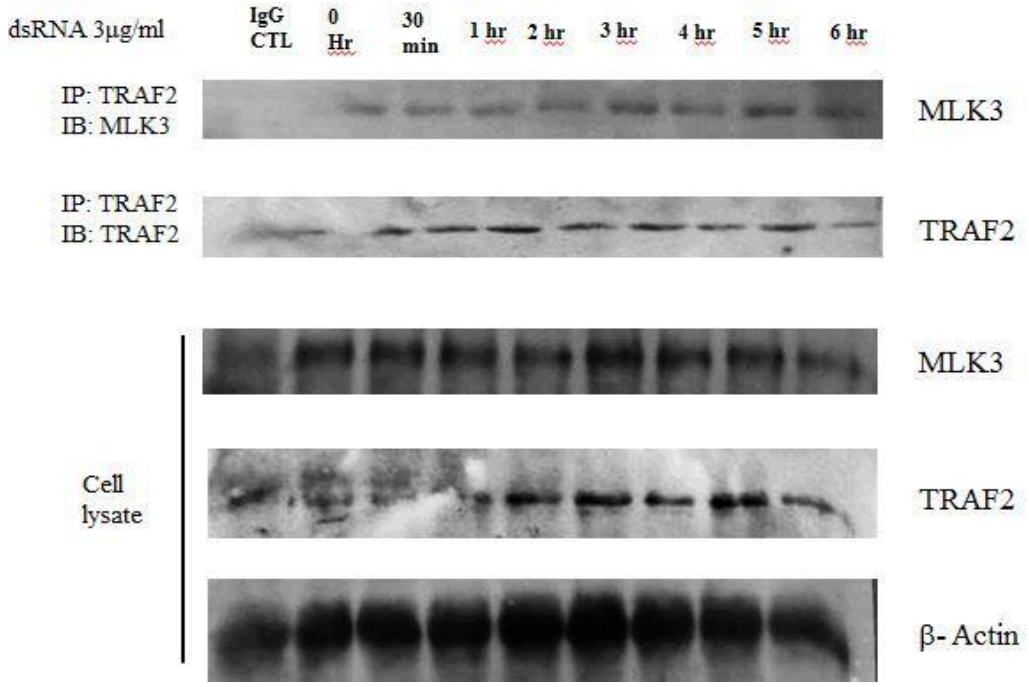


Figure 9: MLK3 andTRAF2 Associate in SKOV3 cells. SKOV3 cells were plated on 10 cm dishes and treated with dsRNA (30 µg) 30 min to 6 h. Endogenous TRAF2 was immunoprecipitated from cell lysates with TRAF2 antibody. TRAF2 and MLK3 in immunoprecipitates and whole cell lysates were evaluated by immunoblot analysis with MLK3, TRAF2 and actin antibody. Rabbit IgG antibody was used as an immunoprecipitation control.

Sample	MLK3-TRAF2 band intensity ratio
CTL	1
30 min	0.863225996
1 hr	0.530437362
2 hr	0.988782049
3 hr	1.035561277
4 hr	1.178945332
5 hr	0.974981304
6 hr	1.684508559

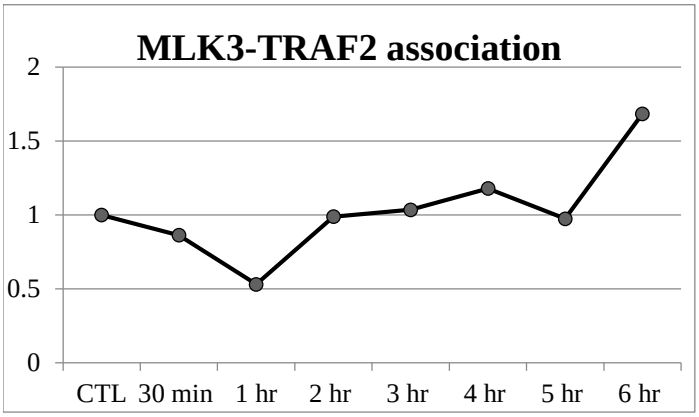


Table 3.1/Figure 10: MLK3-TRAF2 association. Signal intensities from figure 8 were normalized to the control sample for both MLK3 and TRAF2 immunoprecipitation sample sets. The normalized values were then set as a ratio comparing MLK3 to TRAF2 values throughout the time course. The table and graph represent the normalized signal intensity ratios of MLK3 to TRAF2.

3.1.4 MLK3 Interferes with IRF3 activation and translocation

TRAF2, TRAF5, and TRAF6 are essential for the activation of both IKK and TBK1 by MAVS [19]. The roles of TRAF proteins in both NF-κB and IRF3 activation by MAVS are surprising since these TRAF proteins are also recruited to other receptors such as TNF receptor, TLRs, IL-1 receptor, and CD40, which only stimulate NF-κB but not IRF3 [19]. These two major transcription factors, NF-κB and IRF3, are activated following oligomerization of MAVS leading to production of type I IFNs, such as IFNβ. Since MLK3 negatively regulates the NF-κB signaling cascade [58], we hypothesized

that MLK3 might negatively impact activation of IRF3, thereby decreasing IFNβ productivity. FLAG-MAVS was transiently co-transfected with a UAS-luciferase/GAL4-IRF3 system with or without GST-MLK3 in HEK293 cells (Fig 11). The UAS-Gal4 luciferase reporter gene, containing the Gal4 upstream activation sequence (UAS) driving luciferase transcription was co-transfected with a Gal4-IRF3 plasmid. IRF3 nuclear translocation is required for the Gal4 transcriptional initiation of the UAS luciferase reporter, thereby allowing quantitation of active IRF3 that has translocated to the nucleus. As with the overexpression of MDA5 and MAVS, the overexpression of MLK3 had a significant dose dependent inhibitory effect on MAVS-induced Gal4-IRF3 activity.

To further study the inhibitory effect of MLK3 on IRF3 translocation, HEK293 cells were transiently co-transfected with IRF3-GFP with or without FLAG-MAVS, FLAG-TRAF2 and/or GST-MLK3, (Fig 12). Cells overexpressing MAVS and TRAF2 induced IRF3 activation and translocation to the nucleus, as demonstrated by GFP localization to the nucleus. However, with the addition of MLK3, IRF3 nuclear translocation was inhibited, as demonstrated by the absence of GFP in the nucleus. The images were quantified by counting all the cells in the image containing GFP, FLAG and DAPI. The cells with GFP present in the nucleus were then counted as "translocation positive cells" (Table 3.2). Collectively our preliminary results suggest MLK3 has a direct effect on IRF3 signaling, which would provide one explanation for the decrease in IFNβ production in cells expressing GST-MLK3.

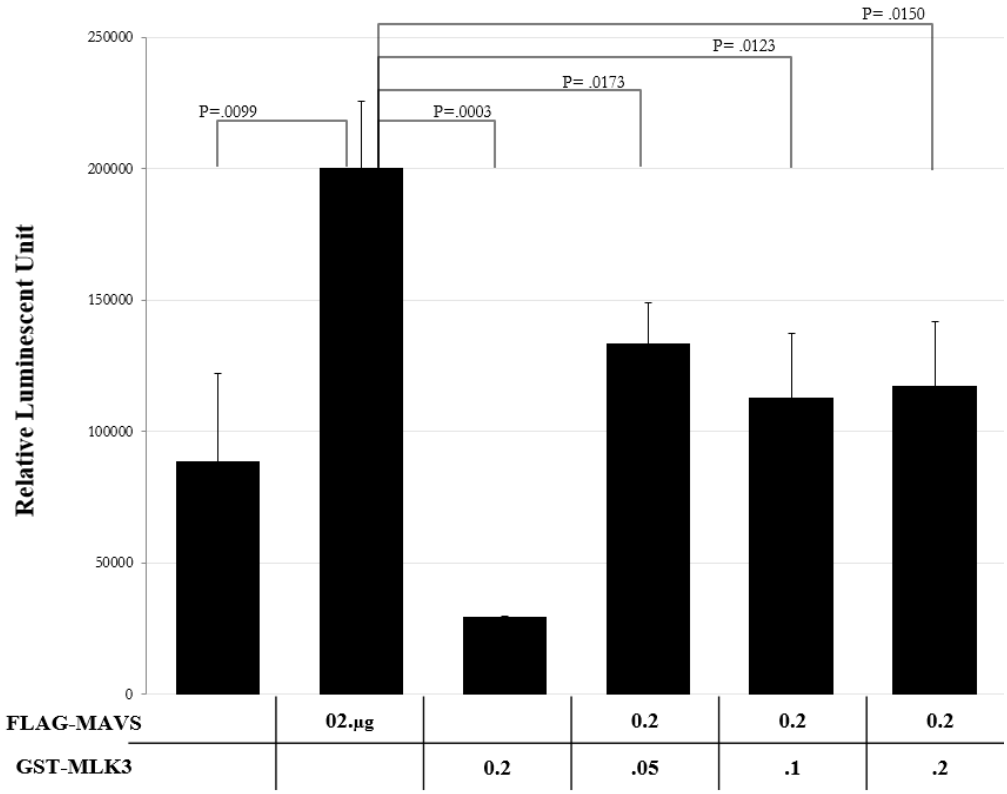


Figure 11: MLK3 Interferes with IRF3 Activation. HEK 293 cells were transiently co-transfected with UAS-luciferase (0.1 µg), IRF3-GAL4 (0.1 µg) and SV40 β-gal (0.1 µg), with or without the indicated amounts of FLAG-MAVS and GST-MLK3. Empty pcDNA vector was used to balance each sample to 1 µg DNA. The cells were harvested 24 h after transfection and luciferase and β-Galactosidase assays performed. Luciferase activity was normalized to the co-transfected β-galactosidase expression construct.

A

.2 µg IRF3-GFP
.2 µg Flag-MAVS
.2 µg Flag-MAVS
.1 µg GST- MLK3
.2 µg Flag-MAVS
.2 µg GST- MLK3
.2 µg Flag-MAVS
.3 µg GST- MLK3
IRF3-GFP
FLAG-MAVS
DAPI

B

.2 µg IRF3-GFP
.2 µg Flag-TRAF2
.2 µg Flag-TRAF2
.1 µg GST- MLK3
.2 µg Flag-TRAF2
.2 µg GST- MLK3
.2 µg Flag-TRAF2
.3 µg GST- MLK3
IRF3-GFP
FLAG-MAVS
DAPI

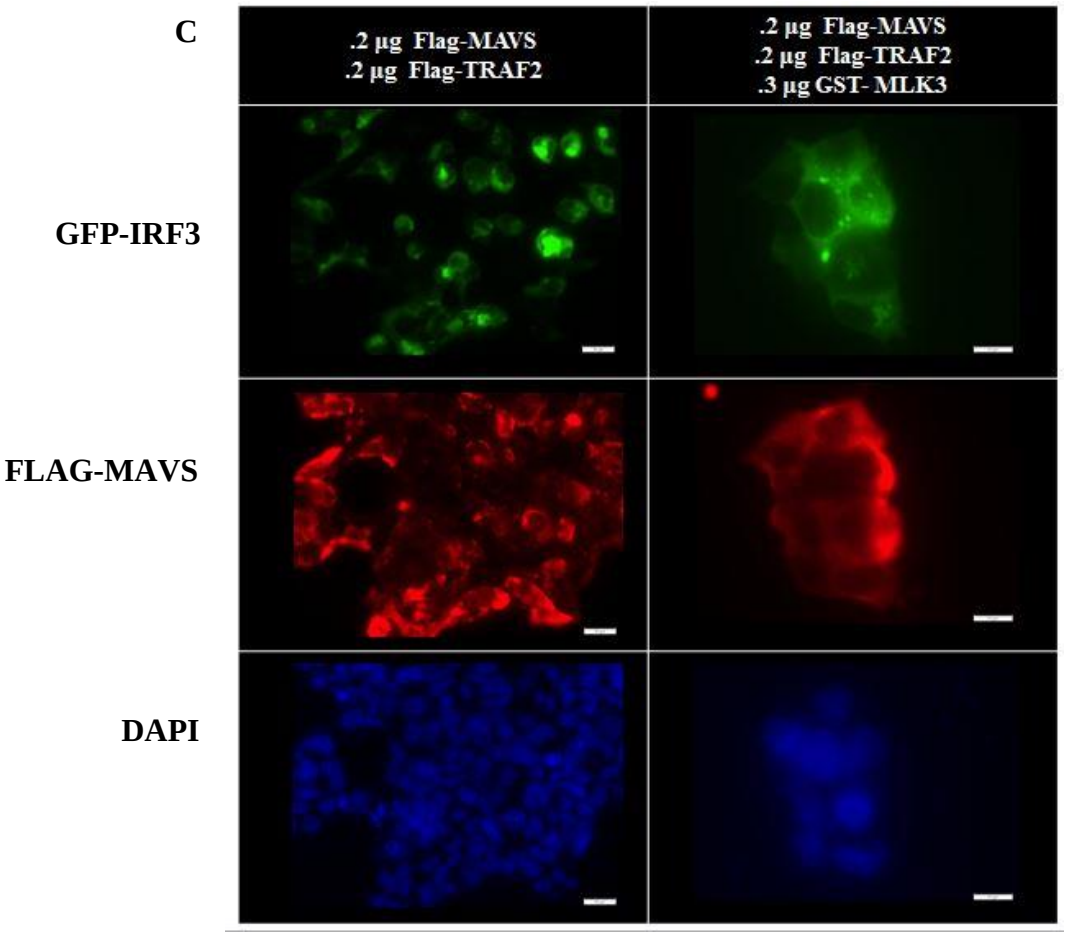


Figure 12: MLK3 Interferes with IRF3 activation. HEK 293 cells were plated on Poly-L-lysine treated coverslips and then transiently transfected with pIRF3-GFP (0.2 ug). Cells were then co-transfected with FLAG-MAVS (0.2 ug) and/or FLAG-TRAF2 (0.2 ug) along with the indicated amounts of GST-MLK3. After 24 h, cells were fixed and incubated with anti-FLAG primary antibody then Goat-anti-Mouse Alexa Fluor 594 secondary. Cells were then treated with Pro-Long Gold anti-fade reagent containing DAPI overnight, sealed then imaged at 60X or 100X using an Olympus IX81 epi-fluorescent microscope.

Sample	total cells	translocation positive
IRF3-GFP	44	0
IRF3-GFP + MAVS	23	4
IRF3-GFP + MAVS + .1 µg MLK3	3	0
IRF3-GFP + MAVS + .2 µg MLK3	10	0
IRF3-GFP + MAVS + .3 µg MLK3	54	0
IRF3-GFP	44	0
IRF3-GFP + TRAF2	50	5
IRF3-GFP + TRAF2 + .1 µg MLK3	8	0
IRF3-GFP + TRAF2 + .2 µg MLK3	3	0
IRF3-GFP + TRAF2 + .3 µg MLK3		
IRF3-GFP	44	0
IRF3-GFP + MAVS + TRAF2	42	18
IRF3-GFP + MAVS + TRAF2 + .3 µg MLK3	7	1

Table 3.2: IRF3-GFP quantification. The images were quantified by counting all the cells in the image containing GFP, FLAG and DAPI. The cells with GFP present in the nucleus were then counted as “translocation positive cells”

Chapter 4

Discussion

MLK3 is a MAP3K family member that is associated with a range of cellular responses including migration, differentiation and apoptosis, each being stimuli specific [32,48,53,55,56]. Upon activation by environmental factors such as inflammatory cytokines, oxidative stress, and growth factors MLK3 transduces the signal to downstream MAP2Ks, including MKKs 1-7 which send the signal to MAPKs; JNK, ERK and p38 which regulate cellular processes [27,57].

MLK3 expression levels are elevated in ovarian, breast, pancreatic, colon, and cervical cancers [28]. In ovarian cancer cells MLK3 is required for proliferation, invasion and matrix metalloproteinases (MMP) expression [52, 53]. In breast cancer cells, MLK3 is necessary for cell migration, invasion of metastasis and tumor growth [50, 51, 61]. A number of studies suggest that targeting of MLK3 could be a therapeutic strategy for treatment or prevention of metastatic disease in breast cancer [51]. MLK3 is also involved in stress responses where it upregulates JNK and p38 signaling leading to an upregulation of apoptosis [27]. Although most of the roles assigned to MLK3 involve upregulation of signaling pathways, an anti-survival role for endogenous MLK3 in NIH3T3, SKOV3 and HEK293 cells through downregulation of NF-κB activation by inhibiting IKK activity has been identified [58]. Few studies have assessed MLK3's role

in innate immune responses. As such we investigated a possible role in the RLH signaling pathway.

4.1 MLK3 inhibits IFNβ promoter activation by MDA5 and MAVS

When a cell encounters a viral intruder, it must respond quickly in order to suppress the spread of infection to healthy cells. Although multiple detection pathways are known, the RLH pathway has been implicated as the most vital based on studies in knock out mice [62]. Upregulation of RLH pathways involves the recognition of single or double stranded RNA by binding proteins such as RIG-I and MDA5. Following RLHs association with MAVS, recruitment of adaptor proteins, such as TRAF2, transduce the signal to transcription factors NF-κB and IRF3 which assist in the transcription of type I IFNs, such as IFNβ [1]. Upregulation of RLH signaling is critical for healthy cell immunity, but uncontrolled signaling can lead to immune deficiencies or septic shock [63].

To determine if MLK3 impacts RLH signaling, we first assessed the effect of MLK3 overexpression on MDA5 or MAVS dependent induction of an IFNβ luciferase reporter in HEK293 cells. These luciferase studies showed that MLK3 overexpression resulted in a significant dose dependent downregulation of IFNβ luciferase activity (Fig 5). MLK3 inhibited both MDA5 and MAVS dependent responses suggesting that it impacted significant components downstream of MAVS. Next, we wished to gain insight into whether MLK3 kinase activity is necessary for this inhibitory effect, we transiently co-transfected our IFNβ luciferase reporter and FLAG-MAVS with or without GST-

MLK3 or kinase-dead MLK3 in HEK293 cells. We observed that kinase dead MLK3 expression did not inhibit IFNβ luciferase activity, suggesting that MLK3's inhibitory role is kinase dependent. This suggests that the kinase domain of MLK3 may play a role in MLK3s activity, assisting in its inhibitory role in the RLH signaling pathway.

To detect if endogenous MLK3 also inhibits RLH responses, we knocked down MLK3 with siRNA and assessed IFNβ mRNA levels in response to dsRNA treatment. Cells transfected with MLK3 specific or non-target siRNAs for 24 h were then treated with dsRNA for 3 or 6 h. We observed a significant increase in the amount of IFNβ mRNA expressed in cells that received the MLK3 siRNA over those that received the non-target siRNA (Fig 7). These data suggest that MLK3 dependent regulation of IFNβ through MDA5 and MAVS signaling is physiologically relevant, although it is not yet certain whether normal, non-cancer cells elicit a similar effect.

4.2 MLK3-TRAF2 association in RLH pathway detected through dsRNA treatment

Collectively our data suggests that MLK3 is likely acting on a signaling protein downstream of MAVS. TRAF2, a regulator recruited downstream of MAVS has been implicated in activation of JNK, NF-κB and IRF3 [59]. Interestingly, the interaction between MLK3 and TRAF2 occurs once cells are stimulated with TNFα and leads to the activation of MLK3, through the TRAF-N domain of TRAF2 and the C-terminal domain of MLK3 [54, 55]. Although three TRAF proteins (TRAF2, TRAF5 and TRAF6) can interact with ectopically expressed MLK3, only TRAF2 can activate MLK3 kinase

activity [54, 55]. TRAF2 can associate with MLK3 in various cell types including SKOV3 and HEK293. We investigated whether MLK3 and TRAF2 associated in SKOV3 cells when stimulated with dsRNA. SKOV3 cells were treated with dsRNA for .5 to 6 h, and endogenous TRAF2 was immunoprecipitated and MLK3 co-precipitation assessed. Immunoblot analysis showed constitutive MLK3-TRAF2 association. Throughout the dsRNA time course the MLK3-TRAF2 association varies within a small range of the normalized control sample, with the exception of the 1 hr treatment time. We hypothesize that during the 1 hr treatment time MLK3 may be out-competed by other signaling factors such as TBK-1 for TRAF2 (Fig 13). These data suggest that the addition of dsRNA may cause the binding competition on TRAF2, however the MLK3-TRAF2 association re-associated with extended periods of dsRNA treatments. It is plausible that MLK3 is recruited to TRAF2 in order to assist in the regulation of IFNβ production, as overexpression of IFNβ can lead to health complications.

4.3 MLK3 Interferes with IRF3 activation

IRF3 has been identified as the central transcriptional factor regulating IFNβ gene expression [14]. Our results suggest that IRF3 regulation by MLK3 must impact IFNβ gene expression. Transiently overexpressed FLAG-MAVS with or without GST-MLK3 resulted in significant inhibition of IRF3 activation and translocation. Consistent with these results, preliminary immunofluorescence data showed nuclear translocation of IRF3 in a subset of cells co-expressing MAVS or TRAF2, but not in cells expressing MLK3 and MAVS or TRAF2. Possibly, MLK3 expression blocks IRF3 activity and translocation into the nucleus by competing with TBK-1 for TRAF2 binding. Although

these data need to be repeated, expanded and quantified (Fig A, B, C). Collectively these data may give further rational to MLK3s ability to inhibit IFNβ gene expression.

4.4 Summary and Future Directions

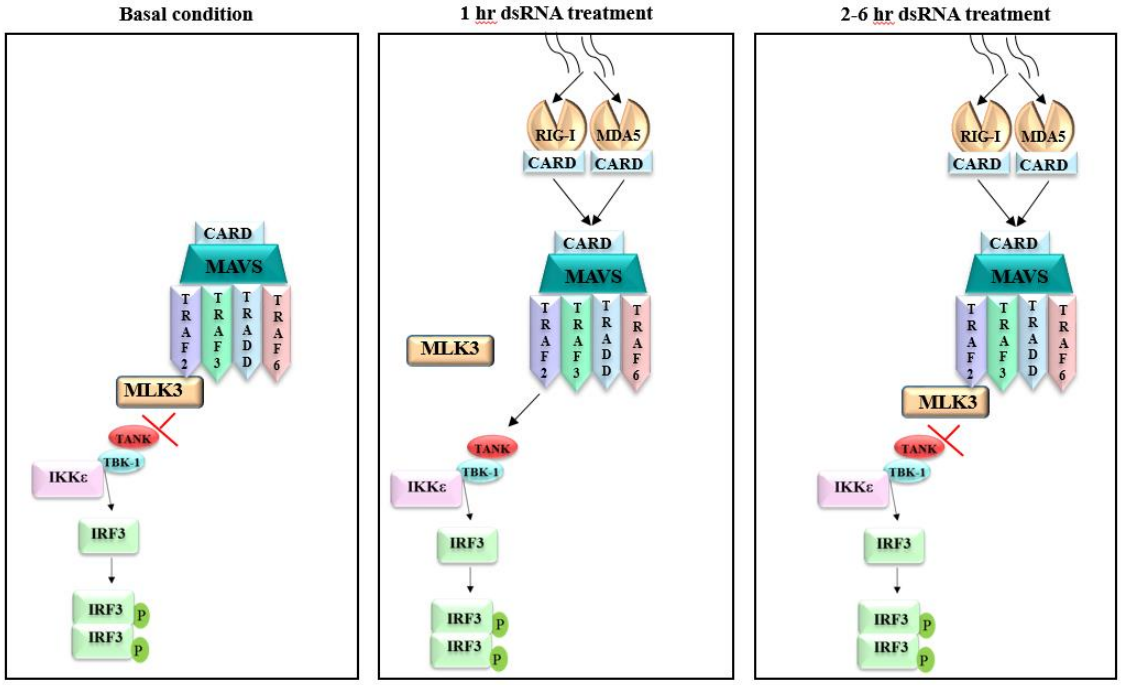


Figure 13: The role of MLK3 in dsRNA signaling. During basal conditions MLK3-TRAF2 constitutively associate, leading to inhibition of downstream transcription factor IRF3. After 1 hr of dsRNA the MLK3-TRAF2 association levels decrease, allowing downstream signaling to occur. For the remaining 6 hrs of the dsRNA time course, a MLK3-TRAF2 association is observed.

Collectively our observations suggest that MLK3 may have a possible role for the inhibition of IFNβ production. Overexpression of MLK3, MAVS and MDA5 suggest an

inhibitory role of MLK3 on IFNβ downstream of MAVS. Additionally we observed that MLK3 kinase activity is required for this inhibitory role on IFNβ production. Our data also suggest that suppression of MLK3 activity through siRNA knockdown leads to an increase in IFNβ mRNA levels when stimulated with dsRNA. Several signal regulators downstream of MAVS are responsible for IFNβ activity, including TRAF2. Our data show stable TRAF2-MLK3 association with or without dsRNA treatment. Our results overexpressing also suggest that MLK3 has an inhibitory role in IRF3 activity and translocation into the nucleus. Immunofluorescence of GFP-IRF3 with or without MAVS, TRAF2 or MLK3 also suggest an inhibitory role for MLK3 in IRF3 activity and translocation into the nucleus. In the future we hope to identify a mechanism for MLK3 dependent inhibition of IFNβ activity. Additionally, the study of other signal regulators downstream of MAVS such as TBK-1 and IKK would be of great interest.

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