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## TRAF2-MLK3 interaction is essential for TNF- $\alpha$ -induced MLK3 activation

Gautam Sondarva<sup>1,5</sup>, Chanakya N. Kundu<sup>2,5</sup>, Suneet Mehrotra<sup>1</sup>, Rajakishore Mishra<sup>1</sup>, Velusamy Rangasamy<sup>1</sup>, Pradeep Sathyanarayana<sup>2</sup>, Rajarshi S. Ray<sup>1</sup>, Basabi Rana<sup>3,4</sup>, and Ajay Rana<sup>1,2,4</sup>

<sup>1</sup>Department of Pharmacology, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153, USA

<sup>2</sup>Division of Molecular Cardiology, The Texas A & M University System Health Science Center, College of Medicine, Temple, Texas 76504, USA

<sup>3</sup>Department of Medicine, Division of Gastroenterology, Hepatology & Nutrition, Stritch School of Medicine, Loyola University Chicago, Maywood, Illinois 60153, USA

<sup>4</sup>Hines Veterans Affairs Medical Center, Hines, Illinois 60141, USA

### Abstract

Mixed Lineage Kinase 3 (MLK3) is a mitogen-activated protein kinase kinase kinase (MAPKKK) that is activated by Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) and specifically activates c-Jun N-terminal kinase (JNK) upon TNF- $\alpha$  stimulation. The mechanism by which TNF- $\alpha$  activates MLK3 is still not known. TNF receptor-associated factors (TRAFs) are adaptor molecules that are recruited to cytoplasmic end of TNF receptor and mediate the downstream signaling, including activation of JNK. Here, we report that MLK3 associates with TRAF2, TRAF5 and TRAF6; however only TRAF2 can significantly induce the kinase activity of MLK3. The interaction domain of TRAF2 maps to the TRAF domain and for MLK3 to its C-terminal half (amino acids 511-847).

Endogenous TRAF2 and MLK3 associate with each other in response to TNF- $\alpha$  treatment in a time-dependent manner. The association between MLK3 and TRAF2 mediates MLK3 activation and competition with the TRAF2 deletion mutant that binds to MLK3 attenuates MLK3 kinase activity in a dose dependent manner, upon TNF- $\alpha$  treatment. Furthermore the downstream target of MLK3, JNK was activated by TNF- $\alpha$  in a TRAF2 dependent manner. Hence, our data show that the direct interaction between TRAF2 and MLK3 is required for TNF- $\alpha$ -induced activation of MLK3 and its downstream target, JNK.

### Keywords

c-Jun N-terminal kinase (JNK); Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ); Mixed Lineage Kinase (MLK3); TNF receptor-associated factors (TRAFs)

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Correspondence should be addressed to Ajay Rana, Department of Pharmacology, Bldg 102, Loyola University Medical Center, 2160 S. First Avenue, Maywood, IL 60153, USA. Tel: 708-216-5761; Fax: 708-216-6596; arana@lumc.edu.

<sup>5</sup>These authors contributed equally to this work.

## Introduction

Mixed Lineage Kinase 3 (MLK3) is a MAPKKK and belongs to MLK family [1]. The MLK family members are characterized by the presence of signature sequences of Serine/Threonine (Ser/Thr) and Tyrosine kinases within their catalytic domain. We and others have shown that some of the MLK members, including MLK3 [2], MLK2 [3] and DLK [4] possess the functional Ser/Thr kinase activities, however, the tyrosine kinase activity of any MLK family member is still not known. Structurally MLK family members are highly conserved in their catalytic domain but they diverge in the C-terminal domain, suggesting that probably each family member might be regulated differentially by different ligands. Although the specific ligands for all MLK family members are not yet known, we reported earlier that MLK3 can be activated by TNF- $\alpha$  [5]. The TNF- $\alpha$  was also able to activate JNK, the downstream target of MLK3, specifically in a MLK3-dependent manner, whereas the activation of other two MAPKs, p38 and ERK was not mediated via MLK3 [5]. Although our studies showed that TNF- $\alpha$  is a ligand of MLK3 [5], however the mechanism by which TNF- $\alpha$  activates MLK3 is still not understood.

TNF- $\alpha$  is a pleiotropic cytokine that renders cellular effects by binding to its cognate receptors, TNFR1 and TNFR2. It has been suggested that upon encounter with trimeric TNF- $\alpha$ , the TNF receptors undergo trimerization, which leads to intracellular signaling [6, 7]. Recently, it has been reported that TNFR proteins self assemble in the absence of ligand, undergoing conformational changes upon ligand engagement that lead to downstream signaling [8]. Upon conformational changes in the TNFRs, the cytoplasmic ends of TNFRs bind to specific adaptor proteins that ultimately transmit the signals to downstream targets [9]. Since TNFR proteins lack intrinsic enzymatic activity, the binding of adaptor proteins to the cytoplasmic end of the receptor was shown to be essential for TNF- $\alpha$  mediated signaling [9]. One of the protein families that bind to both the TNFRs is TRAFs that has been shown to activate JNK and NF- $\kappa$ B pathways upon TNF- $\alpha$  stimulation [10].

Till date, six members of the TRAF family have been described [11-13]. All the TRAFs contain C-terminal TRAF domain that mediates the interaction with TNF receptors and hetero- or homo- dimerization among the TRAF family members [11]. In addition to the TRAF domain, the N-terminus of the TRAF proteins, with the exception of TRAF1, contains RING domain and multiple Zinc finger structures; those are essential for their effector functions [9, 12]. The TRAF family not only mediates TNF receptor family-mediated signaling but may also link the downstream signaling via other receptor like interleukin-1 receptor (IL-1R) [9]. Upon IL-1R receptor engagement, the cytoplasmic end of the receptor recruits a protein complex, including IRAK that binds with TRAF6 and activates JNK, p38, ERK and Src tyrosine kinase [13-16]. Among the six known TRAF proteins, TRAF2, TRAF5 and TRAF6 are reported to activate JNK [17], the downstream target of MLK3 [2] and other MLKs [1]. It is also reported that TRAF proteins activate the MAPK cascades at the level of MAP3K and MAP4K, including ASK1 [18, 19], TAK1 [20], MEKK1 [21], NIK [22] and GCK [23]. Whether, the TRAF proteins also recruit MLK3 for TNFR-mediated activation of downstream JNK is yet to be determined.

In this report, we show that although TRAF2, TRAF5 and TRAF6 can all bind to MLK3, however, only TRAF2 can activate the MLK3 kinase activity. The interaction between TRAF2 and MLK3 was mediated via the TRAF domain of TRAF2, and the C-terminal domain of MLK3. The endogenous TRAF2 and MLK3 also associate with each other, in response to TNF- $\alpha$  treatment and TRAF2 associated MLK3 possessed significantly higher kinase activity compared to total MLK3 pool. The association between TRAF2 and MLK3 was essential for TNF- $\alpha$ -induced MLK3 activation, since competition with the TRAF domain of TRAF2 was able to attenuate MLK3 activation in a dose-dependent manner. We also show that TRAF2 was essential for TNF- $\alpha$ -induced JNK activation in a MLK3-dependent manner. Together, these data show a role of TRAF2 in TNF- $\alpha$ -induced MLK3 activation, and its downstream signaling to JNK pathway.

## Results

### MLK3 and TRAF proteins associate in vivo

To determine whether TRAF proteins play any role in the activation of MLK3 via TNFRs, we first attempted to identify the TRAF protein(s) that binds with ectopically expressed MLK3. The human HEK-293 cells were transiently transfected with equal amounts of GST-tagged MLK3 along with either Flag-tagged or Myc-tagged TRAF expression vectors, as indicated (Fig. 1). The TRAF proteins were immunoprecipitated either by anti-FLAG (for TRAF2, 5 and 6) or by anti-Myc (for TRAF1 and 3) antibodies and any associated recombinant GST-tagged MLK3 was detected by anti-GST antibody. Our results showed that TRAF2, 5 and 6 associated with ectopically expressed MLK3, however TRAF1 and 3 failed to co-immunoprecipitate with MLK3 (Fig.1). Taken together, these results indicate that MLK3 associates with TRAF2, 5 and 6 under over expressed condition.

### TRAF2 regulates TNF- $\alpha$ -induced MLK3 kinase activity

Interaction between TRAF2 and some MAP3K members, such as ASK1 [18, 19], TAK1 [20] and MEKK1 [21] has been shown to induce their kinase activities. We examined which of the TRAF proteins (i.e. TRAF2, 5 and 6) that showed interaction with MLK3 (Fig.1) in fact can activate MLK3 kinase activity. HEK-293 cells were transiently transfected with a constant amount of MLK3 and the three TRAF expression vectors, as shown in Fig. 2. As controls, some cells were also transfected with TRAF expression vectors alone, to rule out the contribution of any contaminating kinase during the kinase assay. Recombinant MLK3 was precipitated by GSH-pull down and subjected to kinase assay using MLK3 specific substrate, SEK1 (K-R) protein, expressed in bacteria. MLK3 was about 3.5 fold more active in the cells, where TRAF2 was transfected (Fig. 2A, compare lanes 2 with 4) where as MLK3 was not significantly activated in cells that were transfected with TRAF5 and 6 (Fig. 2A). The expression levels of MLK3 and TRAFs were almost equal in all our transfection experiments. These results suggest that TRAF2 might be the adaptor protein that recruits MLK3 to the TNFR. To further confirm that our results were not due to transfection artifact, mouse embryonic fibroblasts (MEFs) from wild type and TRAF2 deficient mice were utilized to examine whether TRAF2 is required for TNF- $\alpha$ -induced MLK3 activation. The wild type (TRAF2<sup>+/+</sup>) and TRAF2 deficient (TRAF2<sup>-/-</sup>) MEFs were treated with 10nM of TNF- $\alpha$  for 30 or 60 minutes and the endogenous MLK3 kinase activity was measured.

Endogenous MLK3 was activated about 4.5 fold following 30 or 60 minutes stimulation by TNF- $\alpha$  in wild type and not in TRAF2 deficient MEFs (Fig. 2B, compare lanes 4 and 5 with 8 and 9). The time period for TNF- $\alpha$  treatment was chosen based on our results as shown in Fig. 3. Taken together these results conclusively demonstrate that TRAF2 is necessary for TNF- $\alpha$  mediated MLK3 activation.

### **Association between endogenous MLK3 and TRAF2 is regulated by TNF- $\alpha$**

Given that TRAF2 protein can associate (Fig.1) with and activate ectopically expressed MLK3 (Fig. 2A), we planned to test the association between TRAF2 and MLK3 under more physiological condition. The Jurkat T cells were chosen for these experiments, since these cells respond very well to TNF- $\alpha$  treatment and express considerable amounts of endogenous MLK3. To examine the endogenous interaction between MLK3 and TRAF2, Jurkat T cells were starved in low serum (0.2% FBS) containing medium for 12 hours and then treated with human TNF- $\alpha$  for different period of times. The endogenous TRAF2 was immunoprecipitated and any associated MLK3 in the immune-complex was blotted with antibody against MLK3. Endogenous MLK3 co-immunoprecipitated with TRAF2 in a time dependent manner and the interaction was maximal at 30 minutes of TNF- $\alpha$  treatment and was undetectable by 120 minutes (Fig. 3A). To further confirm that the endogenous interaction between MLK3-TRAF2 was not due to any artifacts of non-specific interaction, MEFs from wild type and TRAF2 deficient mice were treated with murine TNF- $\alpha$ , similar to Jurkat T cells. Endogenous TRAF2 was immunoprecipitated and blotted for any associated MLK3, similar to Jurkat T cells. The association between endogenous TRAF2 and MLK3 was only observed in TRAF2 immunoprecipitates from wild type MEFs, but not in TRAF2 deficient MEFs in response to TNF- $\alpha$  treatment (Fig. 3B). This association between MLK3 and TRAF2 was also higher in wild type MEFs at 30 minutes of TNF- $\alpha$  treatment (Fig. 3B), as observed in Jurkat T cells. Therefore, it can be concluded that the interaction between MLK3 and TRAF2 is physiological and is regulated by TNF- $\alpha$ .

### **Mapping of in vivo interaction domains between MLK3 and TRAF2**

The sequence analysis of TRAF family proteins reveal several conserved domains, such as RING, Zinc finger and TRAF domains, which are reported to mediate protein-protein interactions. To identify the domain(s) on TRAF2 proteins that interact with MLK3, several TRAF2 deletion constructs were utilized, as shown in Fig. 4A. These TRAF2 mutant constructs were transfected into HEK-293 cells along with MLK3 expression vector, as indicated in Fig. 4B. First, the expression levels of all TRAF2 mutants, including the full length proteins were analyzed, and then the cell lysates with equal expression of TRAF2 mutant proteins were immunoprecipitated using anti-FLAG antibody. The association between MLK3 and TRAF2 mutant proteins was detected using an antibody against the GST-tag present on recombinant MLK3. As per our expectation, MLK3 interacted with full length and other TRAF2 mutant proteins, except the TRAF2 mutant protein, where the entire TRAF domain was deleted (Fig. 4B, lane 4). Interestingly, the interaction between entire TRAF domain and MLK3 was the least (Fig. 4B, lane 5) whereas it was highest with the N-terminus of the TRAF domain (Fig. 4B, lane 7). Our result is consistent with one other protein, where it has been reported that isolated TRAF domain also does not interact with ASK1; even though the deletion of TRAF domain from TRAF2 fails to interact with full

length ASK1 [19]. However, our results, clearly suggest that TRAF2 interact with MLK3 via TRAF-N domain (Fig. 4B).

Similar to TRAF2, MLK3 also contains several conserved domains, which have been shown to mediate protein-protein interactions [24]. Several deletion mutants (Fig. 4C) of MLK3 protein were created to map the specific site on MLK3, required for interaction with TRAF2. These constructs were transfected in HEK-293 cells along with Flag-tagged, full length TRAF2 expression vector. The interaction between TRAF2 and MLK3 mutant proteins were determined by immunoprecipitating TRAF2 by anti-FLAG antibody and blotting with anti-GST antibody for associated MLK3 mutant proteins. As per our earlier observation (Fig. 1), full length MLK3 interacted with TRAF2 and all other mutant proteins, except the C-terminal domain of MLK3 failed to interact with full length TRAF2 (Fig. 4D, lane 10). These results suggest that MLK3 interact with TRAF2 via its C-terminal regulatory domain.

### **The association between TRAF2-MLK3 is necessary for TNF- $\alpha$ -induced MLK3 activation**

TRAF2 is an adaptor protein and lacks any intrinsic kinase activity and thus we wanted to know how TRAF2 activates MLK3 kinase activity. One potential way by which TRAF2 might activate MLK3 is via protein-protein interaction in response to TNF- $\alpha$  treatment. To test whether direct interaction of TRAF2 with MLK3 activates MLK3 kinase activity by TNF- $\alpha$ , Jurkat T cells were treated with TNF- $\alpha$  for different time points, as indicated in Fig. 5A. First, we optimized the conditions to immunoprecipitate equal amounts of total MLK3 and TRAF2 associated MLK3 by using MLK3 and TRAF2 antibodies respectively (Fig. 5A). Once we optimized how much of cell lysates were needed to immunoprecipitate equal amounts of MLK3 by both the antibodies, we immunoprecipitated comparable amounts of total and TRAF2 associated MLK3 as shown in Fig. 5A, and measured MLK3 kinase activity by using SEK1 (K-R) protein. As envisioned, our results showed about 4 fold activation of TRAF2-associated MLK3 when compared to equal amount of total MLK3 after 30 minutes of TNF- $\alpha$  treatment (Fig. 5A, compare lanes 5 with 6). These results clearly suggest that TNF- $\alpha$ -induced TRAF2-MLK3 interaction is required for MLK3 activation. To further confirm that the interaction between TRAF2-MLK3 indeed is required for induction of MLK3 kinase activity, we attempted to compete off this interaction by using the TRAF-N domain that we determined to be the minimal domain on TRAF2 for MLK3 interaction (Fig. 4B). Constant amount of MLK3 and increasing quantity of cDNA expressing TRAF-N domain (AA, 272-358) were transfected in HEK-293 cells and treated with either 10nM of TNF- $\alpha$  for 30 minutes, or left alone, as controls (Fig. 5B). Equal amount of MLK3 was immunoprecipitated from these cells and subjected to kinase assay. Confirming our endogenous interaction data (Fig. 5A), the MLK3 kinase activity was gradually attenuated/competed off by increasing dose of TRAF-N domain in TNF- $\alpha$  treated cells but not in untreated cells (Fig. 5B). These results clearly suggest that TNF- $\alpha$  induces interaction between TRAF2 and MLK3 and this interaction leads to MLK3 activation.

### **TRAF2 and MLK3 are necessary for TNF- $\alpha$ -induced JNK activation**

Earlier we have shown that MLK3 was necessary for TNF- $\alpha$ -induced JNK activation [5] and MLK3 specifically mediated TNF- $\alpha$ -induced JNK activation and not other MAPKs [5]. To examine whether TRAF2 mediates TNF- $\alpha$  action on JNK via MLK3, we took advantage of

TRAF2 deficient MEFs. These cells were transfected with TRAF2 expression plasmids or used without transfection as controls. Two days post-transfection, these cells were treated with TNF- $\alpha$  for 30 or 60 minutes and JNK kinase activity was measured using bacterially expressed GST-c-JUN as the substrate. The basal activity of JNK in these cells was very low upon TNF- $\alpha$  treatment in absence of endogenous TRAF2 (Fig. 6A, lane 2) and was slightly elevated upon ectopic expression of TRAF2, without TNF- $\alpha$  treatment (lane 3). However, JNK activity was increased about 3.5 fold in these cells, when TRAF2 was ectopically expressed, and treated with TNF- $\alpha$  for 30 or 60 minutes (Fig. 6A). The MLK3 kinase activity was also estimated from these cells, which showed activation profile similar to that of JNK (Fig. 6B). Based on these results and our previous published results [5], we conclude that TRAF2 is necessary for MLK3-mediated JNK activation by TNF- $\alpha$ .

To further confirm that MLK3 is required for TNF- $\alpha$ -induced JNK activation via TRAF2, normal MEFs (i.e. TRAF2<sup>+/+</sup>) were used to knock down the endogenous MLK3 by transducing these cells with lentivirus, containing MLK3 specific shRNA. Cells transduced with LacZ shRNA were used as controls (Fig. 6C). These cells were treated with murine TNF- $\alpha$  for 30 min or left alone as controls. Western Blot analysis with anti-MLK3 antibody, clearly showed about 95% knock down of endogenous MLK3 by MLK3 specific shRNA (Fig. 6C, lower panel, lane 3 and 4) but not by LacZ shRNA (lanes 5 and 6). Equal amount of cell lysates were then blotted with phospho-JNK antibody to determine the activation status of endogenous JNK in these cells. Our results showed that TNF- $\alpha$  indeed activated endogenous JNK in control cells with endogenous MLK3 (Fig. 6C, upper panel lanes 2 and 6), however the activation of JNK by TNF- $\alpha$  was attenuated significantly when MLK3 was knocked down by MLK3 specific shRNA (see lane 4). Collectively these results convincingly prove that TRAF2-MLK3 pathway mediates the TNF- $\alpha$ -induced JNK activation.

## Discussion

We have shown earlier that MLK3 was activated by TNF- $\alpha$  [5]. In this study, we also reported that TNF- $\alpha$ -induced MLK3 activation, specifically activated JNK but not p38 or ERK MAPKs [5], suggesting that TNF- $\alpha$ -induced MLK3 activity was actually specific for JNK activation. Here, we provide a mechanism by which TNF- $\alpha$  activates MLK3 and its downstream target JNK.

The family member of TRAF adaptor proteins have been implicated in cytokine-mediated downstream signaling, where TRAF2, TRAF5 and TRAF6 have been shown to activate the JNK pathway [17]. Our initial expectation was that MLK3 might interact with all three TRAF proteins, which are already implicated in the activation of JNK, which will lead to induction of MLK3 catalytic activity. Our results clearly suggest that all three TRAF proteins (i.e. TRAF2, TRAF5 and TRAF6) can interact with ectopically expressed MLK3 (Fig. 1), but only TRAF2 can activate MLK3 kinase activity (Fig. 2A). It remains a possibility that under specific physiological conditions and in response to specific stimuli, TRAF5 and 6 proteins might also lead to MLK3 activation, which is yet to be determined. The interaction between TRAF2 and MLK3 is physiological based on the fact that

endogenous TRAF2 can interact with MLK3 in response to the ligand of MLK3 i.e. TNF- $\alpha$  (Fig. 3), in a time dependent manner.

TRAF2 interaction with other MAP3Ks has previously been shown to cause their activation [18-21], but how the interaction per-se leads to increase in the catalytic activities of these kinases is still not known. We also observed that the interaction between MLK3 and TRAF2 was essential for TNF- $\alpha$ -induced activation of MLK3 because the TRAF2-associated MLK3 was 4.5 fold more active compared to the total cellular pool of MLK3 (Fig. 5A).

Furthermore, we also confirmed that the activation of MLK3 by TNF- $\alpha$  was via TRAF2 interaction because MLK3 activation was attenuated in a dose dependent manner by the TRAF-N protein (Fig. 5B) that interacts with MLK3 (Fig. 4A). Thus collectively, we can conclude that TRAF2-MLK3 interaction is essential for MLK3 activation by TNF- $\alpha$ . During review of this manuscript, similar findings were reported by Korchnak et al [25]. We reported earlier that in addition to TNF- $\alpha$ , the bioactive membrane lipid, ceramides also activates MLK3 [5] and therefore one potential mechanism by which TRAF2 interaction with MLK3 can lead to its activation might be via recruiting MLK3 to the membrane. When MLK3 is recruited at the membrane, the ceramides generated via TNF- $\alpha$ -induced sphingomyelinase activation [26] might modulate MLK3 kinase activation, which is yet to be determined. We also examined any association between MLK3 and TRAF2 by ceramide in Jurkat cells. The ceramide concentration that activated the endogenous MLK3 in Jurkat cells did not induce any interaction between TRAF2 and MLK3 (data not shown), suggesting that mechanisms of MLK3 activation by TNF- $\alpha$  and ceramides are distinct.

TRAF2 has been shown to cause activation of both JNK and NF- $\kappa$ B pathways [17]. Although, now it is known that TRAF2 primarily activates JNK pathway rather than NF- $\kappa$ B, as the MEFs from TRAF2 deficient mice were defective in JNK activation but not in NF- $\kappa$ B pathway activation [27]. One report also implicated MLK3 in the activation of NF- $\kappa$ B pathway in T cells by activating IKKs [28]; however we were unable to reproduce similar effect of MLK3 on NF- $\kappa$ B activation in T cells. Interestingly other MAP3K members, like ASK1 and MEKK1 are also activated via TNF- $\alpha$ -induced TRAF2 interaction and have been reported to activate their downstream JNK pathway [18, 19, 21, 29]. However, studies in mice deficient for these kinases could not support an essential role of these kinases in TNF- $\alpha$ -induced JNK activation, suggesting some other MAP3K member might be essential for TNF- $\alpha$ -induced JNK activation [30, 31]. Thus, so far, it can be concluded that MLK3 is a primary MAP3K in TNF-JNK pathway, which was also confirmed by an earlier report in MLK3 knockout MEFs [32]. In addition, we also observed that MLK3-induced JNK activation does require TRAF2 (Fig. 6A).

Based on our current data and several published results, we propose a model for JNK and NF- $\kappa$ B pathway activation via TNFRs-TRAF2 pathway. The TNF- $\alpha$ -induced JNK and NF- $\kappa$ B pathways bifurcate at the level of TRAF2, where MLK3 interacts with TRAF2 upon TNF- $\alpha$  stimulation and phosphorylates MKK4 and MKK7 (two MAPKK members in JNK pathway) to phosphorylate and activate downstream JNK that finally translocates to the nucleus and regulate AP1-mediated transcription. Since TRAF2 deficiency purely down regulates JNK and not NF- $\kappa$ B pathway, TNF- $\alpha$  might utilize other TRAFs, such as TRAF5 to activate NIK, which eventually leads to NF- $\kappa$ B activation. In addition, the death domain

containing protein RIP is required for TNF- $\alpha$ -induced NF- $\kappa$ B activation. Since we did not observe any NF- $\kappa$ B activation in Jurkat T cells upon MLK3 over expression (data not shown), we can conclude that TNF- $\alpha$ -induced interaction between MLK3 and TRAF2 primarily induce JNK pathway and not NF- $\kappa$ B.

In conclusion, our data provide an insight into the mechanism by which TNF- $\alpha$  activate MLK3. Our biochemical data clearly show that TRAF2-MLK3 interaction is necessary for MLK3 and its downstream target JNK activation. The interaction between MLK3 and TRAF2 is physiological because these proteins interact endogenously upon TNF- $\alpha$  treatment, in a time dependent manner and the activation of MLK3 was specifically blocked by the mutant of TRAF2 that binds with the C-terminus of MLK3 protein. We are currently in the process of determining in details, how the interaction between TRAF2 and MLK3 leads to MLK3 activation.

## Materials and Methods

### Cell Culture and Treatments

Human embryonic kidney 293 (HEK293) cells, TRAF2 deficient (TRAF2<sup>-/-</sup>) and normal Mouse Embryonic Fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). Jurkat T cells were maintained in RPMI medium containing 4.5g/L glucose, 10mM HEPES, 1.0mM sodium pyruvate, 10% FBS and 50 $\mu$ M  $\beta$ -ME. For TNF- $\alpha$  treatment, either murine TNF- $\alpha$  (Roche Applied Science, Mannheim Germany) for cell lines of murine origin, or human TNF- $\alpha$  (Roche Applied Science, Mannheim, Germany) for cell lines of human origin, were used, as indicated. The cells were starved in 0.2% serum containing media for 12 hours prior to treatment with 10nM TNF- $\alpha$  for the indicated periods of time.

### Plasmids, Transfection and MLK3 knockdown

The Flag-tagged TRAF2 clones were obtained from Dr. James R. Woodgett (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada) as described [18]. Myc-tagged TRAF1 and TRAF3 were obtained from Dr. Bharat Agarwal (MD Anderson Cancer Center, Houston, Texas) and all other TRAFs (i.e. TRAF5 and 6) were from Dr. Woodgett's laboratory [18]. The MLK3 clones were constructed in our laboratory, as described earlier [24, 33]. For cDNA transfection, in most cases, 1 $\mu$ g of various cDNAs were taken, otherwise as indicated. The various cell types used were transfected with the appropriate expression vectors using LipofectAMINE (Invitrogen, Carlsbad, CA) reagent as per manufacturer instruction. The endogenous MLK3 was knocked down using MLK specific shRNA. The shRNA oligos were synthesized commercially (Invitrogen, Carlsbad, CA),

Upper strand:

CACCGGCTGGAAACGCGAGATCCCGAAGGATCTCGCGTTTCCAGCC,

Lower strand:

AAAAGGCTGGAAACGCGAGATCCTTCGGGATCTCGCGTTTCCAGCC



The upper and lower strands were annealed and sub-cloned into entry vector pENTR/H1/TO vector (Invitrogen, Carlsbad, CA) and recombinant lentivirus was produced using Virapower T-Rex Lentiviral expression system (Invitrogen, Carlsbad, CA) following manufacture's instruction. 20 MOI were used to knock down the endogenous MLK3 in MEFs.

### Immunoblot Analysis

For immunoblotting, equal protein content of cell extracts or the immunoprecipitated protein samples were taken. The proteins were separated on denaturing SDS-PAGE and transferred onto polyvinylidene difluoride membrane (PVDF) and blotted with antibodies as indicated. Antibodies used were: anti-FLAG (Sigma, St. Louis, MO); anti-GST (Millipore, Billerica, MA); anti-TRAF2 (Cell Signaling, Danvers, MA); anti-JNK (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-phospho JNK (Promega, Madison, WI). The antibody for immunoprecipitating endogenous JNK was provided by Dr. Joseph Avruch (Massachusetts General Hospital, Boston, MA). For immunoprecipitating endogenous MLK3, the antibody against the C-terminus peptide of MLK3 was developed in our laboratory, as described in [24, 33]. For blotting endogenous MLK3, the antibody against the N-terminal peptide of MLK3, developed in our laboratory was used.

### Immunoprecipitation and Kinase Assay

Jurkat T cells, TRAF2<sup>+/+</sup>, TRAF2<sup>-/-</sup> MEFs and HEK293 cells were lysed in lysis buffer as described previously [2]. The cell extracts were clarified by centrifugation at 15,000 × g for 5 min, and protein contents were estimated using the Bradford method. Immunoprecipitation and glutathione S-transferase (GST) pull-down assays were performed either by specific antibodies or glutathione-Sepharose beads, respectively. After thorough washing, the immunoprecipitates were either processed for immunoblotting or used for kinase assay as described previously [2, 5].

### Acknowledgements

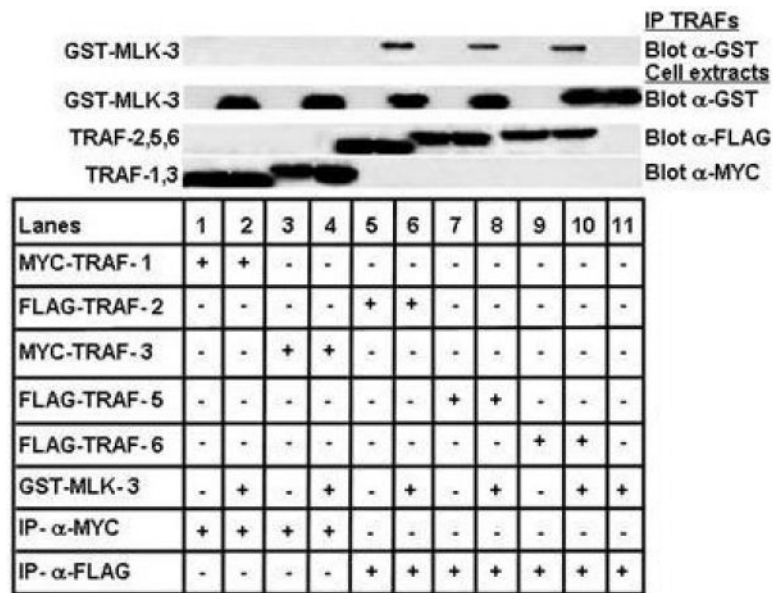
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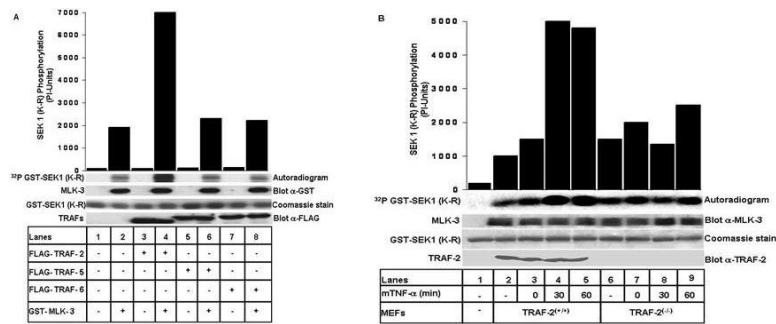
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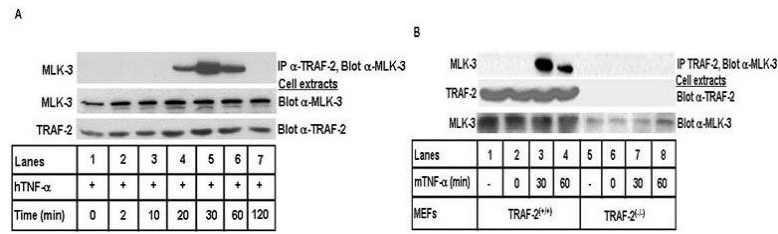


**Figure 1.** MLK3 associates with TRAF proteins. HEK-293 cells were transiently transfected with GST epitope-tagged MLK3 along with either Flag or Myc epitope-tagged TRAF expression vectors, as indicated. The TRAF proteins were immunoprecipitated using antibodies against the specific epitopes and any associated MLK3 was detected by using anti-GST antibody. The expression of MLK3 (middle panel) and TRAF proteins (lower two panels) were detected using antibodies against specific epitopes, as indicated.

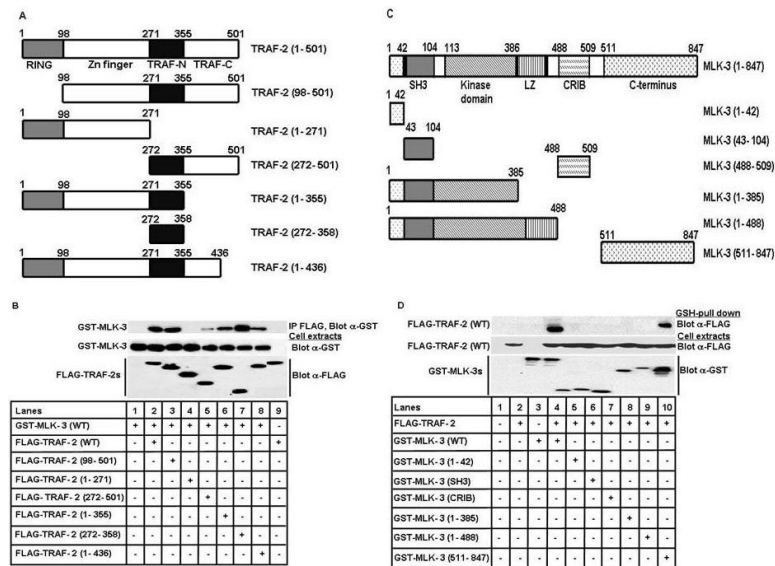


**Figure 2.**

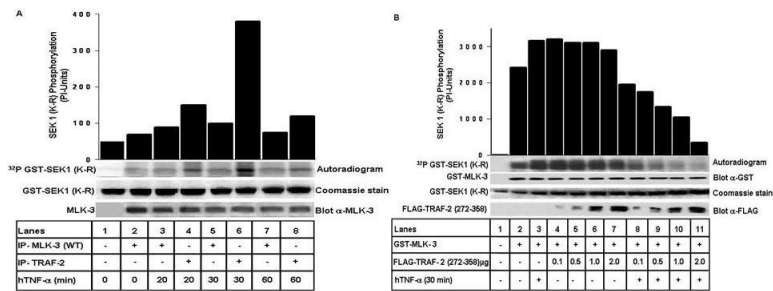
TRAF2 is necessary for TNF $\alpha$ -induced MLK3 activation. **(A)** Overexpression of TRAF2 causes activation of MLK3. HEK-293 cells were transiently transfected with GST epitope-tagged MLK3 and Flag epitope-tagged various TRAF expression vectors, as indicated. The cells were starved for 12 hours in 0.2% serum before harvesting 48 hours post-transfection. The recombinant MLK3 was pulled down by reduced glutathione sepharose (GSH) beads and subjected to kinase assay using bacterially expressed, SEK1 (K-R) protein as a specific substrate. The equal expression of MLK3 (second panel from top) and TRAF proteins (bottom panel) were detected by antibodies against epitopes, as indicated. The equal quantity of GST-SEK1 (K-R) substrate protein was judged by Coomassie stain. **(B)** TRAF2 is essential for MLK3 activation by TNF- $\alpha$ . The mouse embryonic fibroblasts (MEFs) from wild type (lanes 2-5) and TRAF2-deficient (lanes 6-9) mice were grown in serum containing complete media and then starved for 12 hours in 0.2% serum containing media before TNF- $\alpha$  (10nM) treatment for 30 or 60 minutes. The endogenous MLK3 was immunoprecipitated from these cells using MLK3 specific antibody and the immunoprecipitate were subjected to kinase assay as detailed above.

**Figure 3.**

TNF- $\alpha$  induces the endogenous association of MLK3 with TRAF2. **(A)** MLK3 and TRAF2 associate endogenously in response to TNF- $\alpha$  in Jurkat cells. The Jurkat T cells were starved for 12 hours in 0.2% serum containing media and treated with 10nM of TNF- $\alpha$  for indicated periods of time. The endogenous TRAF2 protein was immunoprecipitated and immune-complex protein samples were blotted with anti-MLK3 antibody. **(B)** The endogenous interaction between MLK3 and TRAF2 is specific. The MEFs from wild type and TRAF2 deficient mice were grown and treated with TNF- $\alpha$  as described above and the endogenous TRAF2 was immunoprecipitated and any associated MLK3 protein was detected by anti-MLK3 antibody.

**Figure 4.**

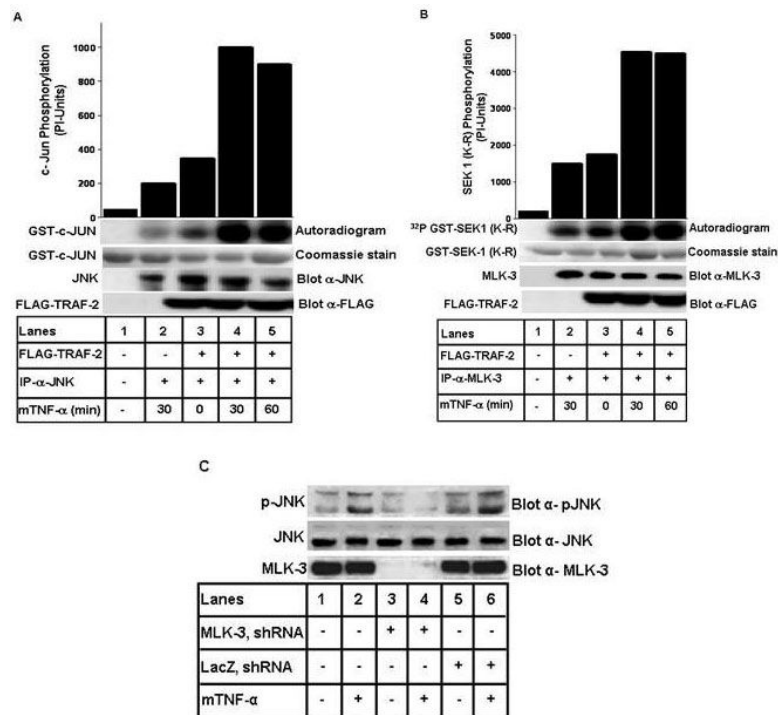
Mapping the interaction domains of TRAF2 and MLK3. **(A)** TRAF2 mutants were used to determine the interaction domain(s). The domain structure of full-length TRAF2 is shown, with amino acid number. The various TRAF2 mutants used are presented diagrammatically. **(B)** TRAF2 interacts with MLK3 via TRAF domain. HEK-293 cells were transfected with GST-tagged MLK3 and indicated Flag-tagged TRAF full length or truncated mutant constructs. The TRAF proteins were immunoprecipitated by anti-FLAG antibody and any associated MLK3 was detected by anti-GST antibody (upper panel). The expression of MLK3 (middle panel) and TRAF mutant (lower panel) proteins were detected by antibodies against the tag-epitopes. **(C)** MLK3 mutants used to determine the interaction domains(s). The domain structure of full length MLK3 is shown; with amino acid number. The various MLK3 are presented diagrammatically. **(D)** The C-terminus domain of MLK3 interacts with TRAF2. HEK-293 cells were transfected with full length Flag-tagged TRAF2 and GST-tagged MLK3 deletion mutant or full length expression vectors, as indicated. The MLK3 proteins were pulled down with GSH beads and any associated TRAF2 protein was detected with anti-FLAG antibody.



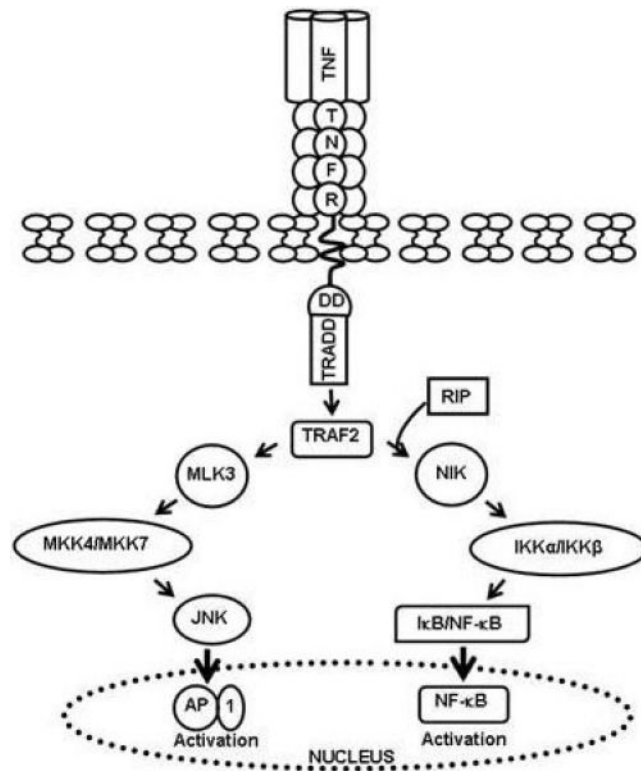
**Figure 5.**

TNF- $\alpha$  induces an association between TRAF2 and MLK3 and activates MLK3 kinase activity. **(A)** Association of TRAF2 with MLK3 increases its kinase activity. Jurkat T cells were starved for 12 hours as indicated in Fig. 3 and were treated with 10nM of TNF- $\alpha$  for indicated periods of time. Cells were lysed and comparable amounts of endogenous total MLK3 and MLK3 associated with TRAF2 were individually immunoprecipitated and MLK3 kinase activities were measured using SEK1 (K-R) as substrate. **(B)** Interaction between TRAF2 and MLK3 is necessary for TNF- $\alpha$ -induced MLK3 kinase activation. HEK-293 cells were transfected with MLK3 and increasing concentrations of TRAF2 mutant constructs (i.e. TRAF2 AA 272-358) to compete off the binding between full length endogenous TRAF2 and recombinant MLK3. Some of these cells were treated with TNF- $\alpha$  as indicated for 30 minutes. The recombinant MLK3 was immunoprecipitated and kinase activity was detected as described in Fig. 2.



**Figure 6.**

TRAF2 and MLK3 are necessary for TNF- $\alpha$ -induced JNK activation. **(A)** JNK is the downstream target of MLK3, specifically induced by TNF- $\alpha$ -TRAF2 pathway. The TRAF2 deficient MEFs (i.e. TRAF2<sup>-/-</sup>) were treated with 10nM of TNF- $\alpha$  for 30 minutes (lane 2) or reconstituted with full length TRAF2 expression vectors and then treated with TNF- $\alpha$  for 30 or 60 minutes (lanes 4 and 5). The endogenous JNK was immunoprecipitated and the kinase activity was measured using GST-Jun as the substrate. **(B)** Cell extracts as prepared in (A) were used to immunoprecipitate endogenous MLK3 and kinase activity was estimated as described in Fig. 2. **(C)** MLK3 is necessary for TNF- $\alpha$ -induced JNK activation. The endogenous MLK3 in normal MEF cells was knocked down by transducing lentivirus containing MLK3 specific shRNA, as described in Materials and Methods. As controls, some cells were transduced with LacZ shRNA or left alone. TNF- $\alpha$  treatment was done as in (A); and total cell lysates were blotted with indicated antibodies.



**Figure 7.**

A model of MLK3 activation by TRAF2 via TNF-pathway. The pro-inflammatory cytokine TNF- $\alpha$  binds to its receptor, TNFR that allows death domain containing adaptor protein TRADD to associate with the death domain (DD) of TNFR at the cytoplasmic end. The TRAF2 binds with TRADD and MLK3. This protein-protein interaction allows MLK3 activation and its downstream target JNK via MKK4 and MKK7 (two MAPKK). The other details about NF- $\kappa$ B activation by TRAF2 is described in the text.